Interaction of tryptophan-related compounds with ABCG2 transporter: comprehensive analysis of tryptophan metabolome in milk

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Abstract

Milk is the main source of the essential amino acid tryptophan and its metabolites for sucklings. The main pathway in tryptophan metabolism is the kynurenine pathway that generates several bioactive metabolites with relevant health effects, including prevention of neurodegenerative disorders. ABCG2 transporter participates in milk secretion of different compounds. Our aim was to provide a comprehensive overview of the *in vitro* and *in vivo* interactions between tryptophan-related compounds and ABCG2. Tryptophan metabolome was analysed in milk and plasma from wild-type and Abcg2$^{-/-}$ mice using liquid chromatography-tandem mass spectrometry. Our results show that Abcg2 mediates the secretion of kynurenic acid, kynurenine, anthranilic acid and xanthurenic acid into milk. Complementary metabolome analysis revealed that the bovine ABCG2 Y581S polymorphism increases kynurenine concentration in cow milk. All these results show the relevance of the interaction of ABCG2 and tryptophan-related compounds present in milk and its potential effect on health, mainly in postnatal development.

1. Introduction

Milk contains a number of nutrients and bioactive ingredients that play an important role in health. Some of these important nutrients and bioactive ingredients of milk are L-tryptophan (Trp) and its metabolites. It is worth noting that breast milk is the only source of this essential amino acid in breast-fed infants (Schneider, Mutungi, & Cubero, 2018). Trp is an aromatic amino acid critical for protein synthesis; in addition to this essential role, it is the precursor of several bioactive compounds with important physiological roles (Cervenka, Agudelo, & Ruas, 2017). Trp metabolism proceeds mainly by kynurenine (KYN) and serotonin (5HT) pathways (Fig. 1), and the main metabolites generated are related to broad spectrum effects associated with neurodegerative disorders, pain syndromes and autoimmune diseases (Vécsei, Szalárdy, Fülöp, & Toldi, 2013). Probably the best-known pathway is Trp conversion to 5HT and finally to melatonin. Both compounds have important roles in the central nervous system (CNS). 5HT is an important neurotransmitter involved in the control of adaptive responses in the CNS. Melatonin is a neurohormone and a powerful free-radical scavenger, therefore it is an important antioxidant (Reiter et al., 2017). In addition, it plays an essential role in regulating circadian rhythms related to physiological functions such as sleep and blood pressure (Boutin, Audinot, Ferry, & Delagrange, 2005). Nevertheless, more than 95% of Trp is metabolized through the Kynurenine Pathway (KP) in the liver (Badawy, 2019). This pathway generates a range of metabolites, collectively known as kynurenines, involved in immune response, inflammation and neurotransmission (Stone, Stoy, & Darlington, 2013). Most of them are neuroactive and show relevant roles in the regulation of NMDA (N-methyl-D-aspartate) receptor function and the production of free radicals (Nayak & Buttar, 2016). The presence of some of these metabolites in the diet could have important effects on biological processes. For example, an excess of L-KYN in diet could affect neuronal development in young (Pocivavsek, Wu, Elmer, Bruno, & Schwarcz, 2012).

Thus, the content of Trp metabolites in milk might have important effects on human health. The presence of a large number of metabolites and xenobiotics in 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/BCRP mediates the active secretion of xenobiotics and several vitamins into milk (van Herwaarden et al., 2007). ABCG2 is present in a multitude of tissues

which affect the body distribution of a wide range of molecules including chemotherapeutic (mitoxantrone, methothexate) and non-chemotherapy drugs (nitrofurantoin, fluoroquinolones), as well as endogenous and dietary compounds such as flavonoids, porphyrins, estrone-3-sulfate and uric acid (Safar, Kis, Erdo, Zolnerciks, & Krajcsi, 2019). However, its role in the regulation of the milk content of Trp metabolites has not been explored.

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 an SNP encoding a substitution of a Ser by Tyr at aminoacidic position 581 (Y581S) described as an *in vitro* and *in vivo* gain-of-function polymorphism (Otero, 2015; Real et al., 2011), which is directly involved in milk quality affecting the presence of ABCG2 substrates in cow milk (García-Lino et al., 2019). In humans, several genetic analyses have demonstrated that single nucleotide polymorphisms (SNPs) leading to ABCG2 deficiency are essential in the pathogenesis of hyperuricemia and gout (Woodward et al., 2009). The Q141K variant yields to decreased ABCG2 protein expression and influences risk of hyperuricemia and gout. Because of this, the Q141k variant has been studied as a potential therapeutic target (Woodward et al., 2009). Although there is some evidence linking hyperuricemia to changes in Trp levels (Liu et al., 2011), and Dankers et al. (Dankers et al., 2013) have suggested that kynurenic acid (KYNA) could interact with human ABCG2, the interaction between ABCG2 and Trp metabolites has not been fully established.

The aim of this work was to study the interaction of Trp-related compounds with ABCG2 transporter through the analysis of Trp metabolome in plasma and milk of wild-type and Abcg2$^{-/-}$ mice. The outcome was corroborated by *in vitro* transport studies with cells overexpressing murine Abcg2. A complementary Trp metabolome analysis was performed in plasma and milk from cows carrying the polymorphism Y581S and non-carrier animals.

2. Materials and methods

2.1. Standards and chemicals

Reference standards of leucine (Leu), isoleucine (Ile), Trp, 5HT, melatonin, serotonin (5HT), 5-hydroxyindolacetic acid (5HIAA), KYN, KYNA, xanthurenic acid (XA) and anthranilic acid (AA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kynurenic acid-d5, phenylalanine-d5 and serotonin-d5 were supplied by Toronto Research Chemicals (Toronto, Canada). Tyrosine-d4, tryptophan-d5, 5-hydroxyindolacetic acid-d4, and kynurenine-13C6 were from Alsachim (Illkirch-Graffenstaden, France). All the other chemicals were analytical grade and 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$^{-/-}$ and wild type female mice aged between 8 and 17 weeks, all of them >99% FVB genetic background (n = 9 – 12), were kindly provided by Dr. A. H. Schinkel (The Netherlands Cancer Institute, Amsterdam, the Netherlands), and were kept in a temperature-controlled environment with a 12-h light / 12-h dark cycle. Pups of approximately 10 days old were separated from their mothers approximately 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 anesthesia with isoflurane. Blood samples were collected by cardiac puncture under anesthesia with isoflurane and centrifuged immediately at 3000 g for 15 min. At the end of the experiment, the mice were killed by cervical dislocation. Plasma and milk samples were stored at -20 ºC until LC-MS/MS analysis.

