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ABCG2 transporter plays a key role in the biodistribution of melatonin and its main metabolites

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Abstract

The ATP-binding cassette G2 (ABCG2) is an efflux transporter expressed in the apical membrane of cells from a large number of tissues, directly affecting bioavailability, tissue accumulation, and secretion into milk of both xenobiotics and endogenous compounds. The aim of this work was to characterize the role of ABCG2 in the systemic distribution and secretion into milk of melatonin and its main metabolites, 6-hydroxymelatonin, and 6-sulfatoxymelatonin. For this purpose, we first showed that these three molecules are transported by this transporter using in vitro transepithelial assays with MDCK-II polarized cells transduced with different species variants of ABCG2. Second, we tested the in vivo effect of murine Abcg2 in the systemic distribution of melatonin and its metabolites using wild-type and Abcg2$^{-/-}$ mice. Our results show that after oral administration of melatonin, the plasma concentration of melatonin metabolites in Abcg2$^{-/-}$ mice was between 1.5 and 6-fold higher compared to the wild-type mice. We also evaluated in these animals differences in tissue accumulation of melatonin metabolites. The most relevant differences between both types of mice were found for small intestine and kidney (>sixfold increase for 6-sulfatoxymelatonin in Abcg2$^{-/-}$ mice). Finally, melatonin secretion into milk was also affected by the murine Abcg2 transporter, with a twofold higher milk concentration in wild-type compared with Abcg2$^{-/-}$ lactating female mice. In addition, melatonin metabolites showed a higher milk-to-plasma ratio in wild-type mice. Overall, our results show that the ABCG2 transporter plays a critical role in the biodistribution of melatonin and its main metabolites, thereby potentially affecting their biological and therapeutic activity.

KEYWORDS
ATP-binding cassette transporters, biological transport, lactation, melatonin, mice, tissue distribution

Laura Álvarez-Fernández and Alex Gomez-Gomez should be considered joint first authors of this study.

Oscar J. Pozo and Gracia Merino should be considered joint senior authors of this study.

1 | INTRODUCTION

Melatonin is a neurohormone centrally produced by the pineal gland at night from tryptophan via serotonin, but local production is also found in several other tissues.[1] This molecule is involved in the regulation of circadian rhythms[2,3] and participates in a variety of other physiological processes,[4] although some of its claimed biological actions remain controversial.[5] Anyway, exogenous melatonin is widely used for multiple therapeutic purposes.

After oral supplementation, melatonin reaches the gastrointestinal tract and is absorbed into the portal circulation to be taken up by the liver. Once there, melatonin is first hydroxylated by cytochromes P450 to 6-hydroxymelatonin (6-OH-Mel)[6,7] and other hydroxylated metabolites such as 4-hydroxymelatonin (4-OH-Mel) and 2-hydroxymelatonin (2-OH-Mel).[8] After this Phase I metabolism, 6-OH-Mel is further metabolized to form sulfate or glucuronide conjugates, with 6-sulfatoxymelatonin (6-SO-Mel) as the main Phase II metabolite (Figure 1).[9] In addition, the hydroxylated metabolites are also formed in the small intestine[6,9] and further absorbed.

The small size and the amphiphilic nature of melatonin allows it easily to interact with membranes and to cross them by a passive diffusion mechanism. However, its interaction with active membrane transporters, including ATP-binding cassette (ABC), remains unexplored. ABCG2 represents the major route for active secretion of toxins, drugs, vitamins, and natural compounds into milk[10] and is also expressed in the apical membrane of cells from important organs for xenobiotic disposition such as the liver, intestine, kidney, and blood–brain barrier, among others.[11] It is involved in modulation of absorption, distribution, and excretion of drugs and environmental chemicals as well as endogenous and dietary compounds,[12] affecting their tissue distribution.[13,14] Interactions between the ABCG2 efflux transporter and some tryptophan-related metabolites from the kynurenine pathway have already been described by our group using targeted metabolomic analysis.[15] Our previous findings support the role of ABCG2 in the secretion into milk of tryptophan, kynurenic acid, kynurenine, anthranilic acid, and xanthurenic acid, but melatonin metabolites were not considered and only endogenous levels were analyzed.[15]

This study aimed to evaluate the role of ABCG2 in tissue distribution and secretion into milk of melatonin and its main metabolites, 6-OH-Mel, and 6-SO-Mel using wild-type and Abcg2$^{-/-}$ mice exogenously administered with melatonin. In vitro transport studies with cells overexpressing the transporter were used to correlate in vitro and in vivo results and to check the interaction of these molecules with different species variants of ABCG2.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Reference standards for melatonin as well as the buffer 4-(2-hydroxyethyl)−1-piperazineethanesulphonic acid (HEPES) and Lucifer Yellow were purchased from Sigma-Aldrich. 6-OH-Mel and 6-SO-Mel were supplied by Carbosynth and 4-OH-Mel and 2-OH-Mel from Toronto Research Chemicals. Ko143 was acquired from Tocris, isoflurane (Isovet®) from Braun VetCare, and oxytocin (Facilpart®) from Syva.

