Check for updates

Received: 1 October 2021 | Revised: 18 February 2022 | Accepted: 28 February 2022

DOI: 10.1111/jvp.13050

ORIGINAL ARTICLE

JOURNAL OF Veterinary Pharmacology and Therapeutics WILEY

Urinary cortisol metabolites are reduced in MDR1 mutant dogs in a pilot targeted GC-MS urinary steroid hormone metabolome analysis

Irina Gramer[1] | Emre Karakus[1] | Michaela F. Hartmann[2] | Stefan A. Wudy[2] | Natali Bauer[3] | Andreas Moritz[3] | Zekeriya Aktürk[4] | Joachim Geyer[1]

[1]Institute of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Justus Liebig University Giessen, Giessen, Germany

[2]Steroid Research & Mass Spectrometry Unit, Pediatric Endocrinology & Diabetology, Center of Child and Adolescent Medicine, Justus Liebig University Giessen, Giessen, Germany

[3]Small Animal Clinic, Internal Medicine and Department of Veterinary Clinical Sciences, Clinical Pathophysiology and Clinical Pathology, Faculty of Veterinary Medicine, Justus Liebig University Giessen, Giessen, Germany

[4]Institute of Medicine, Technical University Munich, Munich, Germany

Correspondence

Joachim Geyer, Institute of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Justus Liebig University Giessen, Giessen, Germany.
Email: Joachim.M.Geyer@vetmed.uni-giessen.de

Funding information

This study was supported in part by grants to E.K. from the Scholar Rescue Fund and by the Philipp Schwartz-Initiative of the Alexander von Humboldt-Stiftung. Funds for open access publication are received from Justus Liebig University Giessen.

Abstract

P-glycoprotein (P-gp) is the gene product of the multidrug resistance gene (MDR1, syn. ABCB1) that normally restricts the transfer of cortisol across the blood–brain barrier. In the absence of P-gp, cortisol access to the hypothalamus is increased and, by feedback inhibition, this finally leads to lower endogenous plasma cortisol levels in dogs with homozygous nt230(del4) MDR1 mutation (MDR1$^{-/-}$ mutant dogs). While a previous study only focused on plasma cortisol levels, the present study used urinary steroid hormone metabolites to analyze cortisol metabolism in MDR1$^{-/-}$ mutant dogs. Morning void urine was collected from 23 MDR1$^{-/-}$ mutant and 16 MDR1$^{+/+}$ normal dogs and was subjected to targeted GC-MS steroid hormone metabolome analysis. Seven cortisol metabolites, cortisol itself, and 13 other steroid metabolites were detected. In general, all cortisol metabolites were lower in the urine of the MDR1$^{-/-}$ mutant dogs, with allo-tetrahydro-cortisol and β-cortol reaching the level of significance. In addition, 11-keto-pregnanetriol levels were significantly lower in the urine of the MDR1$^{-/-}$ mutant dogs, indicating that also the 17alpha-OH-progesterone-derived metabolism was altered. In conclusion, the present study provides the first steroid hormone metabolome analysis in the urine of MDR1$^{-/-}$ mutant dogs. Significant differences in the steroid metabolome of MDR1$^{-/-}$ mutant dogs point to a significant role of P-gp for cortisol metabolism and excretion and so indirectly also for hypothalamic–pituitary–adrenal axis regulation in dogs.

KEYWORDS
collie, cortisol, dog, MDR1 mutation, metabolomics, steroid, urine

1 | INTRODUCTION

P-glycoprotein (P-gp) is a multidrug efflux carrier and is encoded by the multidrug resistance gene *MDR1*, also referred to as ATP-binding cassette transporter gene *ABCB1*. Apart from many drugs and other xenobiotics, P-gp transports endogenous steroids such as cortisol (Uhr et al., 2002). The carrier is expressed in organs with excretory function such as liver and kidney and at blood–tissue barriers such as the blood–brain barrier (Schinkel, 1998). Here, P-glycoprotein restricts compound entry into the brain. Indeed, experiments in mice deficient for both murine *mdr1a* and *mdr1b* P-gps (*mdr1a/1b*$^{-/-}$ knockout mice) showed significantly higher

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2022 The Authors. *Journal of Veterinary Pharmacology and Therapeutics* published by John Wiley & Sons Ltd.

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

brain penetration of [^{3}H]cortisol compared with wild-type controls (Mason et al., 2008; Uhr et al., 2002). Glucocorticoid actions particularly that of cortisol facilitate the ability to adapt to stress and recover from illness. Accordingly, cortisol levels are increased in critically ill intensive-care-unit patients, with the highest values being reported for highest illness-severity and highest mortality (Jurney et al., 1987). Stress, such as infection, pain, or physical exercise, activates the hypothalamic–pituitary–adrenal (HPA) axis, involving the secretion of corticotropin-releasing hormone (CRH) by the hypothalamus that then triggers the release of the adrenocorticotropic hormone (ACTH) by the anterior pituitary (Vermes & Beishuizen, 2001). Subsequently, ACTH induces the release of glucocorticoid hormones, being cortisol in humans and corticosterone in rodents, from the adrenal cortex. Finally, increasing glucocorticoid levels exert negative feedback inhibition to terminate the stress-induced HPA axis activation by inhibiting the hypothalamic paraventricular nucleus and the hippocampus (Erkut et al., 1998). Based on this, the total amount of glucocorticoids reaching the hypothalamus from the blood is an essential factor for HPA regulation (Mason et al., 2008; Müller et al., 2003; Uhr et al., 2002).

