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NOTE

Physiology

Comprehensive profiling of lipid metabolites in urine of canine patients with liver mass

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ABSTRACT. Fatty acids are an essential component of mammalian bodies. They go through different metabolic pathways depending on physiological states and inflammatory stimuli. In this study, we conducted a liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based comprehensive analysis of lipid metabolites in urine of canine patients with liver mass. There were significant differences in quantity of some lipid metabolites that may be closely associated with the disease and/or general inflammatory responses, including increased metabolites of prostaglandin E_2 and/or $PGF_{2\alpha}$. We demonstrated that our approach of profiling lipid metabolites in the urine is useful in gaining insights into the disease. These findings may also have an application as a screening test or a diagnosis tool for canine liver mass.

KEYWORDS: dog, liver mass, lipid metabolite, urine

Polyunsaturated fatty acids (PUFAs) are an essential component of the body. They are classified into omega-6 (n-6) and 3 (n-3) according to position of the first double bond in the carbon chain. PUFAs are converted into various bioactive molecules that play key roles in animal health and disease. Arachidonic acid (AA), a member of n-6 PUFAs, is well-known as the precursor of key lipid mediators such as prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs), that are involved in various inflammatory responses in human and animal diseases. The conversion from AA to these eicosanoids is mediated by oxygenases such as cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 epoxygenases (CYP) or non-enzymatic reactions [5, 7, 10, 11, 18, 19]. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), members of n-3 PUFAs, are essential fatty acids that mammals should obtain from dietary food, since these PUFAs cannot be synthesized in the body. EPA and DHA also go through enzyme-dependent and independent reactions that generate multiple lipid metabolites [15].

Liver masses are frequently encountered in canine patients. The most prevalent diagnosis among primary liver tumors is hepatocellular carcinoma. Its prognosis largely depends on macroscopic morphology, namely massive, nodular, or diffuse. The treatment of choice is surgery, but nonresectable, metastatic, or incompletely resected carcinomas show dismal prognosis. Little is known concerning the disease mechanism [8, 22].

Given the large number of lipid mediators and their pathophysiological roles, their comprehensive profiling in specimens from patients or animal models would be a promising tool to understand the diseases. We previously performed a liquid chromatograph–mass spectrometry (LC-MS/MS)-based analysis of lipid metabolites in urine of healthy dogs, covering 117 types of metabolites derived from AA, EPA, and DHA, and listed ones that were constantly detected in all dogs [12]. Here, we expanded the previous study to quantify 158 lipids in urine of canine patients with liver mass in comparison with healthy dogs.

Canine liver mass cases (n=7) 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 control

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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 [12]. All 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 liver mass group is shown in the Tables 1 and 2, respectively. 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 [15]. 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 (Shimadzu) 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 74 lipid metabolites in either or both of healthy and liver mass groups and found some significant differences in quantity (Supplementary Table 1).

The liver mass group showed a significant increase of PGE_2 metabolites: tetranor-PGEM, 13,14-dihydro-15-keto-tetranor-PGE_2 and 13,14-dihydro-15-keto-PGE_2. There was also an upward tendency of PGE_2 itself in the liver mass group (Fig. 1). These results suggest that PGE_2 biosynthesis is upregulated in these canine patients. This result is in line with previous reports suggesting that liver injury induces the COX-2 expression and production of PGE_2 in human, which may affect hepatocarcinogenesis and the prognosis [3, 23]. Similarly, a significant increase of tetranor-PGFM (Fig. 1), the major urinary metabolite of $PGF_{2\alpha}$, indicates upregulated $PGF_{2\alpha}$ biosynthesis in dogs with liver mass as well. A significant increase of 20-hydroxy-$PGF_{2\alpha}$ was also seen in the liver mass group (Fig. 1). Although little is known about its biosynthetic mechanism, it is speculated that this metabolite has relevance to PGE_2 and/or $PGF_{2\alpha}$