Lactating Holstein-breed cows aged between 2 and 5 years and weighing 650 to 1000 kg were used. Their daily milk yield was on average 40 ± 10 kg. The normal milking routine for all the animals involved milk being taken three times each day. The samples were taken at a private farm located at Villalquite, Leon (Spain). The Y581S genotypes were determined

in accordance with the procedure described by Komisarek et al (2009) (Komisarek & Dorynek, 2009). Animals were divided into 2 groups of 8 Y/S 581 heterozygous and 8 Y/Y 581 homozygous cows. No difference was found in age, weight, or milk yield between the two sets of cows. Samples were collected from the first morning milking by mechanical milking. Blood samples were collected from the tail vein and plasma was separated by centrifugation at 3000 x g for 15 min. Plasma and milk samples were stored at -20 ºC until LC-MS/MS analysis.

2.3. Sample preparation for LC-MS/MS analysis

An aliquot of plasma (70 µL for mice and 150 µL for cows) or milk (between 63 and 180 mg for mice and 150 µL for cows) was mixed with 300 µL of acetonitrile in order to precipitate the proteins. After centrifugation, the supernatant was transferred to a clean tube and 50 µL of the internal standard mixture (containing kynurenic acid-d5, phenylalanine-d5, serotonin-d5, tyrosine-d4, tryptophan-d5, 5-hydroxyindolacetic acid-d4, and kynurenine-13C6) were added. The mixture was evaporated at room temperature under nitrogen stream (<10 psi). After reconstitution with 150 µL of water, 10 µL were injected into the system. The standards used for calibration were subjected to the sample procedure.

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 the determination of Trp-related compounds in milk and plasma from mice and cows. The LC-MS/MS system used to perform the analysis was an Acquity UPLC system (Waters Associates, Milford, MA, USA) coupled to a TQS Micro triple quadrupole mass spectrometer (Waters Associates) equipped with an electrospray ionization interface. Chromatographic separation was carried out on an Acquity BEH C18 column (100 mm x 2.1 mm i.d., 1.7 µm) (Waters Associates) at a flow rate of 300 µL/min. Water and methanol both with ammonium formate (1 mM) and formic acic (0.01% v/v) were used as mobile phase solvents. A gradient elution was used for the chromatographic separation of the analytes. The percentage of the organic solvent linearly changed as follows: 0 min, 1%; 0.5 min, 1%; 7 min, 40%; 8.5 min, 90%; 9 min, 90%; 9.5 min, 1%; 12 min, 1%. Analytes were determined by SRM including 2 ion transitions for each analyte.

179 *2.5. Cell cultures*

180 MDCKII cells and their murine Abcg2 transduced subclones were provided by Dr. A.H.
181 Schinkel, Netherlands Cancer Institute, Amsterdam. MDCKII cells stably transduced with
182 both bovine variants (S581 and Y581) of ABCG2 were previously generated by our group
183 (Real et al., 2011). Culture conditions were as previously described (Otero, 2015; Perez et
184 al., 2013).

185 *2.6. Transport studies*

186 Transepithelial transport assays using Transwell plates were carried out as described
187 elsewhere (Otero, 2015; Perez et al., 2013) with minor modifications. Cells were grown for
188 3 days after seeding on microporous polycarbonate membrane filters at a density of 1.0 x
189 10^6 cells per well. To check the tightness of the monolayer, transepithelial resistance was
190 measured in each well using a Millicell ERS ohmmeter (Millipore). Transport medium
191 consisted of Hanks' Balanced Salt solution (Sigma-Aldrich) supplemented with 4-(2-
192 hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES (25 mM). Two hours before the start
193 of the experiment, medium at both the apical (AP) and basolateral (BL) sides of the
194 monolayer was replaced with 2 mL of transport medium without serum, HANKs solution
195 supplemented, either with or without the specific ABCG2 inhibitor Ko143 (1 µM). The
196 experiment started when medium in either the AP or BL compartment was replaced with
197 fresh medium containing the different compounds at a concentration of 10 µM. Cells were
198 incubated at 37 ºC in 5% CO_2 and 100 µL aliquots of culture media were taken at t=1, 2, 3
199 and 4 h, ours and this volume was replaced with fresh medium. The samples were stored at
200 -20 ºC. Concentrations of studied compounds were subsequently determined by HPLC.
201 Active transport across MDCKII monolayers was expressed by the relative transport ratio
202 (R), defined as apically directed transport percentage divided by basolaterally directed
203 translocation percentage, after 4 h.

204 *2.7. High Performance Liquid Cromatography (HPLC) Analysis*

205 Analysis of samples to determine the concentrations of the studied compounds in the
206 transepithelial transport assays were carried out by HPLC analysis.The chromatographic
207 system consisted of a Waters 2695 separation module and a Waters 2998 UV photodiode
208 array detector. Fifty µL of the culture medium was injected directly into the HPLC system.

Separation of the samples was performed on a reverse-phase column (Atlantis® T3 3 µm, 4.6x150 mm). The mobile phase consisted of 0.14 % trifluoroacetic acid:acetonitrile (80:20), the flow rate of the mobile phase was set to 0.8 mL/min, and UV absorbance was measured at 238 nm. The temperature of the samples was 4 ºC. Standard samples were prepared in the appropriate drug-free matrix, yielding a concentration range from 0.039 µg/mL to 10 µg/mL, with coefficients of correlation >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 the compounds. Both were calculated by the method described by Taverniers et al. (Taverniers, I., De Loose, M., Van Bockstaele, 2004).

2.8. Statistical analysis

Comparisons between groups were performed by the Student's t-test (normal variables) and the Mann-Whitney U test (not normally distributed variables). All analyses were carried out on the assumed significance level of $p \leq 0.05$ using SPSS Statistics software (v. 24.0; IBM, Armonk, New York, NY, USA). The results are shown as mean ± standard deviation (SD).

3. Results

3.1. Determination of Trp-related compounds in milk from Abcg2$^{-/-}$ and wild-type mice

To examine the role of ABCG2 in the transport of Trp metabolites into milk, we collected milk and plasma samples of wild-type and Abcg2$^{-/-}$ female mice.

The targeted LC-MS/MS method included 10 analytes. These Trp-related analytes were divided into three different classes based on their biological origin (Fig. 1): (a) large neutral amino acids (LNAA) competitors of Trp; (b) metabolites belonging to the serotonin pathway; (c) metabolites from the kynurenine pathway.

All the compounds were detected in all plasma and milk samples, with the exception of Trp in milk and melatonin in plasma and milk (Table 1). There were no differences in plasma between wild-type and Abcg2$^{-/-}$ mice for any compound. Nevertheless, a higher secretion into milk concentration of KYN, KYNA, XA, AA and 5HIAA was observed in wild-type compared to Abcg2$^{-/-}$ mice (Table 1). These differences were especially high for XA and KYNA, with a concentration in milk of almost 10-fold higher (75 ± 40 ng/mg versus 7.8 ± 4.7 ng/mg) and 5-fold higher (42 ± 8 ng/mg versus 9.1 ± 3.0 ng/mg) in wild-type and Abcg2$^{-/-}$ mice, respectively. Higher milk-to-plasma ratios were also obtained for these five metabolites in wild-type compared to Abcg2$^{-/-}$ mice, except for 5HIAA. These data indicate that Abcg2 plays a substantial role in the secretion of KYN, KYNA, XA and AA into milk.