2.2 | Cell cultures

The polarized Madin-Darby canine kidney epithelial cells (MDCK-II, parental) and their murine Abcg2 and human ABCG2-transduced subclones were provided by Dr. A. H. Schinkel (Netherlands Cancer Institute). Transduced MDCK-II cells with the ovine and bovine variants of ABCG2 were previously generated by our research group.[16,17] Cells were cultured as described previously.[16,17]

2.3 | In vitro transport studies

Transport assays were carried out as described previously[18,19] with minor modifications, using Hanks' balanced salt solution (Sigma-Aldrich) supplemented with

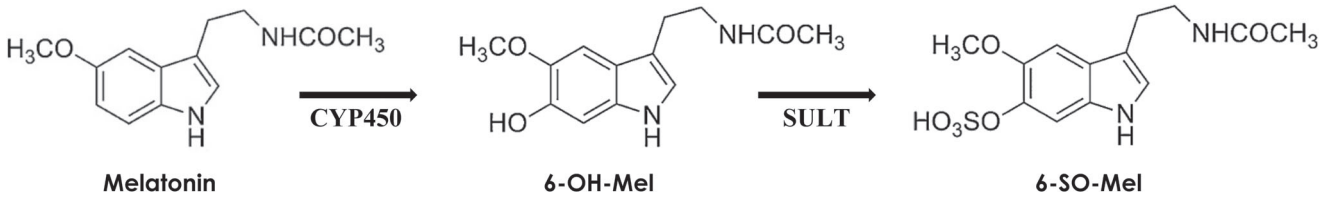

FIGURE 1 Chemical structures of melatonin and its main metabolites, 6-OH-Mel, and 6-SO-Mel. SULT: sulfotransferase.

HEPES (25 mM). Concentration of the studied molecules was determined by HPLC as described below. Active transport across monolayers was expressed as the relative efflux transport ratio, referring to the initial concentration and defined as the apically directed transport percentage divided by the basolaterally directed translocation percentage after 4 h. Transport proficiency was simultaneously checked by testing a typical ABCG2 substrate (danofloxacin 10 μM) (Supporting Information: Table S1).[20]

2.4 | Animal experiments

Animals were handled according to institutional and ARRIVE guidelines complying with European legislation (2010/63/EU). Experimental procedures were approved by the Animal Care and Use Committee of the University of León (ULE_011_2016). Animals used were Abcg2$^{-/-}$ and wild-type mice, all of >99% FVB/N genetic background, generated and kindly supplied by Dr. A. H. Schinkel.

Plasma and tissue distribution assays and milk secretion experiments were performed as described previously[18] in the morning. Melatonin solution was prepared by dissolving the powdered commercial compound in 10% (v/v) ethanol, 40% (v/v) polyethylene glycol, and 50% (v/v) saline and was orally administered to male mice for plasma and tissue distribution analysis, and intravenously to female mice for milk secretion assays.

2.5 | Quantification of melatonin and its metabolites from the in vitro studies by HPLC

HPLC analysis was used to determine the concentration of the tested compounds in transport assays based on previous work.[15] Mobile phase consisted of acetonitrile: orthophosphoric acid 30 mM, pH 4.8 (30:70, v/v) with a flow rate of 0.8 ml/min, and UV absorbance measured at 310 nm. Quantification was performed by comparison with standard samples. The limit of quantification (LOQ) was in the range of 0.01–0.03 μg/ml for all compounds.

2.6 | Preparation of samples from the in vivo studies for LC-MS/MS analysis

Plasma (70 μl) and milk samples (63–180 mg) were mixed with 300 μl acetonitrile. After centrifugation, 50 μl of melatonin-d4 (50 ng/ml) was added to the supernatant. The mixture was evaporated, reconstituted with 150 μl water and 10 μl was injected into the LC-MS/MS system.

Tissue samples were homogenized in a mixture of 400 μl of formic acid at 0.1% (v/v) and 800 μl of acetonitrile per 50 mg of organ. The homogenate was centrifuged and 20 μl of the supernatant was mixed with 25 μl of melatonin-d4 (50 ng/ml) and 100 μl of water, and was directly injected into the LC-MS/MS system.

2.7 | Quantification of melatonin and its metabolites from the in vivo studies by LC-MS/MS

LC-MS/MS was used for the analysis (Waters Associates). Chromatographic separation was achieved on an Acquity BEH C18 column (Waters Associates) at a flow rate of 0.3 ml/min and water and methanol as mobile phases (both with ammonium formate [1 mM] and formic acid [0.01% v/v]). Based on previous work carried out by our group,[21] a gradient program was used for analyte separation. Analytes were determined in the selected reaction monitoring mode including two ion transitions for each analyte: melatonin (233 → 159, 233 → 174), 6-OH-Mel (249 → 158, 249 → 190), and 6-SO-Mel (329 → 190, 329 → 249). The presence of additional hydroxylated metabolites was confirmed by acquiring the transitions 249 → 158 and 249 → 190 for 4-OH-Mel and 249 → 162 and 249 → 189 for 2-OH-Mel. Instrumental LOQs of 0.05, 0.3, and 0.5 ng/ml were obtained for melatonin, 6-SO-Mel, and 6-OH-Mel, respectively.

