J Vet Sci. 2020 Mar;21(2):e23
https://doi.org/10.4142/jvs.2020.21.e23
pISSN 1229-845X·eISSN 1976-555X

Journal of Veterinary Science

Original Article
Biochemistry

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Specific urinary metabolites in canine mammary gland tumors

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OPEN ACCESS

Received: Jul 23, 2019
Revised: Nov 28, 2019
Accepted: Nov 30, 2019

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ABSTRACT

The identification of biomarkers that distinguish diseased from healthy individuals is of great interest in human and veterinary fields. In this research area, a metabolomic approach and its related statistical analyses can be useful for biomarker determination and allow non-invasive discrimination of healthy volunteers from breast cancer patients. In this study, we focused on the most common canine neoplasm, mammary gland tumor, and herein, we describe a simple method using ultra-high-performance liquid chromatography to determine the levels of tyrosine and its metabolites (epinephrine, 3,4-dihydroxy-L-phenylalanine, 3,4-dihydroxyphenylacetic acid, and vanillylmandelic acid), tryptophan and its metabolites (5-hydroxyindolacetic acid, indoxyl sulfate, serotonin, and kynurenic acid) in canine mammary cancer urine samples. Our results indicated significantly increased concentrations of three tryptophan metabolites, 5-hydroxyindolacetic acid ($p < 0.001$), serotonin, indoxyl sulfate ($p < 0.01$), and kynurenic acid ($p < 0.05$), and 2 tyrosine metabolites, 3,4-dihydroxy-L-phenylalanine ($p < 0.001$), and epinephrine ($p < 0.05$) in urine samples from the mammary gland tumor group compared to concentrations in urine samples from the healthy group. The results indicate that select urinary tyrosine and tryptophan metabolites may be useful as non-invasive diagnostic markers as well as in developing a therapeutic strategy for canine mammary gland tumors.

Keywords: Canine mammary cancer; urine metabolites; UHPLC

INTRODUCTION

Currently, there is evidence of an increased incidence of mammary gland tumors in dogs; therefore, the development of novel, fast, and effective diagnostic methods is needed. Up to 50% of localized neoplasms represent malignant tumors [1], and mammary gland tumors are the most abundant cancers and a cause of death in bitches [2]. Age, hormonal exposure, and breed have been shown to be risk factors for mammary cancer. Tumor risk increases with age, and the greatest risk has been shown in dogs aged 7 to 8 years or between 11 and 13 years. Risks associated with hormone therapy such as progestin, estrogen, or a combination thereof have been studied and low-dose progestin alone increased the risk of benign tumors while the hormone combination induced malignant tumors [3,4]. Further studies have indicated a relationship between breast cancer and size and purity in select breeds (poodle, Chihuahua,

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Funding

Realization of this study was supported by the Internal Grant Agency - IGA UVLF 05/2017, Urinary biochemical profile of cancer diseases with potential use in diagnostic practice, Košice, Slovakia.

Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Valko-Rokytovská M, Očenáš P, Salayová A; Data curation: Valko-Rokytovská M, Očenáš P, Salayová A; Formal analysis: Valko-Rokytovská M, Očenáš P, Salayová A, Kostecká Z; Funding acquisition: Kostecká Z; Investigation: Valko-Rokytovská M, Očenáš P, Salayová A, Titková R; Methodology: Valko-Rokytovská M, Očenáš P, Salayová A, Titková R; Project administration: Valko-Rokytovská M, Očenáš P, Salayová A; Resources: Titková R; Software: Valko-Rokytovská M, Očenáš P, Salayová A; Supervision: Valko-Rokytovská M; Validation: Valko-Rokytovská M, Očenáš P, Salayová A; Visualization: Valko-Rokytovská M; Writing - original draft: Valko-Rokytovská M; Writing - review & editing: Valko-Rokytovská M, Očenáš P, Salayová A, Titková R, Kostecká Z.

dachshund, Yorkshire terrier, Maltese, and cocker) with smaller and purer breeds shown to have a more frequent onset of tumor formation [5].