3.2. Effect of the bovine ABCG2 polymorphism Y581S SNP on secretion into milk of Trp-related compounds

A similar analysis was performed on cows carrying the Y581S polymorphism and on non-carrier animals (Table 2). All the analytes were detected in all plasma and milk samples with the exception of AA, which was not detected in any sample, and 5HT, which was not detected in any milk sample. There were no differences in plasma levels between Y/Y 581 and Y/S 581 cows for any compound tested. Significant differences were found only in milk for KYN concentrations (Table 2, Fig. 2). KYN milk concentrations were 2-fold higher in Y/S (4.6 ± 1.8 ng/mL) compared with Y/Y cows (2.4 ± 1.0 ng/mL). The milk-to-plasma ratio for KYN in Y/S cows was 2-fold higher than in Y/Y cows (0.004 ± 0.002 versus 0.002 ± 0.001). This indicates that the polymorphism Y581S affects the *in vivo* active transport of KYN into cow milk.

3.3. Transport of Trp metabolites in MDCKII cell models transduced with murine Abcg2

To corroborate the metabolomic study in mice, a transport assay using parental MDCKII and its subclones transduced with murine Abcg2 was performed to study the role of this protein in the process. KYN, KYNA, XA and AA were tested *in vitro* due to *in vivo* evidence of Abcg2 involved in their milk secretion (Table 2). Trp itself was also included.

In the MDCKII parental cell line, most of the molecules showed similar apically and basolaterally directed translocation (Fig. 3A). However, parental MDCKII cells displayed a high basolaterally directed translocation of KYN and Trp, whereas apically directed translocation was very low, indicating the potential presence of an absorptive KYN and Trp transport process.

In the murine Abcg2-transduced cells (Fig. 3B), we observed for KYN, KYNA and XA increased translocation from the basolateral to the apical compartment, and reduced translocation from the apical to the basolateral compartment compared to parental cells, with high relative transport ratios (AP/BL). For AA, a low transport ratio similar to the parental cells was obtained. The apical transport of KYN, KYNA and XA by Abcg2 was completely inhibited by Ko143, a selective Abcg2 inhibitor (data not shown).

From this, we can confirm that KYN, KYNA and XA are good *in vitro* substrates of murine Abcg2.

3.4. Transport of KYN in MDCKII cell models transduced both bovine variants (Y581 and S581)

To corroborate the role of the bovine Y581S polymorphism in the transport of KYN, transport assays were performed using polarized MDCKII parental cells and their subclones transduced with both bovine ABCG2 variants (S581 and Y581) (Fig. 4). The relative transport ratio of KYN was significantly higher in the S581 expressing cells versus parental cells (1.08 ± 0.25 vs. 0.59 ± 0.19) since apical transport increased and basolateral transport decreased compared with parental cells. However, in the case of cells expressing Y581 cells, no changes were observed in the transport ratio compared with parental cells, indicating that KYN is not transported by this variant. Statistically significant differences were found between transport ratios of both variants of bovine ABCG2, Y581 and S581 (0.42 ± 0.25 vs. 1.08 ± 0.25). Therefore, the differences found between two bovine variants

showed that the Y581S polymorphism affects the *in vitro* transport of KYN, corroborating the differences found *in vivo* between carrier and non-carrier animals.

4. Discussion

In this study a targeted metabolomic analysis was performed to elucidate the role of ABCG2 transport in the active secretion of Trp-related compounds in plasma and milk. The relevance of the bovine Y581S polymorphism on the transport of these compounds present in cow milk was also studied.

Due to the complication in controlling all the animal factors, such as age, nutrition and time of lactation, among others, a study of the association between *in vitro* and *in vivo* results is difficult, especially in cows (Otero, 2015). However, this LC-MS/MS metabolomic study permits a comprehensive estimation of the effect of ABCG2 in Trp-related pathways in plasma and milk in mice and cows. What is of relevance, and an advantage over other studies analysing a limited number of metabolites (Cubero et al., 2005; Laeger, Görs, Metges, & Kuhla, 2012), is the fact that a large number of compounds were determined.

The amount of L-Trp in humans is essential for maintaining health, and one of the most common sources of Trp in the human diet is milk (Richard et al., 2009). Furthermore, the biological activities of its derivative compounds, mainly as neuroactive molecules, adds to the importance of this complete study. Focussing, therefore, on the relevance of these neuroactive compounds in the diet, we performed the metabolome analysis to elucidate the role of ABCG2 transport in active secretion into milk of Abcg2$^{-/-}$ and wild-type lactating mice. Differences in the concentrations of KYN, KYNA, XA and AA were observed in the milk-to-plasma ratio between wild-type and Abcg2$^{-/-}$ mice, which suggests the involvement of Abcg2 in the milk secretion of these compounds (Table 1). These results were corroborated by transport assays showing that KYN, KYNA and XA are *in vitro* substrates of Abcg2 (Fig. 3). Only KYNA had been previously described as a potential Abcg2 substrate in humans (Dankers et al., 2013). Conversely, AA was not confirmed as an *in vitro* substrate of ABCG2, and due to the positive results observed in the *in vivo* study, we cannot exclude its *in vitro* interaction with the Abcg2 transporter in other experimental conditions.

Binding of substrate to ABCG2 has been shown to be dependent of hydrophobic interactions, mainly between hydrogen bond acceptors (HBAs) present in substrates and hydrogen bond donors (HBDs) present in the transmembrane region of the transporter, among other physicochemical features (Matsson et al., 2007). In adition, Xu *et al.* (Xu et al., 2015) demonstrated that binding of substrates with the ABCG2 transporter increases with the number of HBAs present in the potential substrates. In our case, KYN, KYNA and XA, identified as Abcg2 substrates, gave a higher number of HBAs than Trp, 5HIAA and 5HT and AA (non Abcg2 substrates) (Supplementary material, Table S1).

Furthermore, we examined the importance of the bovine Y581S polymorphism in the transport of Trp-related compounds present in cow milk, as previously reported for milk secretion of fluoroquinolone drugs, antiinflammatory drugs and endogenous and dietary compounds (García-Lino et al., 2019; Otero, 2015). 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 plasma ratio for carrier animals (Otero et al., 2016). Our data show that the transfer of KYN into milk is influenced by the bovine Y581S polymorphism with an almost 2-fold increase in the concentration and milk-to-plasma ratio of KYN in the Y/S animals (Fig. 2). This is the first time that differences between both variants of cows, carriers and non-carriers of the Y581S polymorphism has been observed for Trp-related compounds with neuroactive activity. Furthermore, these results are in agreement with *in vitro* transport studies showing that cells overexpressing the S581 variant transported KYN in the basolateral to apical direction more efficiently than the cells with the Y581 variant (Fig. 4), indicating a higher transport capacity *in vitro* for this variant.