2.8 | Statistical analysis

Comparisons were made using the two-tailed unpaired Student's *t*-test for normally distributed variables and the nonparametric Mann–Whitney *U*-test for not-normally distributed data. All analyses were carried out on the assumed significance level of $p \leq .05$ using SPSS Statistics software v. 26 (IBM). The results are shown as mean ± standard deviation (SD).

3 | RESULTS

3.1 | In vitro transport of melatonin and its metabolites

In the MDCK-II parental cell line (without ABCG2 transduction), melatonin, 6-OH-Mel, and 6-SO-Mel showed a similar transport pattern with equal vectorial

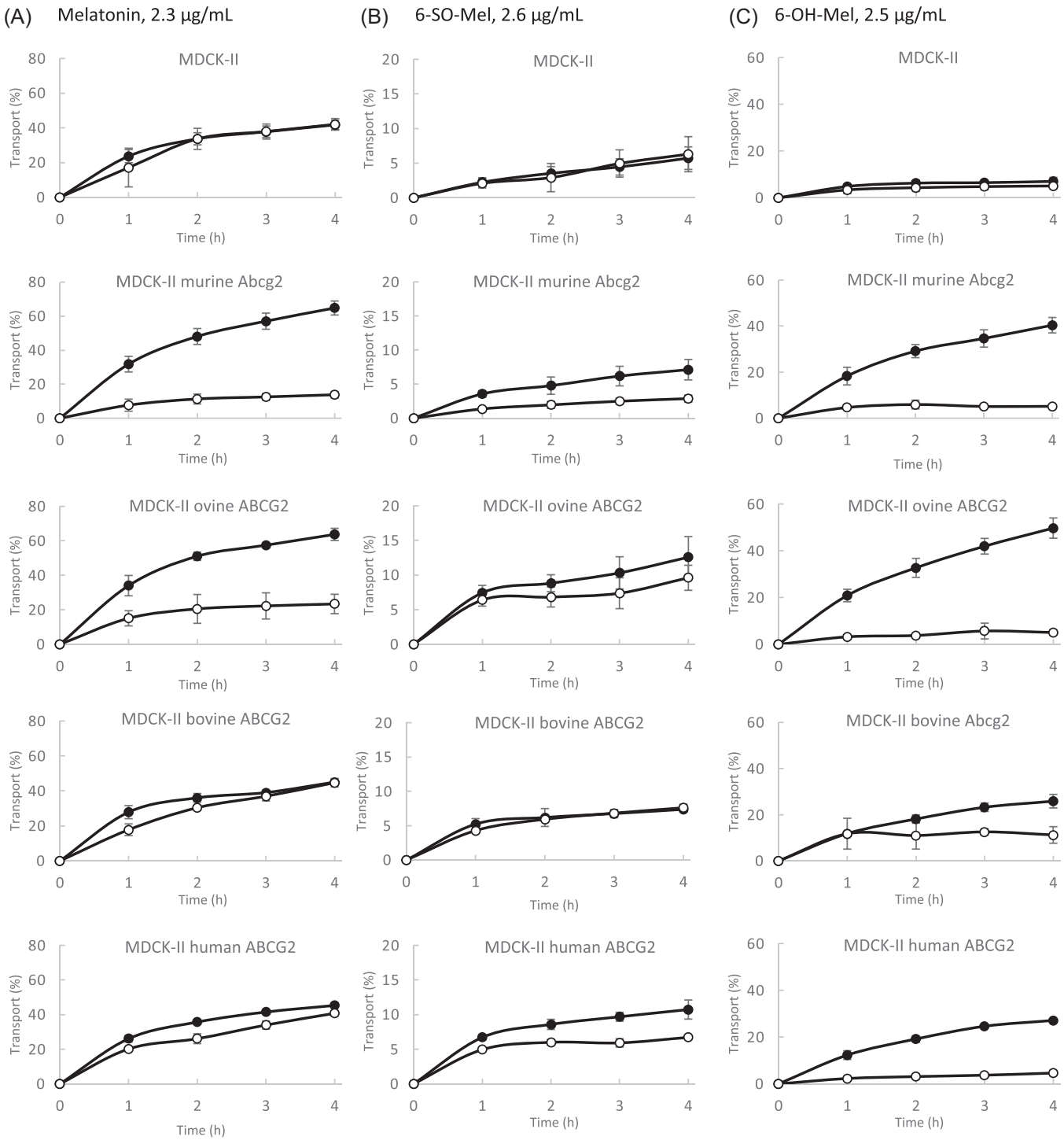

FIGURE 2 Transepithelial transport assay of melatonin at 10 µM (A), 6-SO-Mel at 8 µM (B), and 6-OH-Mel at 10 µM (C) in parental MDCK-II cells and its subclones transduced with murine Abcg2 and ovine, human and bovine ABCG2 transporter. The experiment started when the medium in both sides was replaced with fresh transport solution, with or without the specific ABCG2 inhibitor Ko143 (1 µM) and containing or not the potential substrates melatonin (10 µM), 6-OH-Mel (10 µM), and 6-SO-Mel (8 µM). Aliquots of 100 µl were collected from the opposite compartment where melatonin or its metabolites were added at 1, 2, 3, and 4 h and measured by HPLC. Active transport across MDCK-II monolayers was presented as a percentage of total concentration added to the donor compartment at the beginning of the experiment. Results are presented as mean ± SD. (●) basolateral to apical translocation; (○) apical to basolateral translocation. $n \geq 3$.

translocation (Figure 2). However, differences were observed when comparing cells transduced with diverse ABCG2 species variants with the parental line.