Breast cancer in bitches, as a model of human medicine, is, based on pathological similarities, comparable to human tumors. Prognostic factors for breast cancer diagnosis have included age, pathological tumor size, pathological nodal stage, lymphovascular invasion, histological classification, and expressions of estrogen receptor-alpha, progesterone receptor, human epidermal growth factor receptor 2 (HER2), basal cytokeratin 5/6, and epidermal growth factor receptor. These factors related to invasive breast cancer are similar in humans and canines, as confirmed by biological studies [6].

Metabolomics has great potential in the field of oncology. Predicting, detecting, and monitoring cancers via urine metabolomics is an interesting research object, and, in today's approach to modern medicine, it is a preferred method for early diagnosis. Metabolomics is a non-invasive method that can help in the monitoring of homeostatic imbalances in biological systems. It also provides comprehensive information about potential biomarkers from biological samples (e.g., blood, tissue, saliva, and urine) with relatively simple sample preparation and high-throughput. Rapid non-invasive detection of breast cancer by screening urinary biomarkers can be performed by high-performance liquid chromatography (HPLC)-based analysis. Urine analysis using a validated diagnostic method for select biomarkers within a matrix can promote the need for further screening and early treatment [7].

Amino acids that function as regulators of gene expression and protein phosphorylation cascades also function as cellular signaling molecules [8]. Imbalance of biomarkers, such as tyrosine (TYR) and tryptophan (TRP) metabolites, has been used to suggest the presence of cancer [9], neurological [10] and inflammatory disorders [11]. Elevated urinary TRP metabolite levels have been detected in patients with carcinoid disease [12], bladder cancer [13], and autistic symptoms [14]. The report by Porto-Figueira et al. [15] established a volatomic urinary biosignature from both breast patients and healthy individuals. This study observed that several pathways are upregulated in cancer patients, for example, the phenylalanine pathway in breast cancer. In the study reported by Chen et al. [16], 12 urinary metabolites were identified by performing liquid chromatography (LC)–mass spectrometry as potential biomarkers, including amino acids (phenylalanine, TYR, TRP), organic acids, and nucleosides. The authors observed increased TRP and nucleoside metabolism and also protein degradation in breast carcinoma. The association between TRP and its metabolites, which are present in biological fluids during various pathological disorders, was also confirmed by deriving partial correlation coefficients. The increased presence of plasma free TRP and its metabolites is due to protein breakdown occurring in cancer patients, and there is a close relationship between increased levels of free TRP and its metabolites and the presence of breast cancer [17].

One of the metabolic possibilities for TRP biotransformation (approximately 95%) is the kynurenine pathway in which kynurenic acid is the major metabolite. TRP oxygenase catalysis leads to the destruction of the indole ring, and that catalysis is regulated by adrenocorticosteroids, estrogens, and androgens. It has been suggested that an increase in urinary kynurenic acid, as well as nicotinic acid, levels may be affected by the increased estrogenic activity in breast cancer patients [16].

Urine, a non-invasive source of biomarkers, has potential clinical use in the detection of breast cancer, its diagnosis, and in assessing treatment success after application of

an appropriately selected therapy [18]. The aim of this study was to qualitatively and quantitatively determine relevant biomarker levels in the urine of mammary gland tumor dogs by performing ultra-HPLC (UHPLC) analysis and compare them to levels in healthy control dogs. This method is suitable for relatively simple determination of creatinine, TRP, and TYR and related metabolites in urine samples.

MATERIALS AND METHODS

Sample collection and composition of the study group

The mammary cancer group in the study included 14 female dogs of different breeds, aged from six to sixteen years (**Table 1**). Two of the 14 were large breeds (German shepherds), two were medium breeds (crossbreeds of medium size), and ten were small breeds (Yorkshire terriers, Chihuahuas, and a Shih-Tzu). All procedures involving animals followed the guidelines stated in the Guide for the Care and Use of Animals (protocol number 3323/16-221/3) which was approved by the State Veterinary and Food Administration of the Slovak Republic and by the Ethics Commission of the University of Veterinary Medicine and Pharmacy (Košice, Slovakia). The handling of animals was carried out in a humane manner and in accordance with the guidelines established by the relevant commission. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

All patients underwent surgical intervention and mammary gland tumors were excised. In all patients, histological examination (via staining with Hematoxylin-Eosin) confirmed the diagnosis of adenocarcinoma of the mammary gland (**Table 1**). Prior to surgery, patients were fasted for at least 12 h. Urine samples were obtained from patients before surgery by spontaneous micturition or after sedation (administration of butorphanol and medetomidine) by manual bladder compression.

