Urinary lipid production profile in canine patients with splenic mass

Taiki KIDA1)#, Arisa YAMAZAKI1)#, Tatsuro NAKAMURA1), Koji KOBAYASHI2), Sho YOSHIMOTO3), Shingo MAEDA4), Takayuki NAKAGAWA3), Ryohei NISHIMURA3), Takahisa MURATA1,2)*

1)Department of Animal Radiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
2)Department of Food and Animal Systemics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
3)Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan
4)Department of Veterinary Clinical Pathobiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

ABSTRACT. Polyunsaturated fatty acids (PUFAs), including arachidonic acid (AA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA), are metabolized to various lipid mediators. The profile of these lipid metabolites excreted into the urine reflects inflammatory state of the body and disease conditions. In this study, we quantified 156 types of lipids in urine samples of dogs with splenic mass, using liquid chromatography-tandem mass spectrometry. We found that metabolites of prostaglandin (PG) E2, F2α, and D2, 8-iso-PGF3α, lyso-platelet activating factor, and 14,15-leukotrien C4 significantly increased in urine samples of dogs with splenic mass compared to that of healthy dogs. These observations may reflect general inflammatory responses and will help better understanding of the canine splenic mass.

KEYWORDS: dog, lipid metabolite, splenic mass, urine

Polyunsaturated fatty acids (PUFAs) play essential roles in the body, serving as building blocks of the cell membrane and source of various bioactive molecules. PUFAs are classified into omega-3 such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and omega-6 such as arachidonic acid (AA), based on position of the last double bond in the carbon chain. AA is metabolized to key lipid inflammatory mediators such as prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs). The conversion of AA into these different lipid metabolites is mediated by oxygenases such as cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 epoxygenases (CYP) or enzyme-independent reactions [6, 8, 9, 19]. EPA and DHA are known as essential fatty acids, which mammals need to take from the diet because they cannot synthesize these PUFAs in the body de novo. As with AA, EPA and DHA are also metabolized to various lipid metabolites in an enzyme-dependent or independent manner [14].

Splenic masses are commonly seen in dogs. The differential diagnosis ranges from benign lesions like hematoma to malignancies such as lymphoma, hemangiosarcoma, and histiocytic sarcoma. Surgical resection is the primary method of treatment, and splenectomy is often performed followed by histopathological diagnosis. Benign masses can generally be cured with splenectomy, whereas malignant tumors usually carry poor prognosis with eventual metastasis. Chemotherapy is the only effective treatment option for these malignancies, which generally prolongs survival only for a few months [5, 13, 20, 23]. A better understanding of pathophysiological mechanism of these diseases could lead to identification of more efficacious therapeutics and support of early diagnosis or a screening test. Here, we quantified 158 lipids in urine of canine patients in comparison with healthy dogs, using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to describe the disease-associated profile of urinary lipid metabolites.

Canine splenic mass cases (n=8) with an available urine sample were identified in the Veterinary Medical Center of the University of Tokyo from 2017 to 2018. For comparison, we used the same dataset of lipid metabolites in urinary samples from healthy dogs as the previous study (n=12) which were harvested at Anim Pet Clinic (Tokyo, Japan), since we were not able to obtain another set of healthy samples. These dogs were confirmed to have no abnormality in medical checks including physical examinations, complete blood count, blood serum chemistry, urinary test, chest radiography, and ultrasound, as well as no history of allergy [10, 11]. All

*Correspondence to: Murata T: amurata@mail.ecc.u-tokyo.ac.jp, Department of Animal Radiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

#These authors contributed equally to this work.