For melatonin (Figure 2A), basal to apical translocation highly increased and apical to basal translocation decreased in the murine Abcg2-transduced cells and, to a lesser extent, in the ovine ABCG2-transduced cells, with relative efflux transport ratios significantly higher than in the parental cells (Table 1). When cells with the human and bovine ABCG2 transporter were used, a low transport ratio similar to that obtained for the parental cells was given. These results demonstrate an efficient in vitro transport of melatonin by murine Abcg2, a more moderate transport by ovine ABCG2, and a lack of transport by human and bovine ABCG2 variants.

With regard to 6-SO-Mel (Figure 2B), significant differences in the transport ratio were observed only in the murine Abcg2 and human ABCG2-transduced subclones related to the parental line, indicating that this metabolite is a good in vitro substrate for murine and human variants.

In the case of 6-OH-Mel (Figure 2C, Table 1), a highly efficient apical transport with relative transport ratios significantly higher in all the cells transduced with the different species variants of ABCG2 compared to parental cells was obtained, showing that the 6-hydroxylated form is in vitro transported by all tested species variants.

The specificity of ABCG2-mediated transport was checked using Ko143,[22] a widely known inhibitor of ABCG2. In the cases of compound substrates of ABCG2, relative efflux transport ratios were reduced to values equal to parental cells in the ABCG2-transduced cells with Ko143 (Table 1).

3.2 | Plasma and tissue distribution assays in Abcg2$^{-/-}$ and wild-type mice

Plasma and tissue levels were analyzed in male wild-type and Abcg2$^{-/-}$ mice after oral administration of melatonin.

Melatonin was detected in plasma as well as in all tissues studied (Figure 3A). No significant differences were found in plasma concentration between both types of mice (16.47 ± 9.86 vs. 9.39 ± 4.59 ng/ml in wild-type and Abcg2$^{-/-}$ mice, respectively; $p = .1$). High melatonin concentrations were observed in small intestine and liver whereas it was almost absent in small intestinal content. No significant differences were observed between both types of mice.

Similarly to melatonin, 6-SO-Mel was also detected in plasma and in all studied tissues (Figure 3B). Concentrations were sixfold lower in plasma from wild-type compared with Abcg2$^{-/-}$ mice (3.39 ± 1.11 vs. 21.93 ± 10.71 ng/ml; $p < .001$). Regarding tissues, the main differences were observed in small intestine, small intestinal content, kidney, and testis. Although an almost eightfold lower concentration for 6-SO-Mel was observed in the small intestinal content in the Abcg2$^{-/-}$ male mice compared with wild-type (1.24 ± 0.23 vs. 10.02 ± 3.50 μg/g, respectively; $p = .005$), levels in small intestine were almost sixfold higher in Abcg2$^{-/-}$ male mice (1.27 ± 0.65 vs. 0.23 ± 0.11 μg/g, respectively; $p < .001$). Concentrations were also higher in the kidney (almost sixfold, $p < .001$) and the testis (almost threefold, $p = .006$) of Abcg2$^{-/-}$ mice compared with their wild-type counterpart.

Analytical interferences precluded the detection of 6-OH-Mel in all the matrices evaluated. Instead, two other isomers with exactly the same mass and

TABLE 1 Relative efflux transport ratio, defined as basal to apical translocation percentage divided by apical to basal translocation percentage, at 4 h, for melatonin and its main metabolites (tested at concentrations in the pharmacological range) in MDCK-II cells (parental) transduced with the murine (mAbcg2), ovine (oABCG2), bovine (bABCG2), and human (hABCG2) variants of ABCG2, both in the presence (+Ko143) and absence (−Ko143) of the specific inhibitor of ABCG2, Ko143 (1 μM) ($n \geq 3$).

	Mel (10 μM, 2.3 μg/ml)		6-SO-Mel (8 μM, 2.6 μg/ml)		6-OH-Mel (10 μM, 2.5 μg/ml)	
	−Ko143	+Ko143	−Ko143	+Ko143	−Ko143	+Ko143
Parental	0.99 ± 0.07	1.05 ± 0.06	0.96 ± 0.22	0.97 ± 0.01	1.42 ± 0.35	1.30 ± 0.22
mAbcg2	4.73 ± 0.40*	1.00 ± 0.06**	2.47 ± 0.19*	1.24 ± 0.13**	7.83 ± 0.56*	0.94 ± 0.29**
oABCG2	2.80 ± 0.45*	0.99 ± 0.11**	1.30 ± 0.15	1.02 ± 0.08	10.9 ± 2.66*	1.60 ± 0.13**
bABCG2	1.01 ± 0.05	0.98 ± 0.03	0.97 ± 0.05	0.96 ± 0.04	2.15 ± 0.60*	1.22 ± 0.20**
hABCG2	1.11 ± 0.04	1.02 ± 0.07	1.60 ± 0.24*	1.13 ± 0.08**	6.63 ± 0.75*	1.44 ± 0.20**

Note: Results are presented as mean ± SD. Stock solution of Ko143 (10 mM) was prepared in DMSO.