Apart from *mdr1a/1b*$^{-/-}$ knockout mice, some dogs are deficient for P-gp due to a 4-bp gene deletion mutation in the *MDR1* gene, commonly referred to as MDR1 nt230(del4)/MDR1-1delta mutation. Dogs from the Collie, Longhaired Whippet, Australian Shepherd, Shetland Sheepdog, Silken Windhound, and White Swiss Shepherd breeds are particularly predisposed for this mutation (Gramer et al., 2011) and are suspected to have chronic HPA axis suppression due to higher glucocorticoid penetration into the brain in the absence of P-gp. Indeed, a study by Mealey et al. (Mealey et al., 2007) showed significantly lower basal plasma cortisol levels and significantly lower cortisol concentrations after ACTH administration in MDR1$^{-/-}$ mutant dogs compared with MDR1$^{+/+}$ normal dogs. In addition, plasma ACTH concentrations were significantly lower in the MDR1$^{-/-}$ mutant dogs after dexamethasone administration, clearly indicating that P-gp plays a significant role for HPA axis regulation in the dog. It was suggested that the HPA axis suppression in the MDR1$^{-/-}$ mutant dogs could be linked to a relative adrenal insufficiency. This might reduce the capacity to cope with stress or illness and also might have a poorer disease outcome in MDR1$^{-/-}$ mutant dogs as it is known from human patients with relative adrenal insufficiency of different etiologies (Beishuizen & Thijs, 2001). However, as venipuncture to measure plasma cortisol levels is a stressful procedure for dogs, we aimed in the present study to determine the cortisol levels in MDR1$^{-/-}$ mutant dogs in a completely non-invasive, stress-free procedure. For that, morning void urine was collected from MDR1$^{-/-}$ mutant and MDR1$^{+/+}$ normal dogs and was subjected to targeted gas chromatography–mass spectrometry (GC-MS) steroid hormone metabolome analysis. The second aim of the present study was the comprehensive analysis of the urinary steroid metabolome of MDR1$^{-/-}$ mutant and MDR1$^{+/+}$ normal dogs in order to investigate whether steroids other than cortisol and its metabolites are also dysregulated by the MDR1 mutation in the dog.

2 | MATERIALS AND METHODS

2.1 | Animals and sampling

Dog owners who used the MDR1 genotyping service at our institute were invited to participate in the present study. MDR1 genotyping was performed as reported (Gramer et al., 2011). Inclusion criteria were as follows: (I) dog belongs to the Collie and Australian Shepherd breeds, (II) MDR1 genotype is MDR1$^{+/+}$ normal or MDR1$^{-/-}$ mutant, and (III) dog is free of any diagnosed acute or chronic disease and medication at the sampling time. All dog owners who volunteered to take part in this study were advised to collect the first-morning void urine in appropriate collection vessels during spontaneous voiding of the dogs. Then, the urine was directly transferred to sterile urine cups. Breed, sex, castration status, age, MDR1 genotype, special diet, and general activity of the dogs were recorded in a questionnaire. Urine collection induced no pain, suffering, or damage to the dogs, was non-invasive and stress-free, and did not disturb the normal behavior of the dogs. Urinary creatinine concentrations were determined with an enzymatic test and used for normalization of the urinary steroid metabolite concentrations. Briefly, urine protein was analyzed with the biuret method, and for measurement of urine creatinine, an enzymatic test was applied. Quantitative analysis of urine protein and creatinine was performed with the Pentra 400 ABX Horiba Diagnostics (Axon Lab AG, Reichenbach/Stuttgart, Germany) as reported before (Bauer et al., 2008). In addition, targeted GC-MS analysis of 36 steroid hormones and steroid hormone metabolites was carried out as reported before (Wudy et al., 2007, 2018). Briefly, free and conjugated urinary steroids were extracted from 5 ml urine by solid-phase extraction (Sep-Pak C18 cartridges, Waters), and conjugates were enzymatically hydrolyzed (sulfatase from helix pomatia type H-1, Sigma-Aldrich Chemie). After recovery of hydrolyzed steroids by a second solid-phase extraction, known amounts of internal standards (5α-androstane-3α,17α-diol (Paesel&Lorei), stigmasterol (Sigma-Aldrich Chemie)) were added to each extract before the formation of methyloxime-trimethylsilyl ethers (methoxyamine hydrochloride, Sigma-Aldrich Chemie; trimethylsilylimidazole, Macherey-Nagel). GC was performed using an Optima-1 fused silica column (length, 25 m; film thickness, 0.1 μm; inner diameter, 0.2 mm; Macherey-Nagel) housed in an Agilent 6890 series GC system that was directly interfaced to an Agilent Technologies 5975 inert XL mass selective detector. The derivatized samples were analyzed during a temperature programmed run between 210 and 270 °C. The MS was run in the selected ion monitoring mode. After calibration, values for the excretion of individual steroids were determined by measuring the selected ion peak areas against the internal standard areas. The urine concentrations of the steroid hormones and steroid hormone metabolites (in ng/L) were normalized for the urine creatinine concentrations (in mg/L).

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.2 | Statistics

Statistical analysis was performed using the SPSS version 25.0 software (SPSS Inc.). All variables were tested for normal distribution using the Shapiro–Wilk test. The Student`s *t*-test was used to compare the means of continuous variables and normal-distributed data. When data were skewed, medians were compared with the Mann–Whitney *U* test. A level of $p < .05$ was considered statistically significant.

3 | RESULTS

In the present study, steroid metabolites were analyzed from the first-morning void urine from 39 healthy dogs by the dog owners. Of these 39 dogs, 24 were Collies, and 15 were Australian Shepherds (Table 1). The 24 Collies had the following MDR1 genotypes: MDR1$^{+/+}$ (6 males and 3 females) and MDR1$^{-/-}$ (8 males and 7 females). The 15 Australian Shepherds had the following MDR1 genotypes: MDR1$^{+/+}$ (1 male and 6 females) and MDR1$^{-/-}$ (4 males and 4 females). For all dogs, normal diet and general activity were reported. All data on breed, sex, castration status, and MDR1 genotype are summarized in Table 1.