The healthy control group included 16 female dogs of different breeds from 2 to 14 years old. Healthy control group animals were selected based on the following criteria: absence of any disease, and negative biochemical laboratory test results. Urine samples from healthy control dogs were obtained by spontaneous micturition after 12 h of fasting. Samples were obtained from the Small Animal Clinic of the University Veterinary Hospital, Košice and were stored at −50°C until use.

Table 1. Morphological characteristics of mammary gland tumors in patients (n = 14)

Histopathological type	Stage	Grade	Size (cm)	Consistency	Localization	Age (yr)
Adenocarcinoma	I	II	3 × 4 × 3	Solid	M3, 4 - right side	13
Adenocarcinoma	I	III	3 × 2 × 2	Solid	M3, 4, 5 - bilateral	16
Adenocarcinoma	I	III	3 × 2 × 2	Solid	M4, 5 - bilateral	9
Adenocarcinoma	I	III	3 × 2 × 2	Solid	M3, 4, 5 - bilateral	11
Adenocarcinoma	II	III	2 × 3 × 2	Solid	M4, 5 - bilateral	9
Adenocarcinoma	II	III	1 × 1 × 1	Solid	M2 - left side	14
Adenocarcinoma	III	III	10 × 5 × 6	Solid	M3, 4, 5 - bilateral	6
Cystic papillar adenocarcinoma	I	I	1 × 1 × 1	Solid	M3 - left side	10
Cystic papillar adenocarcinoma	I	III	0.5 (diameter)	Solid	M4 - left side	7
Fibrosarcoma	III	III	10 × 10 × 10	Solid	M4 - left side	10
Low differentiated carcinoma	III	III	10 × 10 × 3	Solid	M5 - right side	7
Mixed carcinoma (with osteoid and myxoid tissue)	II	III	2 × 2 × 2	Elastic	M5 - right side	9
Mixed tumor	II	II	2 × 2 × 2	Elastic	M2 - right side	6
Necrotizing carcinoma	III	III	3 × 4 × 4	Solid	M3, 4, 5 - bilateral	6

https://doi.org/10.4142/jvs.2020.21.e23

Chemicals

Creatinine, TYR, epinephrine (E), 3,4-dihydroxy-L-phenylalanine (L-DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), vanillylmandelic acid (VMA), TRP, 5-hydroxyindolacetic acid (5-HIAA), indoxyl sulfate (IS), serotonin (5-HT), kynurenic acid (KYNA), and formic acid were purchased from Sigma-Aldrich (USA). Methanol of HPLC grade was purchased from Fisher Scientific (Fisher Scientific UK Ltd., UK). Deionized and purified water used in the experiments was from the central water production of the University of Veterinary Medicine and Pharmacy in Košice (RegPur s.r.o., Slovakia). All reagents were HPLC grade.

Instrumentation and chromatography

The separation method was performed according to that in a previously published study [18]. LLC was performed using a UHPLC Dionex UltiMate 3000 RS system (Thermo Fisher Scientific Waltham, USA). The LC system consisted of a quaternary pump, an autosampler, and two detectors (diode array detector [DAD] and fluorescence detector [FLD]). A C18 chromatographic column (Thermo Scientific Acclaim™ 120 C18; Thermo Fisher Scientific, Germany) with 150 mm column length, 3 mm inner diameter, 3 µm particle sizeµ, and 120 Å pore size was used and was thermostatically controlled (37 ± 0.5°C). The collection and evaluation of data were performed using Chromeleon 7.2 Chromatography Data System software. The spectral range for DAD detection was 190–800 nm, and FLD detection was performed at an excitation wavelength (λ_{ex}) of 280 nm and an emission wavelength (λ_{em}) of 350 nm. The injected volume of the standards and samples was 10 µL. The mobile phase of water (containing 0.1% formic acid, A) and methanol (B) was applied under gradient conditions: 0–50% B (0–20 min), 100% A (20–25 min). The flow rate was set at 0.6 mL/min and the overall analysis time was 25 min.