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procedures used in this study followed the Institutional Guideline for the Care and Use of Animals of the University of Tokyo, and all the samples were collected and utilized under the owners’ informed consents obtained at admission. General information on each subject of healthy and splenic mass group is shown in the Tables 1 and 2, respectively. One splenic mass case (ID: 101) provided two urine samples taken and measured at the same time. Therefore, average of the two samples for each metabolite was used for the case. All dogs included in the current study received neither drugs that would directly affect production of lipid mediators, such as steroids and non-steroidal anti-inflammatory drugs, nor radiation therapy. The samples were handled and analyzed as previously described [14]. Urine samples were stored until used at −28°C. The collected samples were centrifuged (20,000 × g, 5 min) and the supernatant was mixed with internal standards (Table 3). After the solid phase extraction (Oasis HLB, Waters, MA, USA), the sample solutions containing lipids fractions were eluted with methanol. The sample solution (5 μL) was injected to liquid chromatography (Nexera 2, Shimadzu, Kyoto, Japan) equipped with mass spectrometer (LCMS-8060, Shimadzu). Lipids (158 targets and 16 internal standards) were analyzed by using LC-MS/MS Method Package for Lipid Mediators version 2 with LabSolutions software according to the manufacturer’s instruction. Relative amount of each metabolite was calculated as the ratio of peak area of each metabolite to that of corresponding internal standard. Resulting value was further corrected by the creatinine concentration measured by LabAssay™ Creatinine (Wako, Osaka, Japan).

In this study, we focused on lipid metabolites that were identified reliably in the urine. Identification of a metabolite was regarded reliable when it was detected in all individual samples within at least either group in comparison. Multivariate statistical analysis was performed using a Wilcoxon rank-sum test with Benjamini-Hochberg false discovery rate cutoff of 0.05 to find metabolites significantly different between two groups with a q-value threshold of 0.05. We identified 75 lipid metabolites in either or both of healthy and splenic mass groups and found some significant differences in quantity (Supplementary Table 1).

Dogs with splenic mass showed a significant increase in a metabolite of PGE₂, 13,14-dihydro-15-keto-tetranor-PGE₂. There was also an upward tendency of other PGE₂ metabolites, 13,14-dihydro-15-keto-tetranor-PGF₁β and tetranor-PGEM. These changes suggest that biosynthesis of PGE₂ is increased in dogs with splenic mass. Similarly, a metabolite of PGF₂α, tetranor-PGF₂ was significantly increased suggesting the upregulation of PGF₂α synthesis (Fig. 1). These results agree in part with a previous report using isolated canine tissues in that the spleen releases PGE₂ and PGF₂α at the resting state and the amount is enhanced upon stimulation, although this study assumed a different setting, i.e. nerve stimuli with electrodes or perfusion with epinephrine or nor epinephrine [2].

Interestingly, a metabolite of PGD₂, 13,14-dihydro-15-keto-tetranor-PGD₂ significantly increased, but another PGD₂ metabolite,

### Table 1. Characteristics of individual healthy dogs

| ID  | Sex | Age (Year, Month) | Breed        | Urine collection method       |
|-----|-----|-------------------|--------------|------------------------------|
| 001 | MC  | 10, 4             | Toy Poodle   | Spontaneous urination        |
| 002 | M   | 4, 2              | Maltese      | Urinary catheter             |
| 003 | FS  | 6, 11             | Pekinese     | Cystocentesis                |
| 004 | FS  | 1, 4              | Toy Poodle   | Spontaneous urination        |
| 005 | FS  | 4, 8              | Toy Poodle   | Cystocentesis                |
| 006 | F   | 3, 9              | Corgi        | Spontaneous urination        |
| 007 | FS  | 13, 9             | Shiba        | Spontaneous urination        |
| 008 | FS  | 8, 2              | Toy Poodle   | Cystocentesis                |
| 009 | FS  | 11, 2             | Miniature Dachshund | Cystocentesis          |
| 010 | F   | 12, 1             | Miniature Dachshund | Cystocentesis          |
| 011 | M   | 4, 8              | Toy Poodle   | Urinary catheter             |
| 012 | FS  | 8, 2              | Mix          | Cystocentesis                |

M, male; F, female; C, castrated; S, spayed.