In total, 36 steroid hormone metabolites (see Table 2) were analyzed via GC-MS and the creatinine-normalized concentration ratios (steroid (in ng/L)/creatinine (in mg/L)) are summarized in Table 3 and Table 4. In addition, a pathway diagram of the steroid metabolism is depicted in Figure 1 to allow better overview.

All cortisol metabolite levels were generally lower in the urine of the MDR1$^{-/-}$ mutant dogs compared with the MDR1$^{+/+}$ normal dogs (Table 3, Figure 1). In the case of allo-tetrahydro-cortisol (α-THF) and β-cortol (β-C), these differences reached the level of significance ($p < .05$). The other cortisol metabolites, being tetrahydro-cortisol (THF; ratio, 0.6; 95% CI, 0.4–0.8), α-cortol (α-C; ratio, 0.8; 95% CI, 0.7–0.9), tetrahydro-cortisone (THE; ratio, 0.4; 95% CI, 0.2–0.6), α-cortolone (α-Cl; ratio, 0.7; 95% CI, 0.6–0.8), and β-cortolone (β-Cl; ratio, 0.5; 95% CI, 0.3–0.7), as well as cortisol itself (F; ratio, 0.8; 95% CI, 0.6–1.0) revealed mean urine concentrations in MDR1$^{-/-}$ mutant dogs at 40–80% of those in MDR1$^{+/+}$ normal dogs (Table 3).

Among the group of 13 other quantifiable steroid metabolites listed in Table 4, only 11-keto-pregnanetriol (11-O-PT) was significantly reduced in MDR1$^{-/-}$ mutant dogs compared with MDR1$^{+/+}$ normal dogs. Other steroid metabolites, being androsterone (AN; ratio, 0.6; 95% CI, 0.4–0.8), etiocholanolone (ET; ratio, 0.7; 95% CI, 0.5–0.9), 11-hydroxyandrosterone (11-OH-AN; ratio, 0.2; 95% CI, 0.0–0.4), 17α-OH-pregnanolone (Po-5β,3α: ratio, 0.3; 95% CI, 0.0–0.6), pregnanetriol (PT; ratio, 0.7; 95% CI, 0.5–0.9), tetrahydro-compound A (THA; ratio, 0.5; 95% CI, 0.2–0.8), and tetrahydro-11-desoxycortisol (THS; ratio, 0.8; 95% CI, 0.6–1.0) revealed mean urine concentrations in MDR1$^{-/-}$ mutant dogs at 20%–80% of those in MDR1$^{+/+}$ normal dogs. Comparable levels (90%–110%) were detected for 5-androstene-3β,17α-diol (A5-3β,17α; ratio, 1.1), 11-hydroxyetiocholanolone (11-OH-ET; ratio, 0.9; 95% CI, 0.8–1.0), and tetrahydro-corticosterone (THB; ratio, 1.0; 95% CI, 1.0–1.0), while the concentrations of dehydroepiandrosterone (DHEA; ratio, 1.2) and 5-androstene-3β,17β-diol (A5-3β,17β; ratio, 1.5) were even higher in the urine of the MDR1$^{-/-}$ mutant dogs (Table 4).

Of note, several steroids could not be detected in any of the urine samples or were below the limit of quantification, thereby precluding statistical analysis. These were estrone (E1), estradiol (E2), estriol (E3), 16α-OH-dehydroepiandrosterone (16α-OH-DHEA), pregnanediol (P5D), allo-tetrahydro-corticosterone (α-THB), 6β-hydroxycortisol (6β-OH-F), and tetrahydro-11-desoxycorticosterone (THDOC). Furthermore, we could detect and quantify some steroids only in the urine of very few dogs, being testosterone (T, one dog, steroid/creatinine ratio =38.0), 17α-OH-pregnanolone-5α (Po-5α3α, three dogs, steroid/creatinine ratio =4.2–5.1), pregnanediol (PD, one dog, steroid/creatinine ratio =45.4), androstenetriol-16α (A5T-16α, three dogs, steroid/creatinine ratio =4.6–8.0), pregnenetriol-17α (P5T-17α, three dogs, steroid/creatinine ratio =2.9–178.6), 20α-dihydrocortisol (20α-DHF, three dogs, steroid/creatinine ratio =11.8–15.6), and 11-keto-androsteron (11-O-AN, 2 dogs, steroid/creatinine ratio =7.63–8.39).

4 | DISCUSSION

Urine is the major excretory pathway for steroid metabolites in dogs (Schatz & Palme, 2001; Williams et al., 2000). About 90% of all steroid hormone metabolites, which are mostly catabolized by reduction and hydroxylation processes, are excreted via the kidneys. Therefore, the urinary steroid metabolite profile mirrors the steroid metabolism of the body (Vitkin et al., 2014) and permits diagnosis of steroid-related disorders (Wudy & Hartmann, 2004). The clinical indications for the measurement of urinary steroid hormone metabolites have been outlined in international guidelines for human medicine (Allende et al., 2014; Kulle et al., 2017;

TABLE 1 MDR1 genotypes, sex, castration status, and breed of the dogs. Urine samples were collected from 39 client-owned dogs that were previously analyzed for the nt230(del4) MDR1 mutation as part of the diagnostic research service at our institute

MDR1 genotype	*n*	Sex	Breeds
MDR1$^{-/-}$	23	7 SF, 4 F, 3 CM, 9 M	15 Collie, 8 Australian Shepherds
MDR1$^{+/+}$	16	1 SF, 8 F, 2 CM, 5 M	9 Collies, 7 Australian Shepherd

Note: Only dogs from the Collie and Australian Shepherd breeds that were diagnosed with either MDR1$^{-/-}$ or MDR$^{+/+}$ were included. All dogs were free of any diagnosed acute or chronic disease and medication at the sampling time.