Standard stock solutions (1 mg/mL) of all 11 analytes were individually prepared in deionized water. The corresponding appropriate working solutions were adequately diluted. Calibration curves for all standards in the urine matrix from the healthy dog group were prepared. Calibration solutions with concentrations ranging from 0.01 to 20 µg/mL were prepared. The number of curve points was n = 8 and every analyte was injected twice. The range of limit of detection (LOD) values for the examined metabolites was 1–15 ng/mL and the limit of quantitation (LOQ) range of values was 4–45 ng/mL. Limits of detection and quantitation have been supplemented by L-DOPA (LOD = 10 ng/mL, LOQ = 31 ng/mL), DOPAC (LOD = 13 ng/mL, LOQ = 40 ng/mL), E (LOD = 11 ng/mL, LOQ = 32 ng/mL) and 5-HT (LOD = 1 ng/mL, LOQ = 4 ng/mL) compared to our previously study [18].

Sample preparation

The thawed and centrifugated samples (10,000 r/min [10,621 rcf] for 5 min; Eppendorf Centrifuge 5430, Germany) at ambient laboratory temperature were filtered by using syringe filters (polyvinylidene difluoride; 0.25 µm pore size) and diluted with mobile phase A to 15% (v/v) for use in UHPLC analysis.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM for Windows Version 5.0 (GraphPad Software, Inc., USA). Ranges and median values are presented. The Mann-Whitney *U* test was used for comparisons within groups. The level of significance was set at $p < 0.05$. Ranges of urine metabolites were calculated according to their log-normal distribution. Spearman's Rank Order Correlation was used to determine the statistical dependence between parameters.

RESULTS

In the present study, we analyzed urine samples from 16 healthy and 14 mammary gland tumor-bearing dogs. The 11 assessed metabolites were simultaneously analyzed by using an optimized HPLC method with DAD and FLD detection (**Table 2**) as was described in our previous study [18]. Quantitative analyses were performed using calibration curves obtained from the standards in the matrix. The concentrations of the monitored metabolites are expressed relative to the measured creatinine level, and median values were calculated (**Table 3** and **Fig. 1**). Compared to the chromatograms of the healthy control group urine, chromatograms of the urine samples from the dogs with mammary gland tumors revealed increased concentrations of TYR, E, L-DOPA, 5-HIAA, IS, 5-HT, and KYNA. In contrast,

Table 2. Chromatographic parameters for urine metabolites derived via DAD or FLD

Detection	Metabolite	Retention time (min)	Regression equation	Standard curve linearity (R^2)
DAD	Creatinine	0.717	y = 0.3889x	0.9997
	DOPAC	6.917	y = 0.6198x	0.9997
	KYNA	9.427	y = 1.0333x	0.9998
FLD	E	1.039	y = 38550.0444x	0.9991
	L-DOPA	1.599	y = 16747.3489x	0.9991
	TYR	2.425	y = 4590.4682x	0.9988
	VMA	3.762	y = 2118.4295x	0.9985
	5-HT	4.139	y = 658758.7562x	0.9994
	IS	6.752	y = 183019.6362x	0.9993
	TRP	7.485	y = 241663.4940x	0.9990
	5-HIAA	8.665	y = 17127.7056x	0.9993

DAD, diode array detector; FLD, fluorescence detector; DOPAC, 3,4-dihydroxyphenylacetic acid; KYNA, kynurenic acid; E, epinephrine; L-DOPA, 3,4-dihydroxy-L-phenylalanine; TYR, tyrosine; VMA, vanillylmandelic acid; 5-HT, serotonin; IS, indoxyl sulfate; TRP, tryptophan; 5-HIAA, 5-hydroxyindolacetic acid.