Our results could have great relevance in the field of nutrition since all of these metabolites show important effects on health and some of them are present in consumed dairy milk. Furthermore, our results could be relevant in the early stages of development where milk is the main food consumed. ABCG2 inhibitors such as drugs (Barrera et al., 2013) and dietary compouds (Miguel et al., 2014) can alter the transfer of these compounds into milk. In fact, these kinds of interactions mediated by ABCG2 have been observed for other ABCG2 substrates. For instance, soy-enriched diet and flaxeed-enriched diet modify *in vivo* milk secretion mediated by ABCG2 of the antimicrobial danofloxacin in sheep (Otero et al., 2018; Perez et al., 2013). Therefore, potentially different concentrations of Trp-related

compounds in consumed milk due to polymorphisms or inhibition of this transporter may affect the intake of these compounds by the offspring or the dairy consumer.

Trp-related compounds generated by KP have an important role in health due to their effects on the CNS since they have been linked to several psychiatric and mental health disorders such as depression and schizophrenia (Chen & Guillemin, 2009). This pathway is also currently involved in immunological processes (Badawy, 2019). KYNA has proved to be a neuroprotective molecule and its neuroprotective role in various experimental models of neurotoxicity has been reported (Majláth, Török, Toldi, & Vécsei, 2016). There is evidence in preclinical models that changes in KYNA concentrations in the brain could influence the development of Huntington's disease (Okamoto et al., 2009) and schizophrenia (Beggiato, Notarangelo, Sathyasaikumar, Giorgini, & Schwarcz, 2018). These compounds may also have effects in other organs. Reports suggest that KYNA could have a protective role in the intestinal mucosa in the framework of obstructive jaundice and may protect from induced ulcers (Glavin, Bose, & Pinsky, 1989). Since we demonstrated that KYNA is an ABCG2 substrate, different milk intake of KYNA due to differences in ABCG2 function may affect the protective role of KYNA in several pathological conditions.

KYN is the main precursor of KYNA. Due to the lower permeability of KYNA in the blood-brain-barrier than presented by KYN, KYN is used as a protective compound in models of Huntington's disease induced by malonate and quinolinic acid (Santamaría et al., 1996). However, despite the benefits in the neurodegenerative diseases provided by KYN administration, several studies report that an exposure to KYN during a vulnerable period of brain development causes cognitive deficits in adulthood related to schizophrenia and other psychiatric diseases (Pocivavsek et al., 2012). In addition, it has been shown that a continuous KYN administration during the prenatal period causes learning and memory deficits in adults rats (Pocivavsek, Thomas, Elmer, Bruno, & Schwarcz, 2014). Since our results show the role of ABCG2 in the presence of KYN in milk, this interaction may play an important role in cognitive development of the offspring.

Our data may have relevant effects in other health fields. Some studies have demonstrated that KYN and KYNA are endogenous ligands of the aryl hydrogen receptor (AhR) (Julliard, Fechner, & Mezrich, 2014). The coupling between AhR and these compounds increases concentration of TGF-β and IL-6 in early sepsis (Wirthgen & Hoeflich,

2015). Also, recent studies suggest that Trp-containing diet might regulate epithelial homeostasis through AhR in ulcerative colitis (Islam et al., 2017).

In conclusion, this work demonstrates that several Trp metabolites are secreted into milk by the Abcg2 transporter. It shows, in addition, that lactating dairy cows carrying the Y581S polymorphism produced milk with higher amounts of KYN compared with non-carriers.

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References

Badawy, A. A. B. (2019). Tryptophan Metabolism: A Versatile Area Providing Multiple Targets for Pharmacological Intervention. *Egyptian Journal of Basic and Clinical Pharmacology*, *9*. https://doi.org/10.32527/2019/101415

Barrera, B., González-Lobato, L., Otero, J. A., Real, R., Prieto, J. G., Álvarez, A. I., & Merino, G. (2013). Effects of triclabendazole on secretion of danofloxacin and moxidectin into the milk of sheep: role of triclabendazole metabolites as inhibitors of the ruminant ABCG2 transporter. *Veterinary Journal*, *198*(2), 429–436. https://doi.org/10.1016/j.tvjl.2013.07.033

Beggiato, S., Notarangelo, F., Sathyasaikumar, K., Giorgini, F., & Schwarcz, R. (2018). Maternal genotype determines kynurenic acid levels in the fetal brain: Implications for the pathophysiology of schizophrenia. *Journal of Psychopharmacology*, *32*(11), 1223–1232. https://doi.org/10.1177/0269881118805492

Boutin, J. A., Audinot, V., Ferry, G., & Delagrange, P. (2005). Molecular tools to study melatonin pathways and actions. *Trends in Pharmacological Sciences*, *26*(8), 412–419. https://doi.org/10.1016/j.tips.2005.06.006

Cervenka, I., Agudelo, L. Z., & Ruas, J. L. (2017). Kynurenines: Tryptophan's metabolites in exercise, inflammation, and mental health. *Science*, *357*(6349), eaaf9794. https://doi.org/10.1126/science.aaf9794

Chen, Y., & Guillemin, G. J. (2009). Kynurenine pathway metabolites in humans: disease and healthy states. *International Journal of Tryptophan Research : IJTR*, *2*, 1–19. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22084578

Cubero, J., Valero, V., Sánchez, J., Rivero, M., Parvez, H., Rodríguez, A. B., & Barriga, C. (2005). The circadian rhythm of tryptophan in breast milk affects the rhythms of 6-sulfatoxymelatonin and sleep in newborn. *Neuro Endocrinology Letters*, *26*(6), 657–661. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/16380706

Dankers, A. C. A., Mutsaers, H. A. M., Dijkman, H. B. P. M., van den Heuvel, L. P., Hoenderop, J. G., Sweep, F. C. G. J., … Masereeuw, R. (2013). Hyperuricemia influences tryptophan metabolism via inhibition of multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP). *Biochimica et Biophysica Acta -*

Molecular Basis of Disease, *1832*, 1715–1722. https://doi.org/10.1016/j.bbadis.2013.05.002

García-Lino, A. M., Álvarez-Fernández, I., Blanco-Paniagua, E., Merino, G., & Álvarez, A. I. (2019). Transporters in the Mammary Gland—Contribution to Presence of Nutrients and Drugs into Milk. *Nutrients*, *11*(10), E2372. https://doi.org/10.3390/nu11102372

Glavin, G. B., Bose, R., & Pinsky, C. (1989). Kynurenic acid protects against gastroduodenal ulceration in mice injected with extracts from poisonous Atlantic shellfish. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *13*(3–4), 569–572.