CM, castrated male; F, female; M, male; SF, spayed female.

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Overview on all steroids analyzed by GC-MS

Abbreviation	Trivial name	Systematic name	LOD [pg]	LLOQ [µg/l]
β-C	β-Cortol	5β-Pregnane−3α,11β,17α,20β,21-pentol	6.3	3.13
α-C	α-Cortol	5β-Pregnane−3α,11β,17α,20α,21-pentol	6.3	3.13
α-Cl	α-Cortolone	5β-Pregnane−3α,17α,20α,21-tetrol−11-one	6.3	3.13
β-Cl	β-Cortolone	5β-Pregnane−3α,17α,20β,21-tetrol−11-one	6.3	3.13
α-THB	Allo-tetrahydro-corticosterone	5α-Pregnane−3α,11β,21-triol−20-one	12.5	6.25
α-THF	Allo-tetrahydro-cortisol	5α-Pregnane−3α,11β,17α,21-tetrol−20-one	12.5	6.25
11-O-AN	11-Keto-androsterone	5α-Androstane−3α-ol−11,17-dione	12.5	6.25
11-OH-AN	11-Hydroxyandrosterone	5α-Androstane−3α,11β-diol−17-one	6.3	3.13
11-OH-ET	11-Hydroxyetiocholanolone	5β-Androstane−3α,11β-diol−17-one	12.5	6.25
11-O-PT	11-Keto-pregnanetriol	5β-Pregnane−3α,17α,20α-triol−11-one	3.1	1.56
16α-OH-DHEA	16α-OH-DHEA	5-Androstene−3β,16α-diol−17-one	25.0	12.50
20α-DHF	20α-Dihydrocortisol	4-Pregnene−11β,17α,20α,21-tetrol−3-one	25.0	12.50
6β-OH-F	6β-Hydroxycortisol	4-Pregnene−6β,11β,17α,21-tetrol−3,20-dione	25.0	25.00
A5-3β,17α	5-Androstene−3β,17α-diol	5-Androstene−3β,17α-diol	12.5	6.25
A5-3β,17β	5-Androstene−3β,17β-diol	5-Androstene−3β,17β-diol	12.5	6.25
A5T−16α	Androstenetriol−16α	5-Androstene−3β,16α,17β-triol	6.3	3.13
AN	Androsterone	5α-Androstane−3α-ol−17-on	6.3	3.13
DHEA	Dehydroepiandrosterone	5-Androstene−3β-ol−17-on	25.0	12.50
E1	Estrone	Estrone	12.5	6.25
E2	Estradiol	Estradiol	6.3	3.13
E3	Estriol	Estriol	6.3	3.13
ET	Etiocholanolone	5β-Androstane−3α-ol−17-on	6.3	3.13
F	Cortisol	4-Pregnene−11β,17α,21-triol−3,20-dione	25.0	12.50
P5D	Pregnanediol	5-Pregnene−3β,20α-diol	8.1	4.20
P5T−17α	Pregnenetriol−17α	5-Pregnene−3β,17α,20α-triol	6.3	3.13
PD	Pregnanediol	5β-Pregnane−3α,20α-diol	12.5	6.25
Po−5β,3α	17α-OH-Pregnanolone	5β-Pregnane−3α,17α-diol−20-one	6.3	3.13
Po−5α,3α	17α-OH-Pregnanolone−5α	5α-Pregnane−3α,17α-diol−20-one	6.3	3.13
PT	Pregnanetriol	5β-Pregnane−3α,17α,20α-triol	1.6	0.78
T	Testosterone	Testosterone	12.5	6.25
THA	Tetrahydro-compound A	5β-Pregnane−3α,21-diol−11,20-dione	25.0	12.50
THB	Tetrahydro-corticosterone	5β-Pregnane−3α,11β,21-triol−20-one	12.5	6.25
THDOC	Tetrahydro−11-desoxycorticosterone	5β-Pregnane−3α,21-diol−20-one	12.5	6.25
THE	Tetrahydro-cortisone	5β-Pregnane−3α,17α,21-triol−11,20-dione	12.5	6.25
THF	Tetrahydro-cortisol	5β-Pregnane−3α,11β,17α,21-tetrol−20-one	12.5	6.25
THS	Tetrahydro−11-desoxycortisol	5β-Pregnane−3α,17α,21-triol−20-one	12.5	6.25

Abbreviations: LOD, limit of detection; LLOQ, lower limit of quantification.

Wood et al., 2008; Wudy et al., 2018), but have been scarcely studied in veterinary species. However, plasma steroid hormone concentrations are important established laboratory parameters in veterinary medicine, too.