Table 3. Levels of metabolites in the urine of study groups as determined by UHPLC and presented as median and interquartile range values (expressed as μmol/mmol creatinine)

Metabolite	Canine mammary gland tumor (n = 14)		Healthy control (n = 16)	
	Median	Interquartile range	Median	Interquartile range
TYR	7.85	7.32	5.50	4.98
	$p = 0.1079$			
E	30.47	34.27	0.37	0.35
	$p = 0.0303^{*}$			
L-DOPA	40.96	32.95	3.53	3.74
	$p = 0.0003^{***}$			
DOPAC	2.39	6.61	4.78	5.78
	$p = 0.4848$			
VMA	2.50	3.35	4.27	3.07
	$p = 0.5167$			
TRP	1.20	1.08	1.51	0.82
	$p = 0.3540$			
5-HIAA	9.83	18.87	2.02	4.11
	$p = 0.0003^{***}$			
IS	31.68	16.08	9.39	11.99
	$p = 0.0091^{**}$			
5-HT	0.45	15.24	0.02	0.06
	$p = 0.0019^{**}$			
KYNA	47.97	66.89	26.99	41.40
	$p = 0.0227^{*}$			

The *p* value of Mann-Whitney *U* test of urine metabolites in canine mammary gland tumor patients versus healthy control. UHPLC, ultra-high-performance liquid chromatography; TYR, tyrosine; E, epinephrine; L-DOPA, 3,4-dihydroxy-L-phenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; VMA, vanillylmandelic acid; TRP, tryptophan; 5-HIAA, 5-hydroxyindolacetic acid; IS, indoxyl sulfate; 5-HT, serotonin; KYNA, kynurenic acid. *Correlation is significant at the 0.05 level; **Correlation is significant at the 0.01 level; ***Correlation is significant at the 0.001 level.

https://doi.org/10.4142/jvs.2020.21.e23

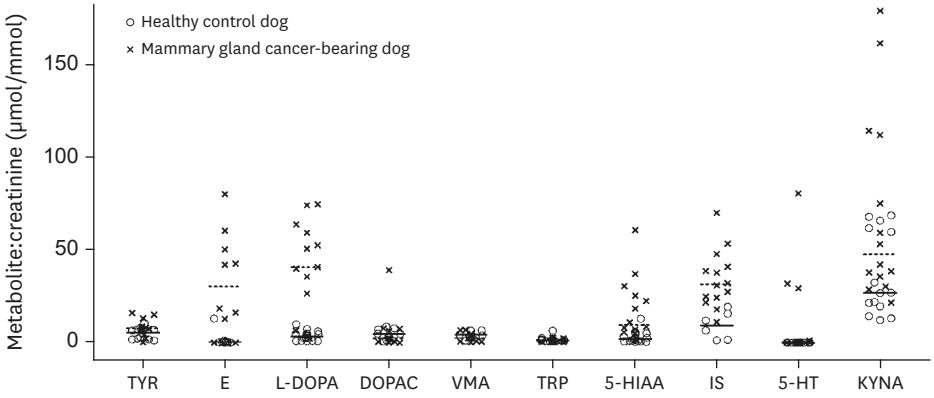

Fig. 1. Urinary metabolite to creatinine ratios in mammary gland cancer and healthy control dogs. Horizontal lines represent median values. TYR, tyrosine; E, epinephrine; L-DOPA, 3,4-dihydroxy-L-phenylalanine; DOPAC, 3,4-dihydroxyphenylacetic acid; VMA, vanillylmandelic acid; TRP, tryptophan; 5-HIAA, 5-hydroxyindolacetic acid; IS, indoxyl sulfate; 5-HT, serotonin; KYNA, kynurenic acid.

lower concentrations were recorded for urinary metabolites TRP, DOPAC, and VMA in dogs with mammary gland tumors compared to the healthy control dogs. Comparison of the median values of metabolites in the two study groups showed significantly higher levels of E (82 times; $p = 0.03$), L-DOPA (11 times; $p = 0.0003$), 5-HIAA (5 times; $p = 0.0003$), IS (3.37 times; $p = 0.0091$), 5-HT (22 times; $p = 0.0019$), and KYNA (1.8 times; $p = 0.022$) in the canine mammary gland group (**Table 3**). Highly significant differences (a p value below 0.001) were recorded between healthy control and the mammary gland tumor dogs for the median values of the L-DOPA and 5-HIAA parameters.