Islam, J., Sato, S., Watanabe, K., Watanabe, T., Ardiansyah, Hirahara, K., … Shirakawa, H. (2017). Dietary tryptophan alleviates dextran sodium sulfate-induced colitis through aryl hydrocarbon receptor in mice. *The Journal of Nutritional Biochemistry*, *42*, 43–50. https://doi.org/10.1016/j.jnutbio.2016.12.019

Julliard, W., Fechner, J. H., & Mezrich, J. D. (2014). The aryl hydrocarbon receptor meets immunology: friend or foe? A little of both. *Frontiers in Immunology*, *5*, 458. https://doi.org/10.3389/fimmu.2014.00458

Komisarek, J., & Dorynek, Z. (2009). Effect of ABCG2, PPARGC1A, OLR1 and SCD1 gene polymorphism on estimated breeding values for functional and production traits in Polish Holstein-Friesian bulls. *Journal of Applied Genetics*, *50*(2), 125–132. https://doi.org/10.1007/BF03195663

Laeger, T., Görs, S., Metges, C. C., & Kuhla, B. (2012). Effect of feed restriction on metabolites in cerebrospinal fluid and plasma of dairy cows. *Journal of Dairy Science*, *95*(3), 1198–1208. https://doi.org/10.3168/jds.2011-4506

Liu, Y., Sun, X., Di, D., Quan, J., Zhang, J., & Yang, X. (2011). A metabolic profiling analysis of symptomatic gout in human serum and urine using high performance liquid chromatography-diode array detector technique. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *412*(23–24), 2132–2140. https://doi.org/10.1016/j.cca.2011.07.031

Majláth, Z., Török, N., Toldi, J., & Vécsei, L. (2016). Memantine and Kynurenic Acid: Current Neuropharmacological Aspects. *Current Neuropharmacology*, *14*(2), 200–209.

https://doi.org/10.2174/1570159X14666151113123221

Marcos, J., Renau, N., Valverde, O., Aznar-Laín, G., Gracia-Rubio, I., Gonzalez-Sepulveda, M., … Pozo, O. J. (2016). Targeting tryptophan and tyrosine metabolism by liquid chromatography tandem mass spectrometry. *Journal of Chromatography A*. https://doi.org/10.1016/j.chroma.2016.01.023

Matsson, P., Englund, G., Ahlin, G., Bergström, C. A. S., Norinder, U., & Artursson, P. (2007). A global drug inhibition pattern for the human ATP-binding cassette transporter breast cancer resistance protein (ABCG2). *The Journal of Pharmacology and Experimental Therapeutics*, *323*(1), 19–30. https://doi.org/10.1124/jpet.107.124768

Miguel, V., Otero, J. A., García-Villalba, R., Tomás-Barberán, F., Espín, J. C., Merino, G., … Álvarez, A. I. (2014). Role of ABCG2 in transport of the mammalian lignan enterolactone and its secretion into milk in abcg2 knockout mice. *Drug Metabolism and Disposition*, *42*(5), 943–946. https://doi.org/10.1124/dmd.113.055970

Nayak, B. N., & Buttar, H. S. (2016). Evaluation of the antioxidant properties of tryptophan and its metabolites in in vitro assay. *Journal of Complementary & Integrative Medicine*, *13*(2), 129–136. https://doi.org/10.1515/jcim-2015-0051

Okamoto, S., Pouladi, M. A., Talantova, M., Yao, D., Xia, P., Ehrnhoefer, D. E., … Lipton, S. A. (2009). Balance between synaptic versus extrasynaptic NMDA receptor activity influences inclusions and neurotoxicity of mutant huntingtin. *Nature Medicine*, *15*(12), 1407–1413. https://doi.org/10.1038/nm.2056

Otero, J. A. (2015). Role of the ABCG2/BCRP transporter and its polymorphisms in the active secretion of molecules into milk and modulation of its activity with natural compounds in ruminants. *Doctoral Thesis*. Universidad de León. https://doi.org/10.18002/10612/11135

Otero, J. A., García-Mateos, D., Alvarez-Fernández, I., García-Villalba, R., Espín, J. C., Álvarez, A. I., & Merino, G. (2018). Flaxseed-enriched diets change milk concentration of the antimicrobial danofloxacin in sheep. *BMC Veterinary Research*, *14*(1), 14. https://doi.org/10.1186/s12917-018-1341-3

Otero, J. A., Miguel, V., González-Lobato, L., García-Villalba, R., Espín, J. C., Prieto, J. G., … Álvarez, A. I. (2016). Effect of bovine ABCG2 polymorphism Y581S SNP on

secretion into milk of enterolactone, riboflavin and uric acid. *Animal*, *10*(02), 238–247. https://doi.org/10.1017/S1751731115002141

Perez, M., Otero, J. A., Barrera, B., Prieto, J. G., Merino, G., & Alvarez, A. I. (2013). Inhibition of ABCG2/BCRP transporter by soy isoflavones genistein and daidzein: Effect on plasma and milk levels of danofloxacin in sheep. *The Veterinary Journal*, *196*(2), 203–208.

Pocivavsek, A., Thomas, M. A. R., Elmer, G. I., Bruno, J. P., & Schwarcz, R. (2014). Continuous kynurenine administration during the prenatal period, but not during adolescence, causes learning and memory deficits in adult rats. *Psychopharmacology*, *231*, 2799–2809. https://doi.org/10.1007/s00213-014-3452-2

Pocivavsek, A., Wu, H. Q., Elmer, G. I., Bruno, J. P., & Schwarcz, R. (2012). Pre- and postnatal exposure to kynurenine causes cognitive deficits in adulthood. *European Journal of Neuroscience*, *35*, 1605–1612. https://doi.org/10.1111/j.1460-9568.2012.08064.x

Real, R., González-Lobato, L., Baro, M. F., Valbuena, S., de la Fuente, A., Prieto, J. G., … Merino, G. (2011). Analysis of the effect of the bovine adenosine triphosphate-binding cassette transporter G2 single nucleotide polymorphism Y581S on transcellular transport of veterinary drugs using new cell culture models. *Journal of Animal Science*, *89*(12), 4325–4338. https://doi.org/10.2527/jas.2011-3841

Reiter, R. J., Rosales-Corral, S., Tan, D. X., Jou, M. J., Galano, A., & Xu, B. (2017). Melatonin as a mitochondria-targeted antioxidant: one of evolution's best ideas. *Cellular and Molecular Life Sciences*, *74*(21), 3863–3881. https://doi.org/10.1007/s00018-017-2609-7

Richard, D. M., Dawes, M. A., Mathias, C. W., Acheson, A., Hill-Kapturczak, N., & Dougherty, D. M. (2009). L-Tryptophan: Basic Metabolic Functions, Behavioral Research and Therapeutic Indications. *International Journal of Tryptophan Research : IJTR*, *2*, 45–60.