*$p \leq .05$, significant differences compared to parental MDCK-II cells (Student's *t*-test, normally distributed data); **$p \leq .05$, significant differences compared to treatment without Ko143 (Student's *t*-test, normally distributed data).

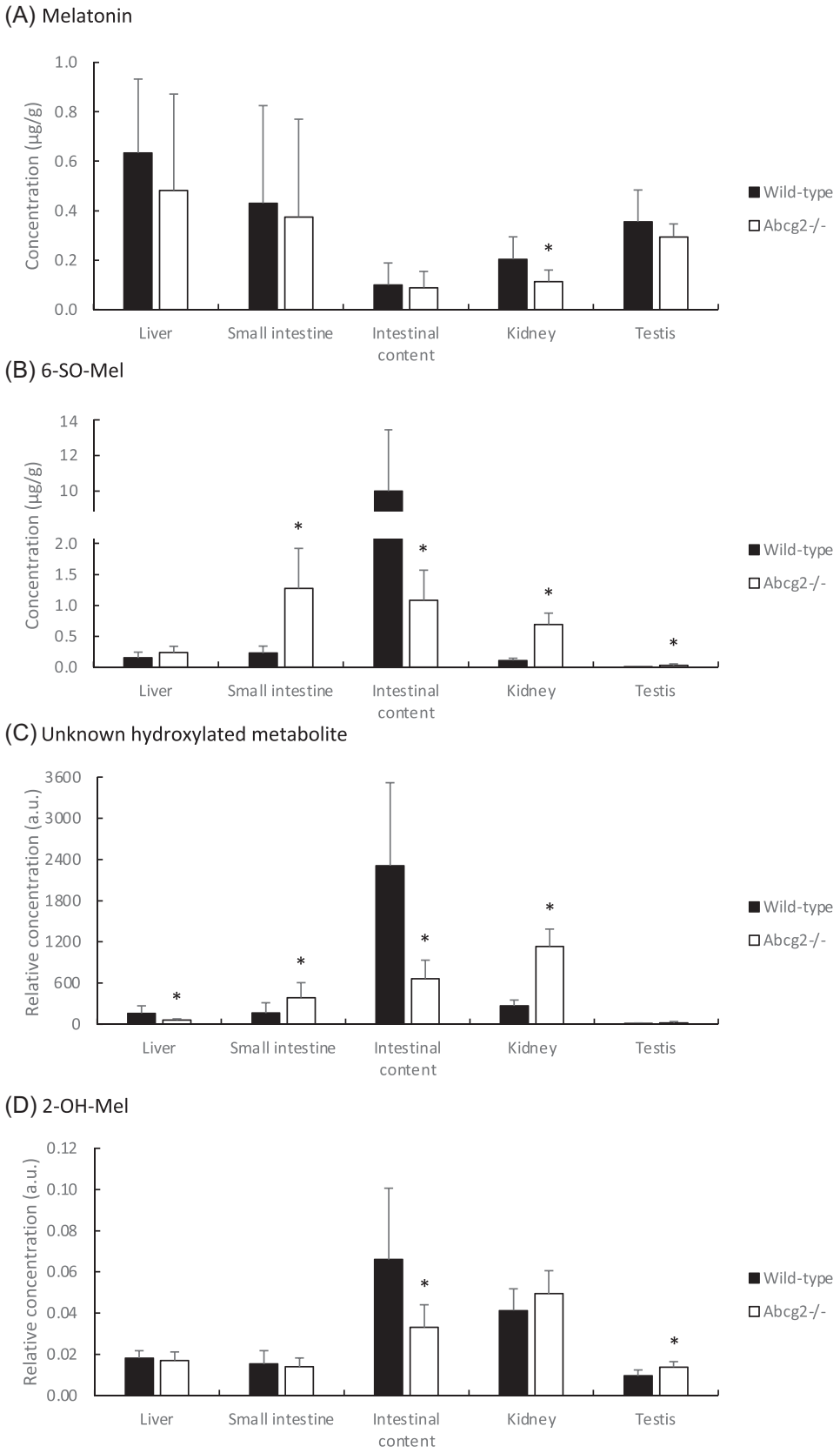

FIGURE 3 Tissue concentration of melatonin (A), 6-SO-Mel (B), and relative abundance of two hydroxylated metabolites (unknown hydroxylated metabolite (C) and 2-OH-Mel (D)) 1 h after oral administration of melatonin (10 mg/kg body weight) to wild-type and Abcg2$^{-/-}$ male mice. Quantification was performed by LC-MS/MS. Data are presented as means ± SDs. a.u.: arbitrary units. *$p < .05$, significant differences versus wild-type mice ($n = 8$) (Student's *t*-test, normally distributed data; Mann–Whitney *U*-test, not normally distributed data; Supporting Information: Table S2)