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 liver mass

ID	Definitive diagnosis	Tumor diameter (cm)	Liver function tests		Sex	Age (year, month)	Breed	Urine collection method
			GPT (U/L)	ALP (U/L)				
001	Hepatocellular carcinoma	7.5 × 6.0 × 6.0 and 4.0 × 3.0 (*1)	335	1,915	M	12, 6	Miniature Dachshund	Urinary catheter
002	Hepatocellular carcinoma	2.7 × 2.6	260	603	MC	13, 0	Maltese	Urinary catheter
003	Hepatocellular carcinoma and adenoma	5.7 × 4.6	501	33,384	M	13, 4	Maltese	Urinary catheter
004	No definitive diagnosis	1.3 × 1.5 (*2)	119	1,523	MC	13, 2	Miniature Dachshund	Urinary catheter
005	Hepatocellular carcinoma	13.0 × 10.0 × 8.0	803	466	M	8, 6	Pomeranian	Urinary catheter
006	Hepatocellular carcinoma	5.2 × 11.5 × 9.6	NA	NA	NA	NA	Beagle	Urinary catheter
007	Hepatocellular carcinoma	9.5 × 5.5 × 5.5	325	13,656	M	8, 8	Mix	Urinary catheter

GPT, glutamic pyruvic transaminase; ALP, alkaline phosphatase; M, male; C, castrated; NA, not available. *1: Two masses were identified in right lateral lobe and left lateral lob, respectively. *2: Multiple masses were identified. Size of the largest one is shown.

synthesis, given its molecular structure [4, 21].

Interestingly, a metabolite of PGD_2, 13,14-dihydro-15-keto-tetranor-PGD_2, significantly increased whereas another metabolite PGJ_2 was in decreasing tendency (Fig. 1). PGD_2 can either be converted to 13,14-dihydro-15-keto-tetranor-PGD_2 and other metabolites by enzymatic reactions [16, 17] or go through non-enzymatic dehydration to generate PGJ_2 [13, 24]. The opposite changes in quantity of these PGD_2 metabolites may reflect a shift in the balance between the two different metabolic pathways in the presence of liver mass.

There was a significant decrease of PGA_1 and a downward tendency of PGA_2 in the liver mass group (Figs. 1 and 2). These cyclopentenone PGs of A series are produced from PGE series through non-enzymatic dehydration, and the reaction is affected by albumin concentration and pH [6, 20]. These factors may be

Table 3. The list of internal standards (IS)

	Name	Concentration (ng/mL)
1	Tetranor-Prostaglandin E metabolite-d_6 (tetranor-PGEM-d_6)	25.0
2	6-keto-Prostaglandin $F_{1\alpha}$-d_4	25.0
3	Thromboxane B_2-d_4 (TXB_2-d_4)	25.0
4	Prostaglandin $F_{2\alpha}$-d_4 ($PGF_{2\alpha}$-d_4)	25.0
5	Prostaglandin E_2-d_4 (PGE_2-d_4)	25.0
6	Prostaglandin D_2-d_4 (PGD_2-d_4)	25.0
7	Leukotriene C_4-d_4 (LTC4-d_4)	25.0
8	Leukotriene B_4-d_4 (LTB4-d_4)	25.0
9	5(S)-Hydroxyeicosatetraenoic Acid-d_8 (5(S)-HETE-d_8)	25.0
10	12(S)-Hydroxyeicosatetraenoic Acid-d_8 (12(S) HETE-d_8)	25.0
11	15(S)-Hydroxyeicosatetraenoic Acid-d_8 (15(S) HETE-d_8)	25.0
12	Oleoyl Ethanolamide-d_4 (OEA-d_4)	0.5
13	Eicosapentaenoic Acid-d_5 (EPA-d_5)	500.0
14	Docosahexaenoic Acid-d_5 (DHA-d_5)	50.0
15	Arachidonic Acid-d_8 (ARA-d_8)	500.0