The present study used GC-MS based urinary steroid metabolome analysis to answer the question if cortisol and its metabolites are less excreted in the urine of MDR1$^{-/-}$ mutant dogs. These dogs showed significantly lower basal plasma cortisol levels in a previous study compared with MDR1$^{+/+}$ normal dogs, suggesting increased suppression of the HPA axis in the absence of P-gp (Mealey et al., 2007). These findings are clearly supported by data from the present study that showed significantly lower urinary concentrations of α-THF and β-C, among generally lower levels of al measured cortisol metabolites in the urine of MDR1$^{-/-}$ mutant dogs (Table 3, Figure 1). Therefore, urinary α-THF and β-C can be regarded as potential non-invasive markers for cortisol levels in MDR1$^{-/-}$ mutant dogs. These

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 3 Urinary cortisol and cortisol metabolites in MDR1$^{-/-}$ and MDR1$^{+/+}$ dogs

Cortisol and cortisol metabolites	MDR1$^{-/-}$		MDR1$^{+/+}$		MDR1$^{-/-}$ / MDR1$^{+/+}$	
	Mean ± SD	*n*	Mean ± SD	*n*	Ratio [95% CI]	*p*-value
F (cortisol)	16.4 ± 7.1	13	19.8 ± 13.6	10	0.8 [0.6–1.0]	.410
α-THF	11.2 ± 3.4	17	20.7 ± 14.9	12	0.5 [0.3–0.7]	.006*
THF	102.6 ± 68.7	23	176.2 ± 210.2	16	0.6 [0.4–0.8]	.217
α-C	12.6 ± 7.9	22	16.1 ± 11.2	12	0.8 [0.7–0.9]	.231
β-C	105.5 ± 63.3	23	221.0 ± 225.5	15	0.5 [0.3–0.7]	.025*
THE	22.7 ± 15.9	22	52.2 ± 88.2	14	0.4 [0.2–0.6]	.170
α-Cl	16.5 ± 9.7	23	22.5 ± 17.3	15	0.7 [0.6–0.8]	.153
β-Cl	170.7 ± 128.9	23	332.9 ± 426.5	16	0.5 [0.3–0.7]	.196

Note: Data represent means ± SD creatinine-normalized urinary steroid concentrations represented by the steroid (in ng/L)/creatinine (in mg/L) ratios. In addition, 95% confidence intervals (95% CI) are provided for the MDR1$^{-/-}$ / MDR1$^{+/+}$ ratios. *Significantly different values with $p < .05$ in MDR1$^{-/-}$ dogs compared with MDR1$^{+/+}$ dogs.

TABLE 4 Urinary non-cortisol steroid hormones and their metabolites in MDR1$^{-/-}$ and MDR1$^{+/+}$ dogs

Steroids and steroid metabolites	MDR1$^{-/-}$		MDR1$^{+/+}$		MDR1$^{-/-}$ / MDR1$^{+/+}$	
	Mean ± SD	*n*	Mean ± SD	*n*	Ratio [95% CI]	*p*-value
AN	9.0 ± 3.2	8	15.6 ± 9.1	8	0.6 [0.4–0.8]	.076
DHEA	14.8 ± 1.5	2	12.0 ± 3.9	3	1.2 [NA]	.421
ET	11.1 ± 4.2	8	17.0 ± 7.1	9	0.7 [0.5–0.9]	.060
A5-3ß,17α	10.1 ± 3.2	7	9.3 ± 2.4	3	1.1 [NA]	.708
A5-3ß,17ß	10.0 ± 0.0	2	6.7 ± 2.1	3	1.5 [NA]	.124
11-OH-AN	4.5 ± 1.2	5	22.6 ± 44.7	8	0.2 [0.0–0.4]	.127
11-OH-ET	26.2 ± 17.8	9	27.6 ± 14.7	8	0.9 [0.8–1.0]	.226
11-O-PT	1.9 ± 0.9	12	7.9 ± 16.5	12	0.2 [0.0–0.4]	.024*
Po−5ß,3α	9.7 ± 7.3	4	34.4 ± 71.5	8	0.3 [0.0–0.6]	.682
PT	5.9 ± 9.0	8	8.2 ± 20.4	12	0.7 [0.5–0.9]	.776
THA	15.1 ± 5.2	6	28.7 ± 27.5	4	0.5 [0.2–0.8]	.781
THB	21.3 ± 19.4	16	21.8 ± 15.7	8	1.0 [1.0–1.0]	.648
THS	7.1 ± 1.6	8	8.8 ± 1.6	4	0.8 [0.6–1.0]	.096

Note: Data represent means ± SD creatinine-normalized urinary steroid concentrations represented by the steroid (in ng/L)/creatinine (in mg/L) ratios. In addition, 95% confidence intervals (95% CI) are provided for the MDR1$^{-/-}$ / MDR1$^{+/+}$ ratios. *Significantly different values with $p < .05$ in MDR1$^{-/-}$ dogs compared with MDR1$^{+/+}$ dogs.

Abbreviation: NA, not available.

findings go in line with the current understanding of chronic suppression of the HPA axis due to increased hypothalamic cortisol feedback regulation in the absence of P-gp in the blood–brain barrier (Figure 2).

The aim of the present study was to analyze cortisol and its metabolites in a completely non-invasive and stress-free environment to avoid measurement artifacts of stress-induced cortisol release, which cannot be ruled out when dealing with dogs for venipuncture or even for intensive clinical monitoring. Therefore, for steroid measurements, we used dog urine collected by the owners in the dogs' natural environment without any stress or intervention. Consequently, this study design had some limitations that need to be clearly stated: the dogs were not subjected to intensive clinical health monitoring, and the dogs' diet and physical activity were not standardized. However, only dogs were included that were free of any diagnosed disease or medication and only dogs with normal diet and general activity were included. Nevertheless, the possible influence of confounding factors such as activity, diet, and subject health at the sampling time cannot be completely ruled out. In addition, the total sample number was too low to carry out additional multivariate regression analyses on possible effects of breed, sex, age, and castration status of the dogs.