Although the sample sizes were small, metabolite levels were compared among the 14 patients according to tumor size and tumor grade. Significantly increased levels of E, L-DOPA, 5-HIAA and 5-HT were recorded in the cases with histological grade III tumors (data not shown). Our results also suggested that tumor size did not affect metabolite levels (data not shown). Based on the results, we did not detect a relationship between tumor size and metabolite levels; however, there appears to be a relationship between tumor grade and metabolite level.

DISCUSSION

Our study was focused on the examination of selected urinary metabolites present in urine during neoplastic processes in dogs with mammary gland tumors. We assessed levels of TYR and its metabolites (L-DOPA, E, DOPAC, VMA), as well as TRP and its metabolites (5-HT, 5-HIAA, KYNA, IS), in urine from healthy dogs and those with mammary gland tumors.

Nam et al. [19] previously reported a decrease in amino acid levels such as TRP, TYR, and phenylalanine in the urine of breast cancer patients based on the results of gas chromatography–mass spectrometry analysis. They identified four metabolic biomarkers (homovanillic acid, 4-hydroxyphenylacetic acid, 5-Hydroxyindolacetic acid, and urea) with significantly different levels in cancer and normal subjects ($p < 0.05$) in their study. Our results showed decreased levels of the urinary TRP and TYR metabolites VMA and DOPAC. On the other hand, the level of TYR in mammary gland tumor-bearing dogs (median 7.85 μmol/mmol creatinine) was higher than that of healthy control group dogs (median 5.50

https://doi.org/10.4142/jvs.2020.21.e23

μmol/mmol creatinine), but the difference was without statistical significance. In mammary gland tumor dogs, urinary concentration of TRP (median 1.20 μmol/mmol creatinine), VMA (median 2.50 μmol/mmol creatinine) and DOPAC (median 2.39 μmol/mmol creatinine) were lower than those of healthy control dogs (TRP, 1.51 μmol/mmol creatinine; VMA, 4.27 μmol/mmol creatinine; and DOPAC, 4.78 μmol/mmol creatinine). However, the increased TYR and decreased TRP, VMA, DOPAC levels showed no statistical significance. Decreased urinary TRP may be associated with the high amino acid demand in cancer metabolism, and amino acid dependence is a common occurrence in cancerous cells [20]. Increased TRP consumption is suggested as a critical factor in progressive cancer [21].

The TRP metabolite, 5-HT is a monoamine neurotransmitter that modulates numerous behavioral and physiological functions. Moreover, 5-HT is a mitogenic factor in cancer cells and acts in an autocrine loop of growth factors, contributing to cell proliferation in aggressive cancers such as lung, bladder, and breast cancers [22]. 5-HT and its metabolite 5-HIAA have been used in the monitoring of disease progression as well as to assess reactions to treatment in patients with carcinoid disease [23] and breast cancer [19]. In our study, we observed a significant increased concentration of 5-HT ($p < 0.01$) and 5-HIAA ($p < 0.001$) in mammary gland tumor dogs compared to those of healthy control dogs.

TRP can be converted to indole through the actions of bacterial tryptophanase, and indole is sulfated into 3-IS in the liver. The IS metabolite is a uremic toxin that can stimulate glomerular sclerosis and interstitial fibrosis [24]. Our data revealed a significant increase ($p < 0.01$) in IS concentration in the urine of canine mammary gland tumor patients. Moreover, a high correlation coefficient (Spearman rank-order correlation test) was detected between TRP and IS ($r = 0.736^{\star}$, $p < 0.05$), assumed to be related to their metabolic relationship.