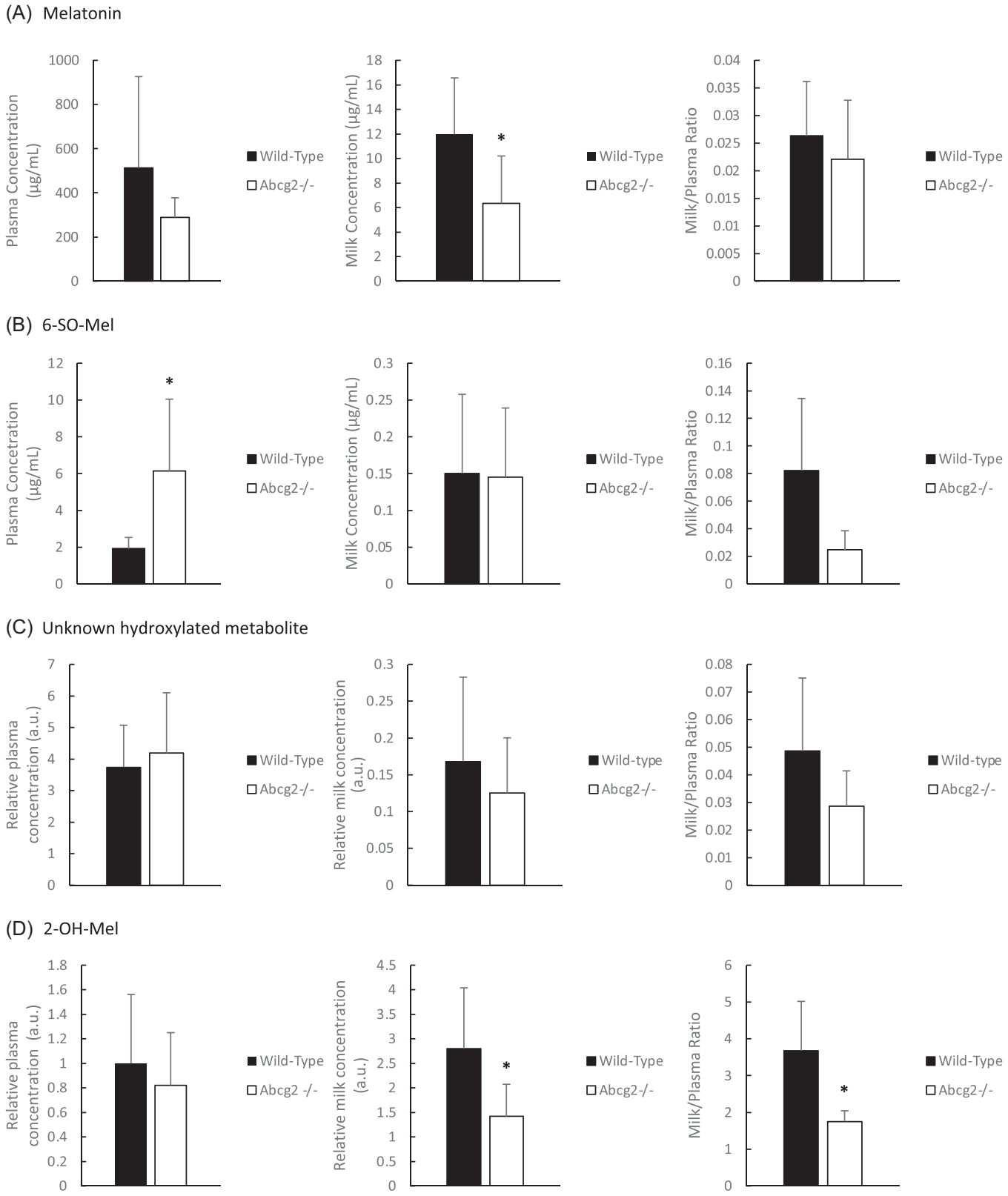

FIGURE 4 Plasma and milk concentration and milk-to-plasma ratio of melatonin (A), 6-SO-Mel (B), and relative abundance of two hydroxylated metabolites (unknown hydroxylated metabolite (C) and 2-OH-Mel (D)) in wild-type and Abcg2$^{-/-}$ female mice after i.v. administration of melatonin at a dose of 10 mg/kg body weight. Plasma and milk samples were collected 30 min after i.v. administration and concentrations were determined by LC-MS/MS. Results are presented as means ± SDs. a.u.: arbitrary units. *$p < .05$, significant differences between both groups of mice ($n = 4$–8) (Student's *t*-test, normally distributed data; Mann–Whitney *U*-test, not normally distributed data; Supporting Information: Table S2).

fragmentation patterns, although with different retention times (one at 3.7 min and the other at 5.6 min), were found, suggesting that they were probably melatonin metabolites hydroxylated in another position. By comparison with the reference material, we confirmed that the late-eluting isomer was 2-OH-Mel. The early eluting one was not associated with any commercially available hydroxylated metabolite. This unknown metabolite shared the fragmentation pattern with both 4-OH-Mel and 6-OH-Mel (Supporting Information: Figure S1) suggesting that the hydroxyl group is present in the indole ring, being 7-OH-Mel the most feasible structure. This hydroxylated metabolite showed the most relevant differences between both types of mice, as shown in Figure 3C. Plasma levels of this hydroxylated metabolite were 1.5-fold lower in samples from wild-type compared with Abcg2$^{-/-}$ mice (310.76 ± 133.64 vs. 463.73 ± 138.61 arbitrary units; respectively; $p = .016$). Regarding tissues, levels of this metabolite were higher in the small intestine of the Abcg2$^{-/-}$ animals, whereas values in the small intestinal content were higher in the wild-type mice. Higher amounts were also observed in the kidney whereas lower levels were found in the liver of the Abcg2$^{-/-}$ compared with wild-type animals.