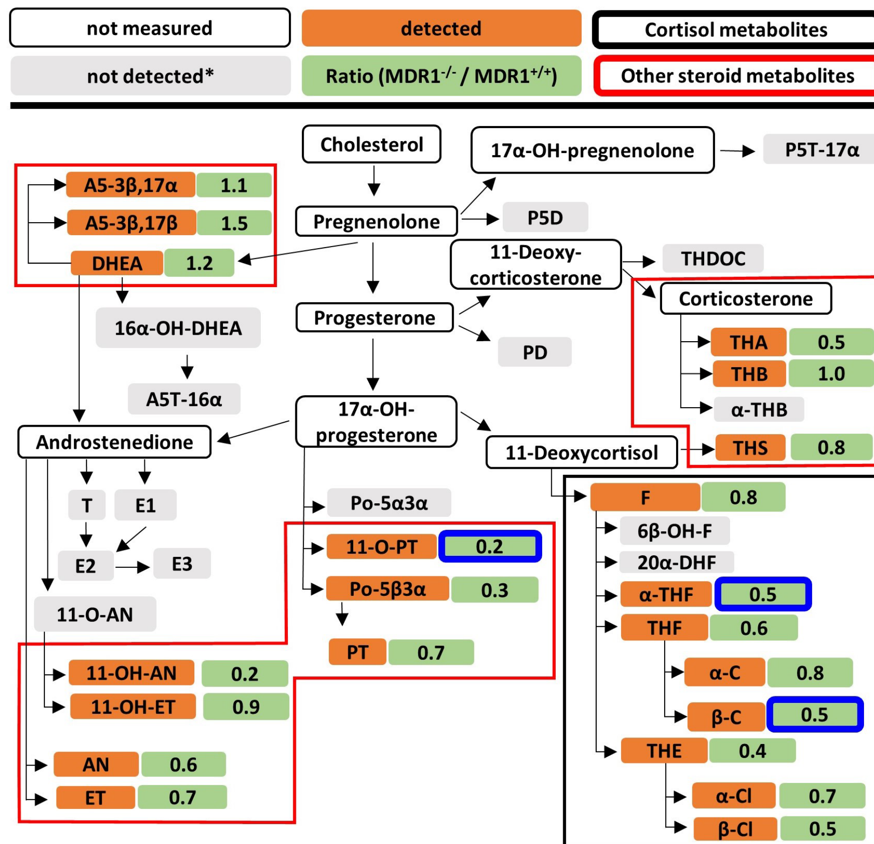

FIGURE 1 Pathways of dog steroid hormone biosynthesis and metabolism. Pathways are partly combined to provide better overview on 36 urinary steroid metabolites measured in the present study. Metabolite abbreviations (detected metabolites in orange and non-detected metabolites in gray), and MDR1$^{-/-}$/MDR1$^{+/+}$ ratios (green) are given. *Not detected or detected in only few dogs. Ringed in blue: Significantly different between MDR1$^{-/-}$ mutant MDR1$^{+/+}$ normal dogs (α-THF, *p*-value = .006; β-C, *p*-value = .025; 11-O-PT, *p*-value = .024). The *p*-values and the 95% CI are given in Table 3 and Table 4. For all abbreviations, please refer to Table 2

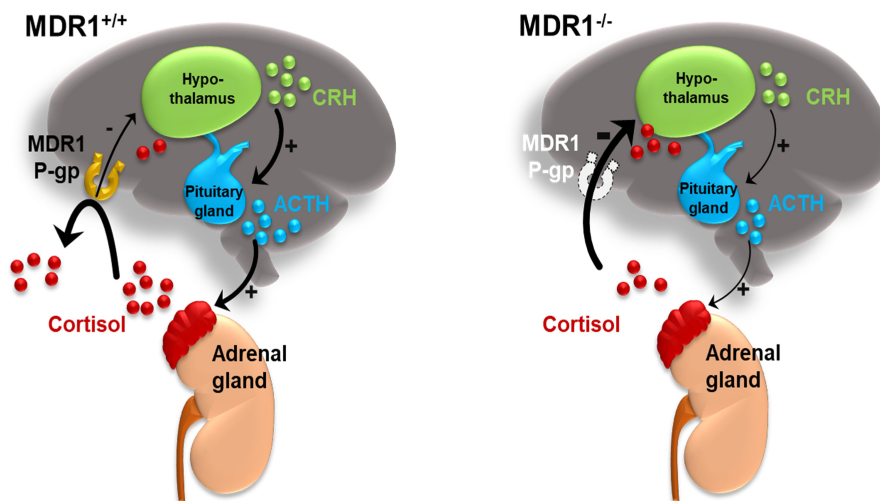

FIGURE 2 Illustration of the increased brain penetration of cortisol in the absence of P-gp (MDR1$^{-/-}$ mutant dogs) and the subsequent suppression of the HPA axis

The second aim of the study was to measure also non-cortisol metabolites in the urine to analyze whether P-gp might also play a role for their regulation and excretion. Only few studies addressed the impact of urinary steroid hormone metabolite excretion on the diagnosis of different diseases in dogs so far. These previous studies measured only cortisol and oxytocin metabolites (Wirobski et al., 2021), or THE and THF (Quilez et al., 2020) from the morning void urine. Another study compared the urinary corticoid-to-creatinine ratio to discriminate healthy from hypercortisolism dogs (Galeandro et al., 2014). A more recent study showed that adrenocortical urinary steroid metabolites could be successfully detected in healthy, septic, and septic shock dogs using GC-MS and revealed that urinary metabolite concentrations of 5α-tetrahydro-cortisol, THE, β-C, α-Cl, β-Cl, and total metabolites were higher in septic shock dogs compared with septic dogs (Boag et al., 2020). Additionally, all steroid metabolites except androsterone exhibited significantly increased levels in septic or septic shock dogs (Boag et al., 2020). On this background, the impact of the comprehensive measurement of 13 steroids and steroid metabolites in the urine of MDR1$^{-/-}$ mutant and MDR1$^{+/+}$ normal dogs has to be emphasized.