Our data revealed a higher KYNA level ($p < 0.05$) in canine mammary gland tumor patients than in healthy control dogs. Chen et al. [16] reported significantly increased TRP metabolites (i.e., indoleacetic acid, KYNA and nicotinuric acid) in patients with breast cancer. TRP is metabolized via the kynurenine pathway, wherein the main product is KYNA. Tryptophan 2,3-dioxygenase (TDO) catalyzes the oxidative cleavage of the indole ring of TRP with such catalysis regulated by adrenocorticosteroids, estrogens, and androgens. Increased KYNA in urine may be due to increased estrogenic activity within the mammary gland tumor. Interestingly, KYNA has a dual role in cancer, either by allowing malignant cells to escape from immune surveillance or by having an antiproliferative effect on cancer cells [25].

L-DOPA may be rapidly decarboxylated to dopamine, which in turn is metabolized to E and norepinephrine. The main end-product of degradation of norepinephrine and E is VMA, whereas the degradation product of dopamine is homovanillic acid. Determination of urinary and plasma catecholamine levels has an important role in the clinical diagnosis of neuroendocrine tumors (such as pheochromocytoma and medullary thyroid carcinoma) [26]. Muthuswamy et al. [27] suggested that E can promote the formation of an immunosuppressive microenvironment in human myeloid cells, as well as in breast and colorectal cancer tissues. Cyclooxygenase-2/tumor-associated factor prostaglandin (COX-2/PGE_2) antagonism may be useful in repairing unwanted modulation of stress-induced immune functions in the cancer microenvironment [27]. In our study, we observed an increased concentration of E (median 30.47 μmol/mmol creatinine, $p < 0.05$) in mammary gland tumor-bearing patients compared to that of control group dogs (median 0.37 μmol/mmol creatinine). A report by Barollo et al. [28] provided experimental evidence of the

overexpression of L-type amino acid transporters in neuroendocrine tumors and offered a molecular basis to clarify the increase in L-DOPA uptake observed in tumor cells. In our study, we confirmed a statistically significant high concentration of the TYR metabolite L-DOPA ($p < 0.001$) in the urine of mammary carcinoma-bearing dogs compared to that of the control group dogs.

In summary, TRP and TYR metabolisms seem to have a close relationship with canine mammary gland cancer. The differences in the intensities of the biomarkers were compared between mammary gland tumor and control dogs and two metabolic biomarkers (L-DOPA and 5-HIAA) were determined to be highly significantly different between the groups (p values < 0.001). In addition, IS and 5-HT were determined to be different at the $p < 0.01$ significance level and two metabolites (E and KYNA) differed with a significance level of $p < 0.05$.

An advantage of the HPLC method used in this study is that it is designed for studies with low sample requirements, minimal sample handling, and few work-up steps. However, the main drawbacks of this method, which could limit the precision of this method, are the sample collection and storage procedures before analysis. Such pre-analytical sample management issues can influence sample quality, which may crucially affect the final result [29]. In our study, the groups of urine samples were quickly frozen and stored in a freezer after collection. Also, the metabolome can be influenced by various endogenous and exogenous factors such as age, breed, diet, presence of disease, and drug exposure [30]. We tried to eliminate or reduce these factors by using a control group composed of different ages and breeds, and also by maintaining a 12 h fasting period in both groups. We are aware that associated diseases and/or drug exposure during surgery may be important factors influencing sample quality; however, those issues were beyond the scope of this study. Regardless, the results of this study represent a basis for future research using larger groups of mammary tumor-bearing and healthy control dogs.

In conclusion, mammary tumors are the most common type of canine neoplasm, with malignant variants detected in up to 50% of cases. There is intense interest in the identification of biomarkers that can distinguish diseased from normal individuals in human and veterinary fields. Non-invasive approaches, such as that used in this study, can reveal imbalances in biological systems and provide comprehensive information useful in disease monitoring. This study was focused on the determination of specific urinary metabolites (TRP and TYR and their metabolites) that are expressed differentially in canine mammary gland tumor-bearing animals. Our results revealed significant increases in the concentrations of the TRP metabolites 5-HT, 5-HIAA, KYNA, IS and the TYR metabolites L-DOPA and E in urine from mammary gland tumor-bearing dogs compared to the levels in healthy control dogs. Thus, monitoring of selected urinary TYR and TRP metabolites may be useful in non-invasive diagnostics as well as in monitoring therapeutic strategies for canine mammary carcinoma.

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