3.3 | Milk secretion assays in Abcg2$^{-/-}$ and wild-type mice

Melatonin was the predominant molecule in both plasma and milk samples 30 min after i.v. administration of melatonin (Figure 4). Similarly to the experiments performed on males (Figure 3A), no significant differences were found in plasma melatonin concentration between both types of animals (513.28 ± 413.11 μg/ml in wild-type vs. 288.24 ± 89.28 μg/ml in Abcg2$^{-/-}$) (Figure 4A). Conversely, milk concentration of melatonin was almost twofold higher in wild-type mice compared with Abcg2$^{-/-}$ lactating female (11.95 ± 4.61 vs. 6.34 ± 3.87 μg/ml, respectively; $p = .027$). However, this significant difference disappeared when the milk to plasma ratio was calculated (0.03 ± 0.01 vs. 0.02 ± 0.01, respectively) (Figure 4A) probably due to high interindividual variability in the plasma concentration.

In the case of 6-SO-Mel, similar milk concentration and milk to plasma ratios were obtained in both types of animals (Figure 4B). Again, high interindividual variability probably precludes a significant difference in the milk to plasma ratio. However, in agreement to the male assay (Figure 3B), plasma concentration was threefold lower (1.92 ± 0.61 vs. 6.14 ± 3.90 μg/ml; $p = .018$) in wild-type mice compared with the Abcg2$^{-/-}$ counterpart (Figure 4B).

Finally, there were no differences in the levels of the unknown hydroxylated metabolite in plasma and milk or in the milk to plasma ratio between wild-type and Abcg2$^{-/-}$ mice. Nevertheless, for 2-OH-Mel, milk levels and the milk to plasma ratio in wild-type mice were around twofold higher compared to Abcg2$^{-/-}$ lactating mice (2.90 ± 1.24 vs. 1.42 ± 0.66, $p = .027$, and 3.68 ± 1.34 vs. 1.75 ± 0.29 arbitrary units, $p = .007$, respectively) (Figure 4C), thus indicating that Abcg2 participates in the secretion into milk of this compound.

4 | DISCUSSION

In this study, we aimed to determine whether ABCG2 is involved in the transport and biodistribution of melatonin and its main metabolites. Our in vivo results support the fact that the murine Abcg2 transporter is involved in the systemic exposure, tissue distribution, and secretion into milk of melatonin metabolites after exogenous administration of melatonin.

Our in vitro results confirm for the first time that melatonin is efficiently transported by murine Abcg2 and ovine ABCG2 (Figure 2A). In the case of the cells transduced with the murine transporter, relative efflux transport ratios (Table 1) were in the same range than the ones previously reported for other indole-based molecules such as kynurenine and kynurenic and xanthurenic acids.[15] However, no ABCG2-mediated transport by human and bovine ABCG2 variants was observed (Figure 2A, Table 1). Since both sheep and wild mice can be considered seasonally breeding animals[23] and melatonin plays an important role in the control of seasonal reproduction, a potential role of ABCG2 in the control of the bioavailability of this hormone, especially in the breeding period of seasonal breeding animals, may be hypothesized. However, further studies are required. Our results have more important research implications. The fact that melatonin is an in vitro substrate of the murine but not of the human variant is something that has to be considered when behavioral assays are conducted in mice and are extrapolated to the human situation.

Interestingly, this species-dependent pattern was not reproduced for melatonin metabolites (Figure 2B,C). An explanation for these disparities regarding species dependency of ABCG2-mediated transport for metabolites compared to melatonin is hard to find. Biological activity of these molecules may contribute in some way since melatonin metabolites significantly support overall antioxidant and anti-inflammatory activities[9] more than

seasonal rhythms, although this second function cannot be ruled out. Potential differences in the efficiency of ABCG2 transduction between subclones cannot be discarded and our outcomes should be considered only in qualitative terms.

To elucidate whether the in vitro declared murine Abcg2-mediated transport of melatonin and its metabolites also plays a role in the vivo situation, plasma and tissue distribution studies were performed using male wild-type and Abcg2$^{-/-}$ mice 1 h after oral administration of 10 mg/kg of melatonin. This setting was selected based on previously reported preclinical studies with melatonin doses ranging from 1 to 10 mg/kg[24] and maximum tissue concentrations observed between 30 min and 1 h after melatonin administration in mice.[25] In fact, the obtained plasma levels of melatonin were within the range of the levels found in most parts of the human body after exogenous administration.[5] Both the parent compound and 6-SO-Mel were detected in plasma and in all the tissues studied (Figure 3). In contrast, 2-OH-Mel and one additional hydroxylated metabolite were detected instead of 6-OH-Mel due to analytical interference. Since previous studies reported that some hydroxylated derivative forms, including 2-OH-Mel, are effective against oxidative stress,[26] we decided not to exclude the unknown one from our analysis.