### Table 2. Characteristics of individual dogs with splenic mass

| ID   | Definitive diagnosis          | Tumor diameter (cm) | Sex | Age (Year, Month) | Breed         | Urine collection method       |
|------|-------------------------------|---------------------|-----|-------------------|--------------|------------------------------|
| 101  | Follicular Marginal Zone Lymphoma | 3                   | MC  | 6, 8              | Toy Poodle   | Spontaneous urination        |
| 102  | Hematoma                      | 7.4 × 5.4 × 5.0     | MC  | 11, 10            | Miniature Dachshund | Urinary catheter             |
| 103  | Hematoma                      | 3 and 6 *           | F   | 14, 5             | Labrador Retriever | NA                          |
| 104  | Hemangiosarcoma               | 5.6 × 4.7 × 3.7     | MC  | 11, 11            | Labrador Retriever | Urinary catheter             |
| 105  | Myelolipoma                   | 4.8 × 4.4 × 3.8     | M   | 13, 8             | Miniature Dachshund | Urinary catheter             |
| 106  | Follicular Marginal Zone Lymphoma | 3.8 × 3.0 × 3.1     | MC  | 12, 1             | Toy Poodle   | Urinary catheter             |
| 107  | Splenic Marginal Zone Lymphoma | 1.7                 | MC  | 15, 0             | Chihuahua    | Urinary catheter             |
| 108  | Fibrohistiocytic Nodule       | 4                   | MC  | 10, 1             | Chihuahua    | Urinary catheter             |

M, male; F, female; C, castrated; NA, not available. *: Two masses were identified.
Table 3. The list of internal standards (IS)

| Name                                                      | Concentration (ng/mL) |
|-----------------------------------------------------------|------------------------|
| 1 Tetranor-Prostaglandin E Metabolite-d₆ (tetranor-PGEM-d₆) | 25.0                   |
| 2 6-keto-Prostaglandin F₁₅-d₄                              | 25.0                   |
| 3 Thromboxane B₂-d₄ (TXB₂-d₄)                             | 25.0                   |
| 4 Prostaglandin F₂α-d₄ (PGF₂α-d₄)                         | 25.0                   |
| 5 Prostaglandin E₂-d₄ (PGE₂-d₄)                           | 25.0                   |
| 6 Prostaglandin D₂-d₄ (PGD₂-d₄)                           | 25.0                   |
| 7 Leukotriene C₄-d₄ (LTC₄-d₄)                            | 25.0                   |
| 8 Leukotriene B₄-d₄ (LTB₄-d₄)                            | 25.0                   |
| 9 5(S) HETE-d₈                                            | 25.0                   |
| 10 12(S) HETE-d₁                                        | 25.0                   |
| 11 15(S) HETE-d₁                                        | 25.0                   |
| 12 Oleoyl Ethanolamide-d₄ (OEA-d₄)                       | 0.5                    |
| 13 Eicosapentaenoic Acid-d₃ (EPA-d₃)                      | 500.0                  |
| 14 Docosahexaenoic Acid-d₃ (DHA-d₃)                       | 50.0                   |
| 15 Arachidonic Acid-d₈ (ARA-d₈)                           | 500.0                  |

Fig. 1. Comparison of lipid metabolites derived from arachidonic acid between healthy group and splenic mass group. Quantity of lipid metabolites with significant difference (*) between healthy group (n=12) and splenic mass group (n=8). Values are represented as ratio of peak area of each metabolite to that of corresponding internal standard, normalized by the creatinine concentration of the sample. Lipid metabolites relevant to pathways above with noticeable changes that did not reach statistical significance are also shown. PG: prostaglandin, LT: leukotriene, HpEPE: hydroperoxy-eicosapentaenoic acid.
PGJ₂ was in downward tendency (Fig. 1). Whereas 13,14-dihydro-15-keto-tetranor-PGD₂ is produced from PGD₂ through enzymatic reactions [16, 18], PGJ₂ is generated by dehydration of PGD₂ in an enzyme-independent manner [12, 22]. Therefore, the balance between these metabolic pathways downstream of PGD₂ may be altered in dogs with splenic mass.