Safar, Z., Kis, E., Erdo, F., Zolnerciks, J. K., & Krajcsi, P. (2019). ABCG2/BCRP: variants, transporter interaction profile of substrates and inhibitors, *15*(4), 313–328. https://doi.org/10.1080/17425255.2019.1591373

Santamaría, A., Ríos, C., Solís-Hernández, F., Ordaz-Moreno, J., González-Reynoso, L., Altagracia, M., & Kravzov, J. (1996). Systemic dl-Kynurenine and probenecid pretreatment attenuates quinolinic acid-induced neurotoxicity in rats. *Neuropharmacology*, *35*(1), 23–28. https://doi.org/10.1016/0028-3908(95)00145-X

Schneider, N., Mutungi, G., & Cubero, J. (2018). Diet and nutrients in the modulation of infant sleep: A review of the literature. *Nutritional Neuroscience*, *21*(3), 151–161. https://doi.org/10.1080/1028415X.2016.1258446

Stone, T. W., Stoy, N., & Darlington, L. G. (2013). An expanding range of targets for kynurenine metabolites of tryptophan. *Trends in Pharmacological Sciences*, *34*(2), 136–143. https://doi.org/10.1016/j.tips.2012.09.006

Taverniers, I., De Loose, M., Van Bockstaele, E. (2004). Trends in quality in the analytical laboratory. II. Analytical method validation and quality assurance. *TrAC Trends in Analytical Chemistry*, *23*(8), 535–552. https://doi.org/10.1016/J.TRAC.2004.04.001

van Herwaarden, A. E., Wagenaar, E., Merino, G., Jonker, J. W., Rosing, H., Beijnen, J. H., & Schinkel, A. H. (2007). Multidrug Transporter ABCG2/Breast Cancer Resistance Protein Secretes Riboflavin (Vitamin B2) into Milk. *Molecular and Cellular Biology*, *27*(4), 1247–1253. https://doi.org/10.1128/MCB.01621-06

Vécsei, L., Szalárdy, L., Fülöp, F., & Toldi, J. (2013). Kynurenines in the CNS: Recent advances and new questions. *Nature Reviews Drug Discovery*, *12*, 64–82. https://doi.org/10.1038/nrd3793

Wirthgen, E., & Hoeflich, A. (2015). Endotoxin-Induced Tryptophan Degradation along the Kynurenine Pathway: The Role of Indolamine 2,3-Dioxygenase and Aryl Hydrocarbon Receptor-Mediated Immunosuppressive Effects in Endotoxin Tolerance and Cancer and Its Implications for Immunoparalysis. *Journal of Amino Acids*, *2015*, 973548. https://doi.org/10.1155/2015/973548

Woodward, O. M., Köttgen, A., Coresh, J., Boerwinkle, E., Guggino, W. B., & Köttgen, M. (2009). Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(25), 10338–10342. https://doi.org/10.1073/pnas.0901249106

Xu, Y., Egido, E., Li-Blatter, X., Müller, R., Merino, G., Bernèche, S., & Seelig, A. (2015). Allocrite Sensing and Binding by the Breast Cancer Resistance Protein (ABCG2) and P-Glycoprotein (ABCB1). *Biochemistry*, *54*(40), 6195–6206. https://doi.org/10.1021/acs.biochem.5b00649

Figure captions

Fig. 1. Tryptophan-related metabolites included in the target LC-MS/MS method. (a) Large neutral amino acids (LNAA) competitors of Trp; (b) Kynurenine pathway and (c) Serotonin pathway. Isoleucine (Ile), leucine (Leu), tryptophan (Trp), kynurenine (KYN), kynurenic acid (KYNA), xanthurenic acid (XA) and anthranilic acid (AA), 5-hydroxyindolacetic acid (5HIAA), serotonin (5HT).

Fig. 2. Plasma concentration (A), milk concentration (B) and milk/plasma ratio (C) of KYN in Y/Y 581 and Y/S 581 cows. Samples were collected at the first routine morning milking. Results are mean; error bars indicated S.D (n=8; ***** $p<0.05$).

Fig. 3. Transepithelial transport of tested compounds (10 µM) in (A) parental MDCKII cells and (B) their murine Abcg2 transduced derivative. (○) translocation from the apical to the basolateral compartment; (●) translocation from the basolateral to the apical compartment. The experiment was started with the addition of each compound to one compartment (basolateral or apical). Aliquots of 100 µL were taken from the opposite compartment at t=1, 2, 3 and 4 h, and measured by HPLC. The fraction of each compound transported to the acceptor compartment was presented as a percentage of the total amount of compound added to the donor compartment at the beginning of the experiment. Results are means, error bars indicated SD (n=3-8). Ratio represents the relative transport ratio (i.e. the apical directed translocation divided by the basolateral directed translocation) at t=4h.

Fig. 4. Transepithelial transport of KYN (10 µM) in polarized MDCKII parental (non-transduced), MDCKII-S581-ABCG2, and MDCKII-Y581-ABCG2 monolayers. The experimental was started (t=0) by replacing the medium in either the apical or basolateral compartment with fresh Hanks supplemented medium containing 10 µM KYN. Aliquots of 100 µL were taken from the opposite compartment at t=1, 2, 3 and 4 h, and measured by HPLC. The fraction of KYN transported to the acceptor compartment was presented as a percentage of the total amount of KYN added to the donor compartment at the beginning of the experiment. Results are means, error bars indicated SD (n=5). Ratio represents the relative transport ratio (i.e. the apical directed translocation divided by the basolateral directed translocation) at t=4h. (○) translocation from the apical to the basolateral compartment; (●) translocation from the basolateral to the apical compartment.

611 Table 1. Levels of Trp-related compounds (ng/mL) in plasma and milk samples from wild-
612 type and Abcg2$^{-/-}$ female mice (n=9-12). Milk to plasma ratio is also represented.

		Wild-type	Abcg2$^{-/-}$	p-value
Plasma	Leu	24400 ± 7229	20937 ± 7925	0.292
	Ile	25209 ± 7670	21507 ± 8275	0.285
	Trp	88660 ± 11302	89831 ± 11302	0.911
	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	Leu	497 ± 238	386 ± 282	0.292
	Ile	1645 ± 515	2051 ± 684	0.250
	Trp	< LOD	<LOD	-
	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	Leu	0.02 ± 0.01	0.08 ± 0.18	0.268
	Ile	0.08 ± 0.02	0.53 ± 1.35	0.265
	Trp	< LOD	< LOD	-
	KYN	0.02 ± 0.01	0.01 ± 0.00	0.012*
	KYNA	6.9 ± 2.6	1.47 ± 0.51	< 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 ± 0.00	0.004 ± 0.00	0.794
	5HIAA	0.17 ± 0.04	0.15 ± 0.05	0.322

613 Results are expressed as means of concentration (ng/mL) ± S.D. * p<0.05 versus wild-type

614

615 Table 2. Levels of Trp-related compounds (ng/mL) in plasma and milk samples from non-
616 carrier (Y/Y) and carrier (Y/S 581) cows (n=4). Milk to plasma ratio is also represented.