Our in vivo results show no significant differences in plasma melatonin concentration between wild-type and Abcg2$^{-/-}$ mice. This lack of an Abcg2-mediated effect in plasma levels of in vitro Abcg2 substrates has been previously reported for other compounds.[20] Several reasons may be behind this fact. On the one hand, the presence of other potential transporters such as solute carriers may interfere in the Abcg2-mediated distribution. On the other hand, the melatonin metabolism itself may help in the regulation of the melatonin content. Our results regarding metabolites show that lower plasma levels were observed in the presence of Abcg2. Although the presence of unknown compensatory mechanisms in Abcg2$^{-/-}$ mice cannot be discarded, these results suggest that Abcg2 is an important determinant of the systemic distribution for melatonin metabolites.

Melatonin was found to be widely distributed, with higher concentrations in the small intestine and liver and almost absent in small intestinal content (Figure 3A), which is in agreement with previous studies in mice.[25] However, similarly to our results in plasma, significant differences between both types of mice were not found in melatonin but rather in its main metabolites (Figure 3B–D). Thus, we found that Abcg2 altered the distribution of melatonin metabolites in important organs.

Regarding the small intestine, higher metabolite concentrations were found in the small intestine of the Abcg2$^{-/-}$ compared to wild-type mice, whereas concentrations in the small intestinal content were higher in the wild-type mice. This indicates that Abcg2 is probably affecting intestinal absorption of metabolites formed in the intestine and/or intestinal excretion of mentioned metabolites formed in the liver after melatonin absorption, with lower levels in the small intestine (tissue) and higher in the small intestinal content of wild-type animals. Moreover, Abcg2 contribution to enterohepatic circulation of these compounds by hepatobiliary excretion cannot be ruled out since higher accumulation in the liver of a hydroxylated metabolite was determined in wild-type mice compared with their knockout counterpart. This is consistent with the higher intestinal luminal content of metabolites in wild-type compared with knockout animals.

In the kidney, Abcg2$^{-/-}$ mice showed a higher accumulation of metabolites than wild-type, which suggests a lower renal elimination of these metabolites and their accumulation in the kidney of knockout animals. Particularly, these results reveal a potential role of Abcg2 in renal excretion of the sulfate metabolite, this being the main route of elimination for this compound.[9] This correlates with the fact that Abcg2 is involved in the urinary excretion of other sulfate derivatives.[27–29] This finding may have important implications for human clinical practice since our results show that 6-SO-Mel is a human in vitro ABCG2 substrate and is used as a urinary marker of melatonin production.[30] Thus, any alteration in ABCG2 expression in the kidney (chemical inhibition, ABCG2 polymorphism) may affect its renal excretion and may lead to misinterpretations of melatonin production.

Our results obtained from testis are also relevant since an almost threefold higher accumulation of 6-SO-Mel was observed in Abcg2$^{-/-}$ mice compared with the wild-type counterpart. These results demonstrate that Abcg2 participates in the transfer of this sulfate metabolite through the blood–testis membrane, as it has also been shown for other natural compounds.[31]

To elucidate the role of Abcg2 in the secretion of melatonin and its metabolites into milk, milk secretion assays with Abcg2$^{-/-}$ and wild-type female mice were performed. Milk concentration of melatonin was almost twofold higher in wild-type compared with Abcg2$^{-/-}$ mice whereas 2-OH-Mel showed a milk to plasma ratio higher in the wild-type compared with Abcg2$^{-/-}$ mice (Figure 4). These results indicate that Abcg2 affects the transfer into milk of melatonin and some of its metabolites and are consistent with other studies which demonstrated that

ABCG2 is involved in the milk secretion of different tryptophan metabolites.[15]

Melatonin concentration in milk has relevant biological implications since this molecule contributes to the development of the gut microbiome.[32] Our findings are of physiological relevance since biological rhythms are not acquired in the first stages of life after birth and pineal melatonin production increases during gestation and breastfeeding.[33] ABCG2 may be involved in the transfer of circadian and seasonal timing from mother to offspring, preparing its neuroendocrine system for the new environment, although the involvement of other factors cannot be excluded. Likewise, this transporter could affect the presence of melatonin derivatives in livestock milk, at least in ovine milk considering the in vitro ovine ABCG2-mediated transport of melatonin and some of its derivatives (Figure 2). Inhibition of ABCG2-mediated transport of other ABCG2 substrates by melatonin derivatives is not very likely since preliminary in vitro assays suggest that ABCG2 is not inhibited at concentrations below 200 μM (unpublished results).

From all the above, we can conclude that Abcg2 affects systemic and tissue distribution of melatonin metabolites, the most influenced organs being the small intestine and the kidney, with higher amounts in Abcg2$^{-/-}$ mice. Therefore, changes in Abcg2 expression or activity may alter tissue concentration of melatonin derivatives and affect their biological and therapeutic effects in different target organs, which could have important health consequences.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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