One of the most obvious differences between the two groups was seen in 14,15-leukotriene C₄ (14,15-LTC₄), which was constantly quantified in samples from splenic mass patients but barely detectable in healthy dog samples. This lipid falls in the category of eoxins, AA-derived metabolites generated through 15-LOX pathway [7]. Compared to the well-known 5-LOX pathway that is responsible for the biosynthesis of leukotrienes, the 15-LOX pathway was only recently reported. The key enzyme, arachidonate 15-lipoxygenase-1 (ALOX15), is highly expressed in reticulocytes, leukocytes, heart, and airway epithelia [3, 15]. Its expression is upregulated in ischemic heart and asthmatic airway, and eosins contribute to the local inflammation [17, 21]. Thus, the increased 14,15-LTC₄ may reflect the accelerated inflammation associated with splenic lesion.

In addition to AA-derived metabolites, there was also a significant increase in quantity of an EPA-derived metabolite, 8-iso-PGF₃α (Fig. 2). This is one of isoprostanes, which are enzyme-independent metabolites of PUFAs oxidized by endogenous reactive oxygen species (ROS). Little is known about the physiological or pathophysiological nature of 8-iso-PGF₃α, although another isoprostane, 8-iso-PGF₂α, has been characterized as a reliable biomarker in human biofluids that indicates oxidative stress of the body in diseases [1, 4]. Lyso-platelet activating factor (lyso-PAF), a precursor of PAF, was also increased in urine samples of dogs with splenic mass (Fig. 2). Lyso-PAF is produced from lyso-phosphatidylcholines in the plasma membrane mediated by phospholipase A₂, and then converted to PAF by lyso-PAF acetyltransferase. This remodeling pathway, but not de novo pathway, is the primary source of PAF in inflammatory conditions [24]. Thus, the current observation may also suggest the state of enhanced inflammation associated with splenic mass.

To our knowledge, this is the first study that reports significant difference in quantity of some lipid metabolites contained in urine samples between healthy dogs and splenic mass patients. Most of these changes in urinary lipid metabolite profile, namely increased metabolites of PGE₂ and PGF₂α, opposite direction of change in two pathways of PGD₂ metabolism and increased 8-iso-PGF₃α and lyso-PAF, were also found in dogs with liver mass in our previous study [11]. Furthermore, we included samples of canine patients with different diagnoses, both benign and malignant, in the splenic mass group. These facts suggest that metabolic changes described above reflect general inflammatory states of the tissues and are not specifically associated with respective diseases. With these general metabolic changes in splenic mass patients placed as a baseline, future studies with much more samples for each specific disease would be able to identify biomarkers that support more accurate and earlier diagnosis of each disease in a minimally invasive way.

One of limitations in the current study is the small sample size we were able to obtain with our best effort. It may have caused us to miss lipid metabolites that could otherwise be found differentially regulated between the healthy dogs and the splenic mass patients, or other potential correlations between tumor size or grade of malignancy and lipid metabolite profile. In addition, there was a significant difference in the age and male/female ratio between the healthy group and the splenic mass group (mean age: 7.4 and 12.0 years, respectively, P=0.00603, Welch’s t test; sample size of male/female: n=3/9 and n=7/1, respectively), as well as difference in breed (Tables 1 and 2) and body weight (not available). Another limitation is the inconsistent procedures of urine sample collection and storage through this study. Samples were harvested by using a catheter, cystocentesis, or spontaneous urination, and stored for different periods of time until being measured. All these differences among samples could potentially have affected the quantity of lipid metabolites.

In conclusion, we analyzed lipid metabolites in urine samples of canine patients with splenic mass and healthy dogs in an unbiased way using LC-MS/MS. We found some significant differences in quantity of lipid metabolites that suggest the enhanced inflammatory states associated with splenic mass. These observations will help better understanding of the canine splenic mass.

POTENTIAL CONFLICTS OF INTEREST. All the authors have no conflicts of interest to declare.

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