Interestingly, among the group of other steroid metabolites 11-O-PT was also significantly lower in the urine of MDR1$^{-/-}$ mutant

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

dogs compared with the MDR1$^{+/+}$ normal dogs. The same was true for AN and ET, even if these values did not fully reach the level of significance. These findings indicated that apart from cortisol metabolism, P-gp might also play a role for the regulation, metabolism, and excretion of other steroid hormones descent from 17α-OH-progesterone and/or androstenedione. However, whether this is also associated with a suppression of the HPA axis must be investigated in follow-up studies, which also measure the plasma concentrations of the relevant steroids with and without ACTH stimulation.

In conclusion, the present study for the first time provides comprehensive steroid metabolome analysis in the urine of MDR1$^{-/-}$ mutant dogs. Significant differences in MDR1$^{-/-}$ mutant dogs indicate that P-gp is involved in cortisol regulation, metabolism, and excretion and seems also to play a role for the urinary excretion of steroid hormones descent from 17α-OH-progesterone. Urinary α-THF and β-C are suggested as potential non-invasive markers for cortisol levels in MDR1$^{-/-}$ mutant dogs.

ACKNOWLEDGMENTS

The authors thank all the dog owners for their support in collecting the urine samples. Open Access funding enabled and organized by Projekt DEAL.

CONFLICT OF INTEREST

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, I.G., S.A.W., J.G.; methodology, I.G., E.K., M.F.H., S.A.W., N.B., A.M., J.G.; investigation, I.G., M.F.H., N.B.; data analysis, I.G., E.K., M.F.H., S.A.W., N.B., A.M., Z.A., J.G.; ressources, S.A.W., A.M., J.G.; writing - original draft preparation, I.G., E.K., M.F.H., N.B., J.G.; writing - review and editing, E.K., M.F.H., S.A.W., N.B., A.M., Z.A., J.G.. All authors have read and agreed to the published version of the manuscript.

ANIMAL WELFARE AND ETHICS STATEMENT

Collection of the urine samples was completely non-invasive, induced no pain, suffering, or damage to the dogs and did not disturb the normal behavior of the dogs. Therefore, ethical review and approval was not necessary. All dog owners were actively involved in the sample collection and consented to the use of the urine samples for the present study.

DATA AVAILABILITY STATEMENT

Data are available on request from the authors.

ORCID

Joachim Geyer https://orcid.org/0000-0003-2663-1858

REFERENCES

Allende, F., Solari, S., Campino, C., Carvajal, C. A., Lagos, C. F., Vecchiola, A., Valdivia, C., Baudrand, R., Owen, G. I., & Fardella, C. E. (2014). LC-MS/MS method for the simultaneous determination of free urinary steroids. *Chromatographia*, *77*, 637–642. https://doi.org/10.1007/s10337-014-2638-4

Bauer, N., Rettig, S., & Moritz, A. (2008). Evaluation the Clinitek status automated dipstick analysis device for semiquantitative testing of canine urine. *Research in Veterinary Science*, *85*(3), 467–472. https://doi.org/10.1016/j.rvsc.2008.02.003

Beishuizen, A., & Thijs, L. G. (2001). Relative adrenal failure in intensive care: An identifiable problem requiring treatment? *Best Practice & Research Clinical Endocrinology & Metabolism*, *15*(4), 513–531. https://doi.org/10.1053/beem.2001.0167

Boag, A. M., Brown, A., Koenigshof, A., Homer, N., Sooy, K., & Jamieson, P. M. (2020). Glucocorticoid metabolism in critically ill dogs (Canis lupus familiaris). *Domestic Animal Endocrinology*, *72*, 106437. https://doi.org/10.1016/j.domaniend.2020.106437

Erkut, Z. A., Pool, C., & Swaab, D. F. (1998). Glucocorticoids suppress corticotropin-releasing hormone and vasopressin expression in human hypothalamic neurons. *Journal of Clinical Endocrinology and Metabolism*, *83*(6), 2066–2073. https://doi.org/10.1210/jcem.83.6.4881

Galeandro, L., Sieber-Ruckstuhl, N. S., Riond, B., Hartnack, S., Hofmann-Lehmann, R., Reusch, C. E. & Boretti, F. S. (2014). Urinary corticoid concentrations measured by 5 different immunoassays and gas chromatography-mass spectrometry in healthy dogs and dogs with hypercortisolism at home and in the hospital. *Journal of Veterinary Internal Medicine*, *28*(5), 1433–1441. https://doi.org/10.1111/jvim.12399

Gramer, I., Leidolf, R., Doring, B., Klintzsch, S., Kramer, E. M., Yalcin, E., Petzinger, E., & Geyer, J. (2011). Breed distribution of the nt230(del4) MDR1 mutation in dogs. *The Veterinary Journal*, *189*(1), 67–71. https://doi.org/10.1016/j.tvjl.2010.06.012

Jurney, T. H., Cockrell, J. L. Jr, Lindberg, J. S., Lamiell, J. M., & Wade, C. E. (1987). Spectrum of serum cortisol response to ACTH in ICU patients. Correlation with degree of illness and mortality. *Chest*, *92*(2), 292–295. https://doi.org/10.1378/chest.92.2.292

Kulle, A., Krone, N., Holterhus, P. M., Schuler, G., Greaves, R. F., Juul, A., de Rijke, Y. B, Hartmann, M. F, Saba, A., Hiort, O. & Wudy, S. A. (2017). Steroid hormone analysis in diagnosis and treatment of DSD: Position paper of EU COST Action BM 1303 'DSDnet'. *European Journal of Endocrinology*, *176*(5), P1–P9. https://doi.org/10.1530/EJE-16-0953