		Y/Y	Y/S	p-value
Plasma	Leu	38067 ± 9127	39692 ± 10952	0.751
	Ile	13559 ± 3439	13315 ± 3283	0.886
	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
	5-HT	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	Leu	432 ± 184	569 ± 291	0.280
	Ile	342 ± 148	441 ± 180	0.248
	Tyr	41 ± 11	64 ± 31	0.071
	Trp	192 ± 74	252 ± 90	0.170
	KYN	2.4 ± 1.0	4.6 ± 1.8	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	Leu	0.01 ± 0.00	0.01 ± 0.00	0.361
	Ile	0.03 ± 0.01	0.03 ± 0.01	0.174
	Tyr	0.009 ± 0.004	0.013 ± 0.006	0.152
	Trp	0.018 ± 0.008	0.020 ± 0.007	0.492
	KYN	0.002 ± 0.001	0.004 ± 0.002	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

617 Results are expressed as means of concentration (ng/mL) ± S.D. * p<0.05 vs wild-type

618

Figure1.ppt
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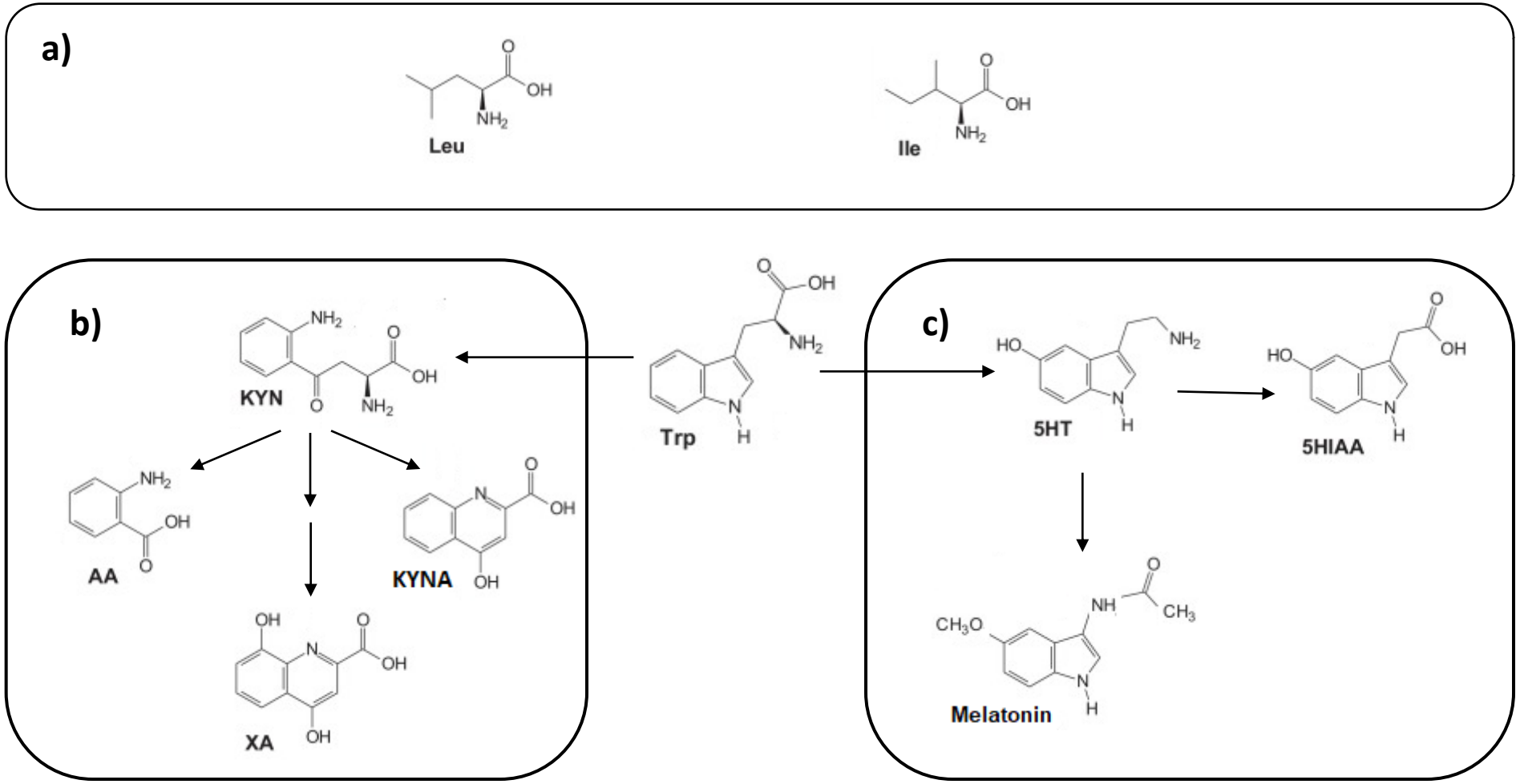

Fig. 1 Garcia-Lino et al.

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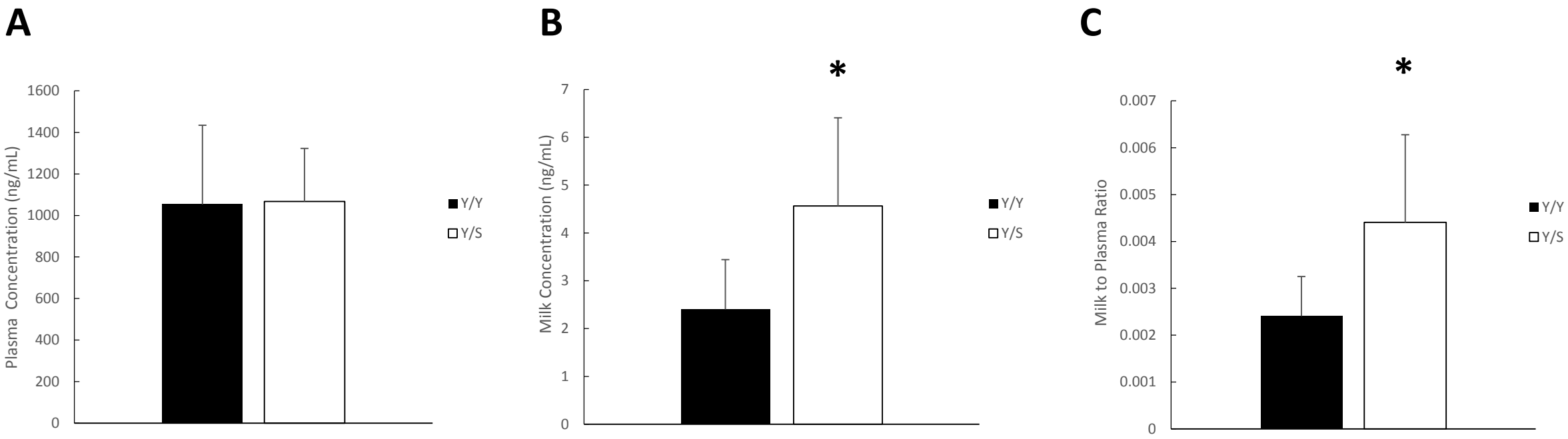

Fig. 2 Garcia-Lino et al.