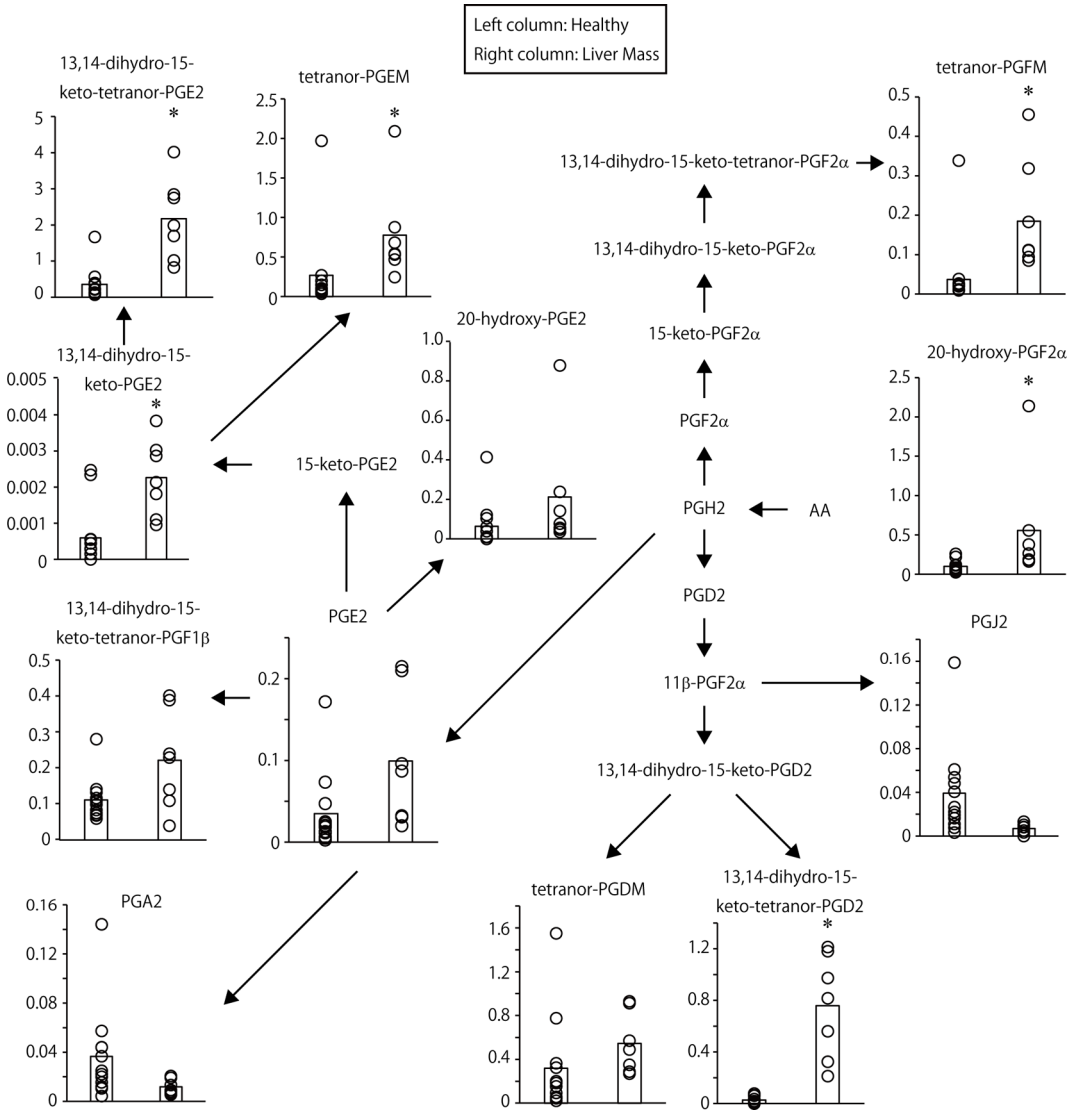

Fig. 1. Comparison of lipid metabolites derived from arachidonic acid between healthy group and liver mass group. Quantity of lipid metabolites with significant difference (*) between healthy group (n=12) and liver mass group (n=7). Values are represented as ratio of peak area of each metabolite to that 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.

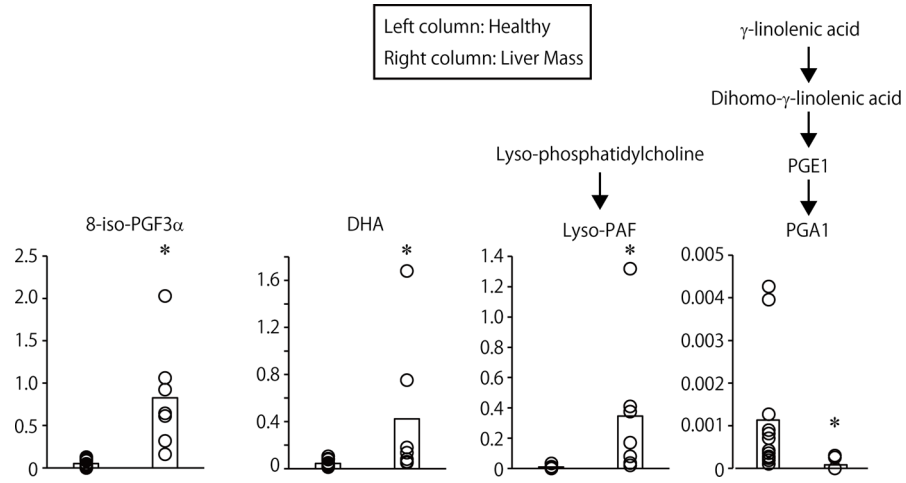

Fig. 2. Comparison of lipid metabolites in other classes between healthy group and liver mass group. Quantity of lipid metabolites with significant difference (*) between healthy group (n=12) and liver mass group (n=7). Values are represented as ratio of peak area of each metabolite to that of corresponding internal standard, normalized by the creatinine concentration of the sample. DHA: docosahexaenoic acid, EPA: eicosapentaenoic acid, PAF: platelet activating factor, PG: prostaglandin.

altered in dogs with liver mass in a way that suppresses the production of PGA series.

An essential n-3 PUFA, DHA was also increased in the liver mass group (Fig. 2). A previous study reported that liver is one of the major sites of accretion in the body for DHA obtained from diet [14]. Another study showed that liver cancer tissues of human contained significantly less DHA than the reference noncancerous tissues [9]. Therefore, one possible explanation for the current observation is that the hepatic uptake of DHA reduced due to lesions associated with liver mass, causing more DHA to remain in the circulation and eventually escape into the urine. There was also an interesting observation regarding EPA, another n-3 PUFA. One of its metabolites, 8-iso-$PGF_{3\alpha}$, was increased in the liver mass group (Fig. 2). This lipid metabolite is categorized as an isoprostane, which is generated through non-enzymatic oxidization of PUFAs by endogenous reactive oxygen species (ROS). Among isoprostanes, 8-iso-$PGF_{2\alpha}$ has been reported as a reliable biomarker of oxidative stress detected in human biofluids under disease conditions [1, 2]. To our knowledge, there is no report describing 8-iso-$PGF_{3\alpha}$ in a pathophysiological context so far. The current finding may also reflect increased oxidative stress associated with liver mass.

Dogs with liver mass also exhibited increased level of lyso-platelet activating factor (lyso-PAF), a precursor of PAF (Fig. 2). PAF is a potent proinflammatory lipid mediator, which is synthesized through two distinct routes, de novo and remodeling pathways. The latter route is responsible for the generation of lyso-PAF and it is the primary source of PAF in response to inflammatory stimuli [25]. Therefore, increased lyso-PAF may also reflect enhanced inflammatory response associated with liver mass.

In the current study, we found some significant changes in urinary lipid metabolite profile in dogs with liver mass compared to healthy dogs. These changes may be specifically associated with the disease and/or general inflammatory responses. Further studies in more detail are needed to precisely describe how these lipid metabolites are involved in canine liver mass, but they could potentially be used as a biomarker in urine samples, that may allow early disease detection and treatment or prognostic prediction in a minimally invasive way.

There are some limitations in this study. Firstly, a small number of subjects may have resulted in suboptimal statistical power. Thus, we may have missed some metabolites that would be otherwise found in different quantity between canine liver mass patients and healthy dogs. The limited sample size also caused noticeable difference in male-female ratio between the groups that may affect the analysis. Secondly, methods used in the present study to collect urine samples were inconsistent, namely through a catheter, by cystocentesis, or spontaneous urination. Besides, samples were stored for different periods until measurement. These differences may affect profile of lipid metabolites in urine samples. Thirdly, as liver mass generally occurs in older animals, there was a significant difference in the age between healthy dogs and liver mass patients in this study (mean: 7.4 and 11.5 years, respectively, P=0.013, Welch's t test), which may affect the amount of lipid metabolites. Larger sample size and a well-controlled procedure in the future studies will not only address concerns above, but also allow additional analyses which would gain further insights into the disease.

In conclusion, the current study demonstrated that our LC-MS/MS-based comprehensive analysis of lipid metabolites in urine is a promising approach to identify multiple metabolic changes of the body in an unbiased way even in a disease with limited previous reports. We revealed changes in lipid metabolite profile of dogs with liver mass for the first time. These findings will help us have a better understanding of the disease and may have an application as a screening test or a diagnosis tool.

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

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