Mason, B. L., Pariante, C. M., & Thomas, S. A. (2008). A revised role for P-glycoprotein in the brain distribution of dexamethasone, cortisol, and corticosterone in wild-type and ABCB1A/B-deficient mice. *Endocrinology*, *149*(10), 5244–5253. https://doi.org/10.1210/en.2008-0041

Mealey, K. L., Gay, J. M., Martin, L. G., & Waiting, D. K. (2007). Comparison of the hypothalamic-pituitary-adrenal axis in MDR1-1delta and MDR1 wildtype dogs. *The Journal of Veterinary Emergency and Critical Care*, *17*(1), 61–66. https://doi.org/10.1111/j.1476-4431.2006.00196.x

Müller, M. B., Keck, M. E., Binder, E. B., Kresse, A. E., Hagemeyer, T. P., Landgraf, R., Holsboer, F., & Uhr, M. (2003). ABCB1 (MDR1)-type P-glycoproteins at the blood-brain barrier modulate the activity of the hypothalamic-pituitary-adrenocortical system: Implications for affective disorder. *Neuropsychopharmacol*, *28*(11), 1991–1999. https://doi.org/10.1038/sj.npp.1300257

Quilez, E., Burchell, R. K., Thorstensen, E. B., Weidgraaf, K., Parbhu, S. E., Lopez-Villalobos, N. & Gal, A. (2020). Cortisol urinary metabolites in dogs with hypercortisolism, congestive heart failure, and healthy dogs: Pilot investigation. *Journal of Veterinary Diagnostic Investigation*, *32*(2), 317–323. https://doi.org/10.1177/1040638719899645

Schatz, S., & Palme, R. (2001). Measurement of faecal cortisol metabolites in cats and dogs: A non-invasive method for evaluating adrenocortical function. *Veterinary Research Communications*, *25*(4), 271–287. https://doi.org/10.1023/a:1010626608498

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Schinkel, A. H. (1998). Pharmacological insights from P-glycoprotein knockout mice. *International Journal of Clinical Pharmacology and Therapeutics*, *36*(1), 9–13.

Uhr, M., Holsboer, F., & Müller, M. B. (2002). Penetration of endogenous steroid hormones corticosterone, cortisol, aldosterone and progesterone into the brain is enhanced in mice deficient for both mdr1a and mdr1b P-glycoproteins. *Journal of Neuroendocrinology*, *14*(9), 753–759. https://doi.org/10.1046/j.1365-2826.2002.00836.x

Vermes, I., & Beishuizen, A. (2001). The hypothalamic-pituitary-adrenal response to critical illness. *Best Practice & Research Clinical Endocrinology & Metabolism*, *15*(4), 495–511. https://doi.org/10.1053/beem.2001.0166

Vitkin, E., Ben-Dor, A., Shmoish, M., Hartmann, M. F., Yakhini, Z., Wudy, S. A. & Hochberg, Z. (2014). Peer group normalization and urine to blood context in steroid metabolomics: the case of CAH and obesity. *Steroids*, *88*, 83–89. https://doi.org/10.1016/j.steroids.2014.07.003

Williams, T. M., Kind, A. J., Hyde, W. G., & Hill, D. W. (2000). Characterization of urinary metabolites of testosterone, methyltestosterone, mibolerone and boldebone in greyhound dogs. *Journal of Veterinary Pharmacology and Therapeutics*, *23*(3), 121–129. https://doi.org/10.1046/j.1365-2885.2000.00267.x

Wirobski, G., Range, F., Schaebs, F. S., Palme, R., Deschner, T., & Marshall-Pescini, S. (2021). Endocrine changes related to dog domestication: Comparing urinary cortisol and oxytocin in hand-raised, pack-living dogs and wolves. *Hormones and Behavior*, *128*, 104901. https://doi.org/10.1016/j.yhbeh.2020.104901

Wood, L., Ducroq, D. H., Fraser, H. L., Gillingwater, S., Evans, C., Pickett, A. J., Rees, D. W., John, R., & Turkes, A. (2008). Measurement of urinary free cortisol by tandem mass spectrometry and comparison with results obtained by gas chromatography-mass spectrometry and two commercial immunoassays. *Annals of Clinical Biochemistry*, *45*(Pt 4), 380–388. https://doi.org/10.1258/acb.2007.007119

Wudy, S. A., & Hartmann, M. F. (2004). Gas chromatography-mass spectrometry profiling of steroids in times of molecular biology. *Hormone and Metabolic Research*, *36*(6), 415–422. https://doi.org/10.1055/s-2004-814565

Wudy, S. A., Hartmann, M. F., & Remer, T. (2007). Sexual dimorphism in cortisol secretion starts after age 10 in healthy children: urinary cortisol metabolite excretion rates during growth. *American Journal of Physiology. Endocrinology and Metabolism*, *293*(4), E970–E976. https://doi.org/10.1152/ajpendo.00495.2006

Wudy, S. A., Schuler, G., Sanchez-Guijo, A., & Hartmann, M. F. (2018). The art of measuring steroids: Principles and practice of current hormonal steroid analysis. *Journal of Steroid Biochemistry and Molecular Biology*, *179*, 88–103. https://doi.org/10.1016/j.jsbmb.2017.09.003

How to cite this article: Gramer, I., Karakus, E., Hartmann, M. F., Wudy, S. A., Bauer, N., Moritz, A., Aktürk, Z., & Geyer, J. (2022). Urinary cortisol metabolites are reduced in MDR1 mutant dogs in a pilot targeted GC-MS urinary steroid hormone metabolome analysis. *Journal of Veterinary Pharmacology and Therapeutics*, *45*, 265–272. https://doi.org/10.1111/jvp.13050

13652885, 2022, 3, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/jvp.13050 by Universitaetsbibl Augsburg, Wiley Online Library on [07/02/2023]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License