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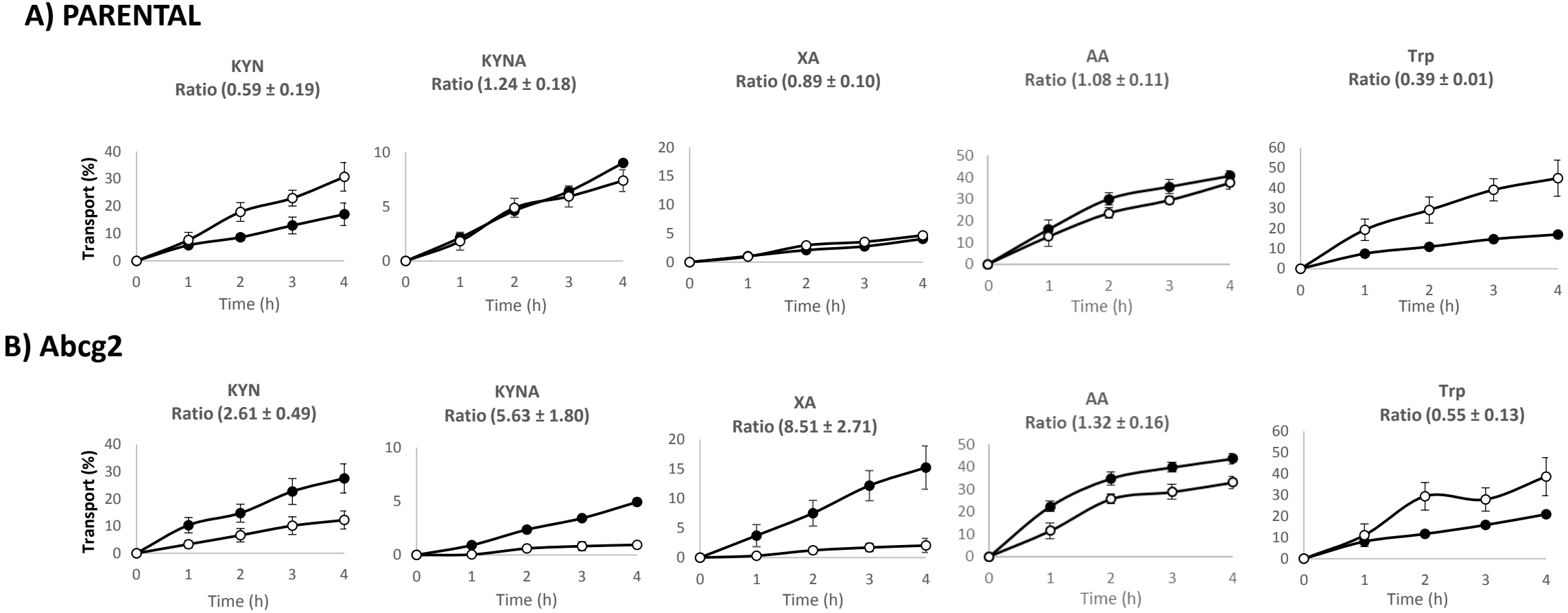

Fig. 3 Garcia-Lino et al.

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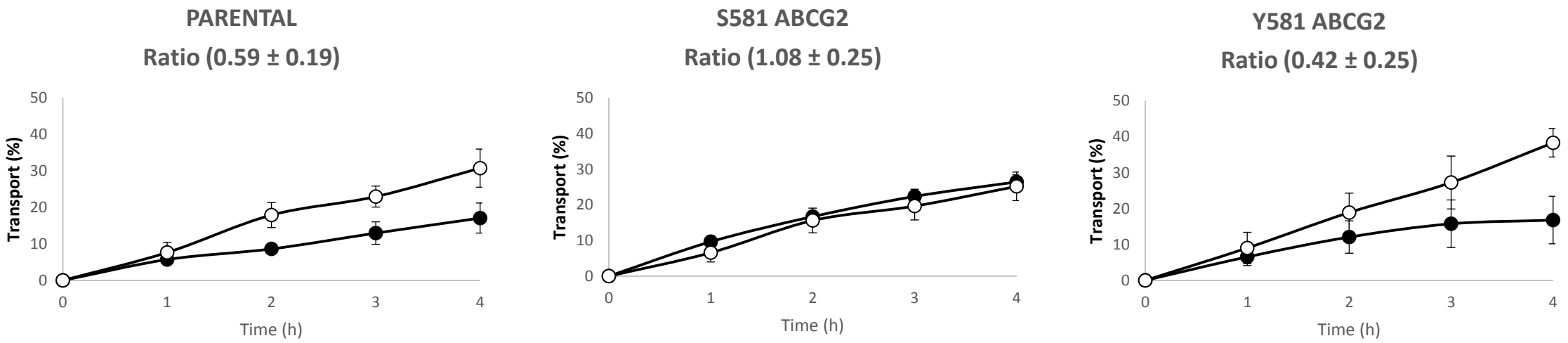

Fig. 4 Garcia-Lino et al.

Supplementary material

Table S1. Hydrogen bond acceptors (HBA) count and hydrogen bond donors (HBD) count for Trp metabolites detected in mouse samples. Data were obtained from U.S. National Library of Medicine (PubChem).

	Hydrogen bond acceptors (HBA) count	Hydrogen bond donors (HBD) count
KYNA	5	3
XA	5	3
KYN	4	2
AA	3	2
Trp	3	3
5HT	2	3
5HIAA	2	2

Graphical_Abstract.pdf
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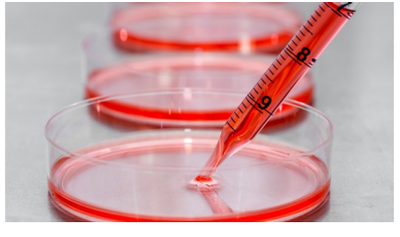
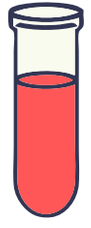
Wild-type and Abcg2-/-

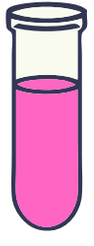
Y581Y and Y581S ABCG2



MDCKII transduced cells



Plasma and Milk Samples



Cell Culture Samples

LC-MS/MS

HPLC

Interactions between ABCG2 and Tryptophan-related compounds

HIGHLIGHTS

LC-MS/MS analysis of tryptophan metabolome in plasma and milk shows ABCG2 interaction

Abcg2 mediates milk secretion of metabolites of the kynurenine pathway in mice

Bovine ABCG2 Y581S polymorphism increases kynurenine concentration in milk

Kynurenine and kynurenic and anthranilic acid are *in vitro* substrates of murine Abcg2

The bovine ABCG2 Y581S polymorphism affects the *in vitro* transport of kynurenine

***Declaration of Interest Statement**

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: