Doctoral Dissertation

Unique lipid profile and its association with mastitis in human milk

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Contents

Abbreviations

AA	arachidonic acid
COX	cyclooxygenase
DHA	docosahexaenoic acid
DOHaD	developmental origins of health and disease
EPA	eicosapentaenoic acid
ESI-MS	electrospray ionization-mass spectrometry
FFA	free fatty acid
FGF	fibroblast growth factor
18-HEPE	18-hydroxyeicosapentaenoic acid
LC	liquid chromatography
5-LOX	5-lipoxygenase
LT	leukotriene
LX	lipoxin
NCD	noncommunicable disease
NSAIDs	non-steroidal anti-inflammatory drugs
PD1	protectin D1
PG	prostaglandin
PLA_2	phospholipase A_2
PPAR	peroxisome proliferator activated receptor
PUFA	polyunsaturated fatty acid
Rv	resolvin

SFA	saturated fatty acid
SPM	specialized pro-resolving mediator
TG	triacylglycerol
TX	thromboxane

I. Introduction

Lipids are essential constituents in food and the body not only because of their high energy value but also their several significances, such as biological membrane components and bioactive substances. Lipids are mainly found in the form of triacylglycerols (TG) in food and stored in biological membranes as phospholipids. Membrane structural lipids are sources of bioactive lipids. For example, steroids derived from cholesterol, platelet-activating factor, sphingosine-1-phosphate, and lysophosphatidic acid derived from phospholipids, pro-inflammatory and specialized pro-resolving lipid mediators derived from polyunsaturated fatty acids (PUFAs) as described below.

PUFAs including arachidonic acid (AA, C20:4, ω-6 PUFA), eicosapentaenoic acid (EPA, C20:5, ω-3 PUFA) and docosahexaenoic acid (DHA, C22:6, ω-3 PUFA) are major constituents of the cell membrane and are critically important as precursors of signal transmitters. The metabolites of AA, EPA and DHA act as bioactive molecules known as lipid mediators. AA-derived lipid mediators often referred to as eicosanoids, play important roles in various physiological and pathophysiological events, acting in an autocrine or a paracrine manner[1]. Although AA-derived prostaglandins (PGs), thromboxane (TX) A_2, and leukotrienes (LTs) have homeostatic functions, their overproduction leads to initiation or exacerbation of inflammation and therefore they are called pro-inflammatory lipid mediators[2] (Fig. 1). On the other hand, AA-derived lipoxins (LXs), EPA-derived resolving (Rv) E series, and DHA-derived RvD series, protectins and maresins are often referred to as specialized pro-resolving mediators (SPMs), which exhibit anti-inflammatory and pro-resolving actions[3–5] (Fig. 2). A proper balance of various lipid mediators and their precursor PUFAs is important for the maintenance of

tissue homeostasis and health, and its disturbance often leads to induction or exacerbation of various diseases.

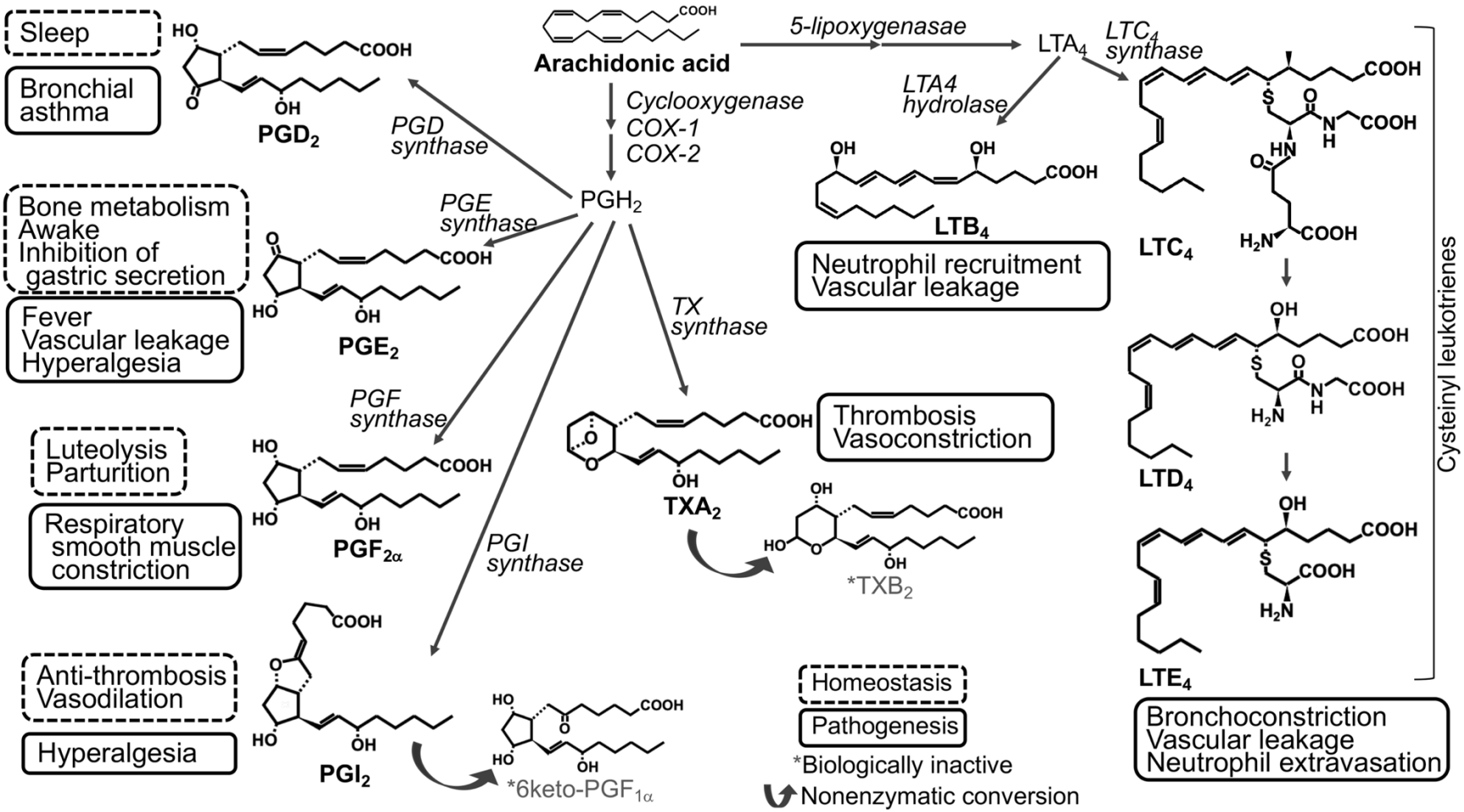

Fig. 1. Proinflammatory lipid mediators.
These lipid mediators bind to specific receptors and exerts various physiological and pathophysiological effects. Enzymes are in *italics*.

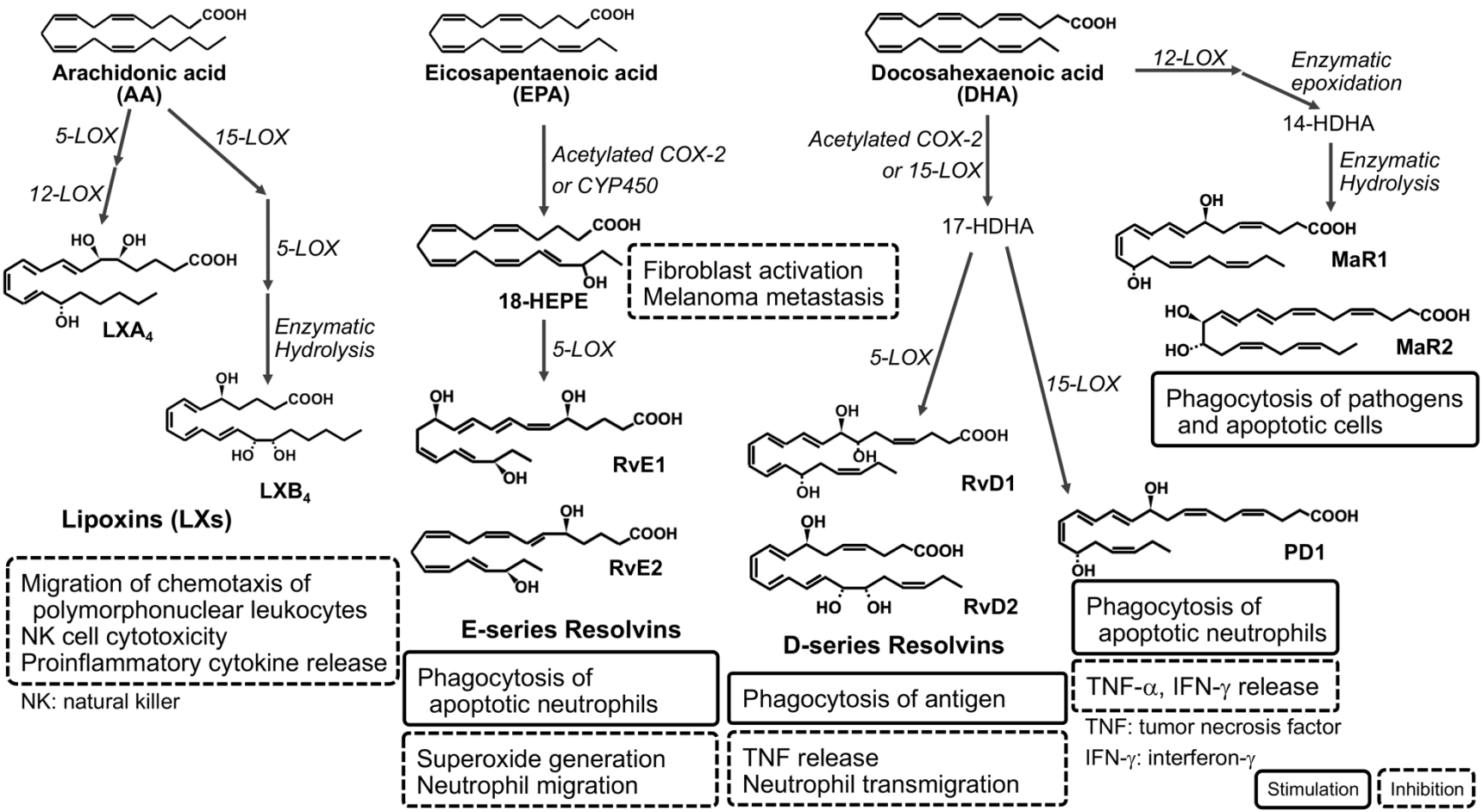

Fig. 2. Pro-resolving lipid mediators.
These lipid mediators are leads for resolution physiology.
Enzymes are in *italics*.

Noncommunicable diseases (NCDs; i.e., lifestyle-related diseases) such as cardiovascular disease, cancer, chronic respiratory disease, and diabetes mellitus are one of the most profound public health issues in the world[1] and are induced by chronic overproduction of pro-inflammatory lipid mediators. Given the pathophysiological roles of pro-inflammatory lipid mediators, inhibition of their overproduction is the therapeutic point of inflammatory disease including NCDs. In clinical treatment, cyclooxygenase (COX), a rate-limiting enzyme of the synthesis of PGs and TXA_2[6], and 5-lipoxygenase (5-LOX), a key enzyme of LTs synthesis, are thought to be the drug target. Nonsteroidal anti-inflammatory drugs (NSAIDs) which are COX inhibitors, and zileuton which is 5-LOX inhibitor are used clinically. However, NSAIDs have been reported to cause side effects such as gastrointestinal adverse effect and cardiovascular risk[7,8], and zileuton has side effects such as liver toxicity[9,10]. Therefore, because novel inhibitors of these enzymes without side effects are needed to prevent and ameliorate chronic inflammatory disorders, we explored functional food ingredients targeting the synthetic pathway of pro-inflammatory lipid mediators. Previously, it was demonstrated that *Dioscorea japonica* extract reduced PGE_2 production in cancer and inflammatory cells[11], and exhibited preventive effects on skin carcinogenesis[12]. The ingredient diosgenin ameliorated LPS-induced liver injury via downregulation of PGE_2 synthetic pathway[13]. In addition, we also demonstrated that red-kerneled rice proanthocyanidin and malabaricane C extracted from nutmeg (*Myristica fragrans*) inhibited the 5-LOX activity, decreased the LTB_4 production, and ameliorated the symptoms of psoriasis-like mouse skin inflammation[14,15]. Thus, the production of lipid mediators regulated by exogenous ingredients will be effective to maintain the health and reduce the risk of the related diseases.

At the time of the neonatal stage, breast milk is the major sauce of nutrition. Human milk provides essential nutrients and bioactive products that support the development of the infant immune system[16]. Lipids constitute approximately 30% of human milk content (other than water) and are important as an energy source. In addition, some PUFAs, such as AA and DHA, are essential components among milk lipids for optimal visual and neurologic development[17]. Overconsumption of SFAs leads to the development of lifestyle-related diseases and replacement of SFAs with PUFAs reduces the incidence of coronary heart disease in adults[2,3], although there is no evidence of the adverse effects of the overconsumption of SFAs on growth and development in child[18]. Exposure of infants to SFAs such as palmitic acid (C16:0) affects the balance of intestinal innate immunity and induces a highly inflammatory environment, thereby leading to induction or exacerbation of atopic dermatitis[19]. Therefore, the content of SFAs and the ratio of SFAs and PUFAs supplied by milk during the very early stage of life could crucially influence the proper development of the immune system in later life. However, only a few reported studies have comprehensively analyzed free fatty acids (FFAs) and their metabolites in human milk. Since milk is not only essential for the health of infants but that for young children and even beyond, comprehensive characterization of the profile of milk lipids is of particular importance.

Infants are incapable of properly digesting long-chain fatty acid TG because of their lower pancreatic lipase activity, and therefore intake of fatty acids in the form of FFAs is favored. In the present study, therefore, we investigated the profiles of long-chain FFAs and their metabolites (*i.e.*, PUFA-derived lipid mediators) in human milk in comparison with cow milk. Additionally, the differences in lipid profiles between normal

and mastitic human milk were analyzed, identifying two particular lipid mediators as potential biomarkers of mastitis.

II. Materials & methods

Milk samples

Human milk samples were obtained from fifty donors, both healthy and with mastitis, at Uchikado maternity center (Kurashiki, Japan) and Tanpopo maternity center (Kurashiki, Japan), and the donors were diagnosed by midwives who recorded lactation age and any symptoms of mastitis. On the basis of breast-related symptoms such as erythema, swelling, pain, heat, induration, axilla lymphadenopathy, accessory breast tissue, engorgement, pus, and systemic fever (37°C or higher), milk provided by the subjects was divided into two groups (normal milk and mastitic milk). The donors were 30.5 ± 0.587 (mean ± SEM) years of age, had an average body mass index of 20.6 ± 0.301 kg/m^2, parity 1.67 ± 0.107 children and with a period after birth ranging from 1 day to 18 months (mean 4.87 ± 0.597 months). Among these baseline characteristics, only the period after birth differed significantly between the normal donors and the mastitic donors (normal, 6.34 ± 0.882 months; mastitis, 3.79 ± 0.760 months). Cow milk samples were obtained from Okayama Prefectural Technology Center for Agriculture, Forestry, and Fisheries, Research Institute for Livestock Science (Okayama, Japan). All samples were stored at -20°C. The protein concentration and the total fat content were determined by the Lowry method and the Röse-Gottlieb method, respectively. The TG concentration was determined using LabAssay™ Triglyceride (FUJIFILM Wako Shibayagi Co., Gunma, Japan) in accordance with the manufacturer's instructions.

All aspects of the human study were approved by the Ethics Committee of Okayama Prefectural University, Okayama, Japan (protocol No. 17-63) and carried out in conformity with the Declaration of Helsinki. Written informed consent was obtained for each donor. All experiments were performed according to Japanese laws and institutional guidelines.

Lipid extraction from milk

The lipid constituents of the milk were extracted using the Bligh and Dyer method and prepared for lipidomic analysis as described previously[20]. For analysis of FFAs, the extract was prepared from 1 mL of milk and 100 pmol of *d5*-labeled EPA was added as an internal standard to calibrate the recovery of fatty acids throughout the procedure. For analysis of PUFA metabolites, the extract was prepared from 10 mL of milk by mixing with 20 mL of ice-cold methanol containing 100 pmol of *d4*-PGE_2 as an internal standard. The mixture was kept at 4°C for 30 min with shaking and then centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was diluted with 70 mL of 0.03% (v/v of water) formic acid and then loaded onto methanol-conditioned Oasis HLB cartridges (Waters Co., Milford, MA) equilibrated with 0.03% (v/v) formic acid. Each cartridge was washed with 0.03% (v/v) formic acid, followed by hexane. Then, the extract eluted with 3 mL of methyl formate was dried under nitrogen and redissolved in 50 mL of 60% methanol for liquid chromatography (LC).

Electrospray ionization-mass spectrometry (ESI-MS)

FFAs and metabolite levels were assessed using a 4000Q-TRAP quadrupole-linear ion trap hybrid mass spectrometer (AB Sciex, Framingham, MA) with LC capability (NexeraX2 system; Shimadzu Co., Kyoto, Japan) [20]. Ten microliters of the prepared sample was applied to a C18 column (Kinetex C18, 2.1 x 150 mm, 1.7 mm, Phenomenex, Inc., Torrance, CA) coupled for ESI-MS/MS. For FFA analysis, the sample applied to the column was separated using a step gradient with mobile phase A (acetonitrile:methanol:water, 1:1:1 (v/v/v) containing 5 mM phosphoric acid and 1 mM ammonium formate) and mobile phase B (2-propanol containing 5 mM phosphoric acid

and 1 mM ammonium formate) at a flow rate of 0.2 mL/min at 50°C. For PUFA metabolite analysis, the sample was separated using a step gradient with mobile phase C (water containing 0.1% acetic acid) and mobile phase D (acetonitrile:methanol, 4:1; v/v) at a flow rate 0.2 mL/min at 45°C. Signature ion fragments for each target lipid were monitored and quantified by multiple reaction monitoring. Lipids were identified by the multiple reaction monitoring transition and retention times based on the peak area of the multiple reaction monitoring transition and the calibration curve for an authentic standard of each compound.

Statistical analysis

Data are expressed as the mean ± SEM. Comparisons of two groups were performed using Wilcoxon-Mann-Whitney rank sum test and Welch's test at a significance level of $p < 0.05$. Associations between milk FFAs or PUFA metabolites and lactational month were analyzed using Spearman correlation.

III. Results

III-1. Comparison of lipid profiles between human milk and cow milk

Proportion of total fat, protein TG, and long-chain FFAs in normal human and cow milk

Both human milk and cow milk contained approximately 45 mg/mL total fat (Fig. 3A). The protein concentration in human milk (12.6 ± 0.524 mg/mL) was 55% lower than that in cow milk (28.1 ± 2.31 mg/mL) (Fig. 3B). The TG content of human milk (1.81 ± 0.175 g/dL) was 1.6-fold higher than that of cow milk (1.11 ± 0.114 g/dL) (Fig. 3C).

The concentrations of long-chain FFAs in human milk and cow milk were 997 ± 197 mM and 53.6 ± 3.04 mM, respectively. The ratio of TG long-chain FFAs in human milk was 10-fold higher than that in cow milk (Fig. 3D). The proportions of SFAs and PUFAs differed between human milk and cow milks. Human milk contained almost the same amounts of SFAs and PUFAs, while cow milk contained 2.4-fold higher levels SFAs than PUFAs (p=0.0022) (Fig. 3E). The ratio of PUFAs to total FFAs in human milk was 1.8-fold higher than that in cow milk (Fig. 3E). In human milk, the total content of ω-3 PUFAs (EPA+DHA) was almost the same as that of ω-6 PUFA (AA) with an ω-3/ ω-6 ratio of 1.32 ± 0.154 ((EPA+DHA)/AA), whereas in cow milk, the content of ω-3 PUFAs (EPA+DHA) was only a quarter that of ω-6 FFA (AA) (Fig. 3F). The ratio of SPMs (LXA_4, LXB_4, and EPA/DHA metabolites)/pro-inflammatory lipid mediators (AA metabolites except for LXA_4, LXB_4) was significantly higher in human milk than in cow milk (p<0.0001, Fig. 3G).

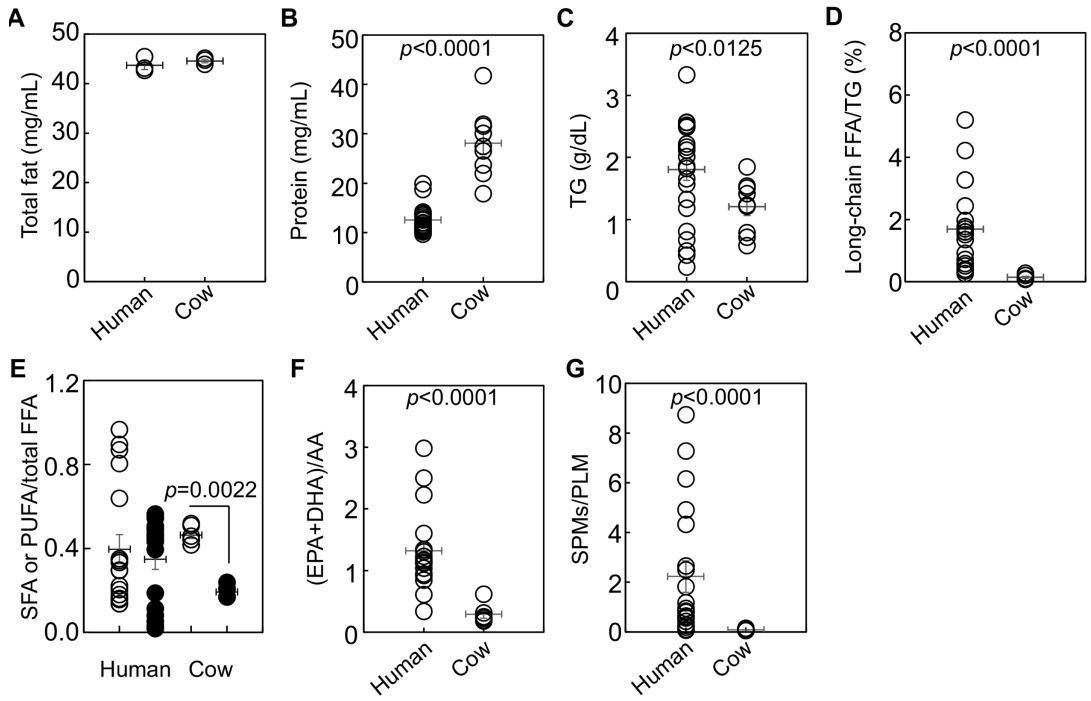

Fig. 3. Differences between human and cow milk constituents.

Total fat levels in bulk milk samples were determined using the Röse-Gottlieb method. Data were compiled from three experiments (A). Milk protein concentration was measured by the Lowry method (B). TG concentration in milk samples was determined using LabAssay^{TM} Triglyceride (FUJIFILM Wako Shibayagi Co., Gunma, Japan) in accordance with the manufacturer's instructions (C). Long-chain FFA/TG ratio (D), SFA or PUFA/total FFA ratio (E: clear circles, SFA; solid circles, PUFA), (EPA+DHA)/AA ratio (F), SPM/pro-inflammatory lipid mediator (PLM) ratio (G). For calculation of the long-chain FFA/TG ratio (D), the amount of each long-chain FFA (g/dL) was calculated from the concentration and molecular weight. SPM is the total of LXA_4, LXB_4, 18-HEPE, RvE1, PD1, RvD1 and RvD2. PLM is the total of PGD_2, PGE_2, $PGF_{2\alpha}$, TXB_2, 6keto-$PGF_{1\alpha}$, LTB_4, LTC_4, LTD_4 and LTE_4. Human milk, n=23, cow milk, n=9 (B and C); human milk, n=18, cow milk, n=6 (D-F); human milk, n=20, cow milk, n=9 (G). Values are shown as means ± SEM. Data were analyzed by Wilcoxon-Mann-Whitney rank sum test.

In terms of the quantity of individual lipids, human milk showed the following long-chain FFA profile: oleic acid (C18:1) ≥ linoleic acid (C18:2) ≥ stearic acid (C18:0) > C16:0 > α-linolenic acid (C18:3, ω-3 PUFA) ≥ others, and contained 5.40 ± 0.943 μM AA, 2.94 ± 0.592 μM EPA and 3.34 ± 0.686 μM DHA (Fig. 4A and B). Cow milk showed the following long-chain FFA profile: C18:1 > C16:0 > C18:0 ≥ C18:2 > C20:4 > others, and contained 1.13 ± 0.177 μM AA, 0.240 ± 0.0392 μM EPA, and 0.0631 ± 0.0177 μM DHA (Fig. 4C and D).

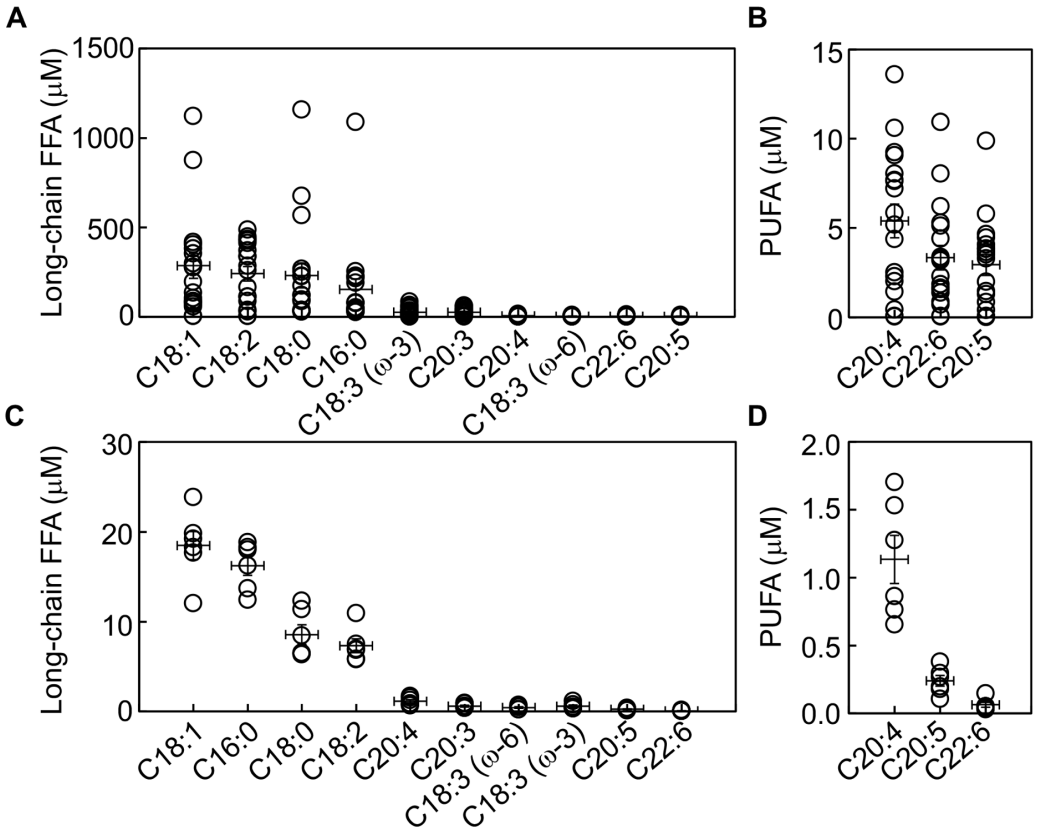

Fig. 4. Levels of long-chain FFA in human milk and cow milk.

Levels of Long-chain FFA in normal human milk (A and B) and cow milk (C and D). Data are presented as the mean ± SEM. n=18 (A and B), n=6 (C and D), n.d.: not detected.

Profiles of PUFA metabolites in human milk and cow milk

Fig. 5 shows the profiles of PUFA metabolites in human milk and cow milk. Human milk contained various AA metabolites in the amount order PGE_2 (0.633 ± 0.151 nM) > PGD_2 (0.396 ± 0.101 nM) > LXB_4 (0.226 ± 0.105 nM) > LXA_4 (0.181 ± 0.0639 nM) > others (Fig. 5A); cow milk contained 6-keto-PGF_{1a} (1.67 ± 0.381 nM) > PGF_{2a} (1.34 ± 0.507 nM) > TXB_2 (0.329 ± 0.0850 nM) > PGD_2 (0.240 ± 0.0413 nM) > others (Fig. 5D). Cysteinyl LTs were not detected in human milk but were substantially present in cow milk (0.00439 ± 0.00116–0.131 ± 0.0360 nM).

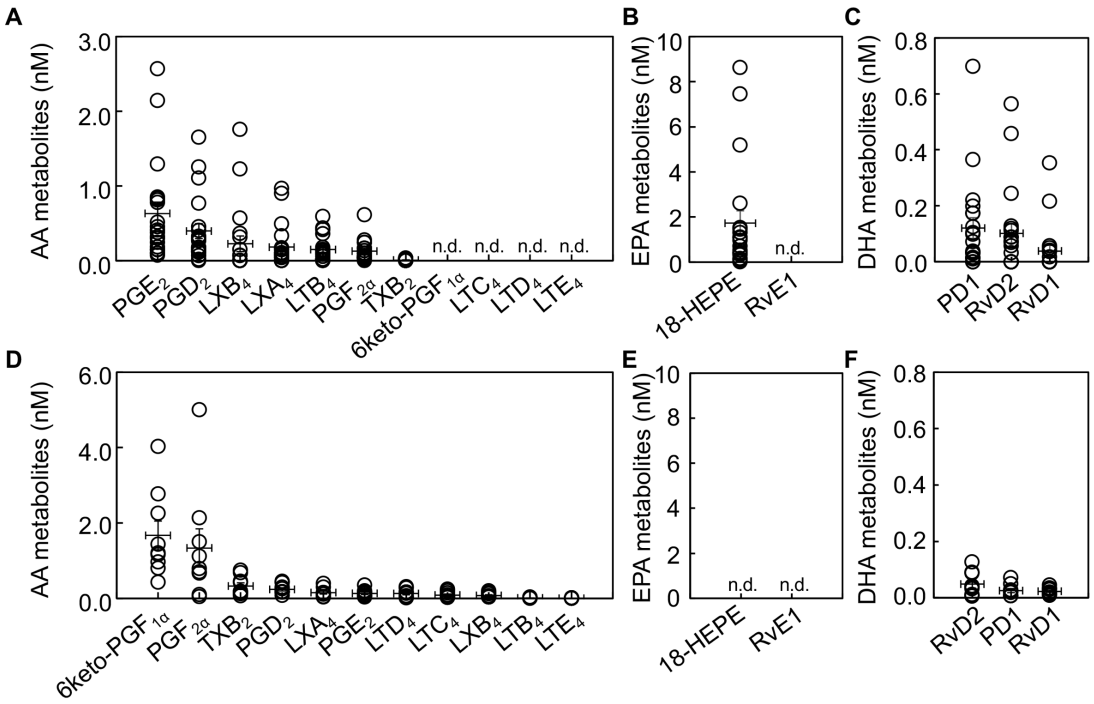

Fig. 5. Levels of long-chain PUFA metabolites in human milk and cow milk.

Levels of AA metabolites (A and D), EPA metabolites (B and E) and DHA metabolites (C and F) in normal human milk (A-C) and cow milk (D-F). Data are presented as the mean ± SEM. n=20 (A-C); n=9 (D-F), n.d.: not detected.

Interestingly, 18-hydroxy eicosapentaenoic acid (18-HEPE), one of the EPA metabolites, was the most abundant metabolite in human milk (1.72 ± 0.551 nM) but undetectable in cow milk (Fig. 5B and E). Several DHA metabolites, protectin D1 (PD1), RvD1 and RvD2, were detected in human milk (0.0386 ± 0.0199–0.120 ± 0.0373 nM), but their levels were much lower in cow milk (less than 0.0486 ± 0.0143 nM) (Fig. 5C and F).

Changes in the amounts of long-chain FFA and PUFA metabolites in human milk during lactation period

The amounts of SFAs, such as palmitic acid and stearic acid, were constant in normal human milk throughout the study period. In contrast, ω-6 PUFAs (C20:4, C18:3 (ω-6), and C18:2) and C20:3 continued to decrease as the lactation period progressed (R^2>0.5, r<-0.7, p<0.001), and ω-3 PUFAs (C20:5, C22:6 and C18:3 (ω-3)) showed a similar tendency (R^2>0.4, r<-0.6, p<0.004) (Fig. 6). However, the contents of PUFA metabolites were not significantly changed throughout the study period (Fig. 7).

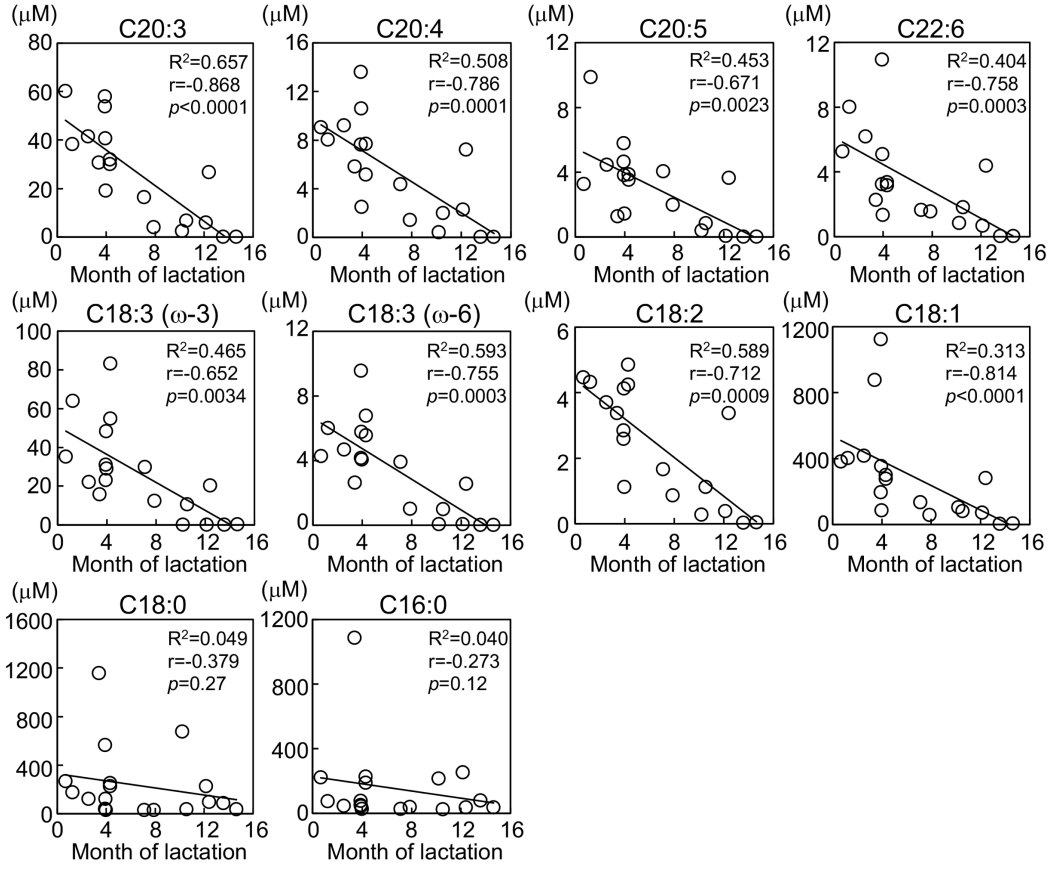

Fig. 6. Changes in levels of long-chain FFA in normal human milk during the lactation period.

Data were analyzed by Spearman's rank correlation test. n=18 in each graph.

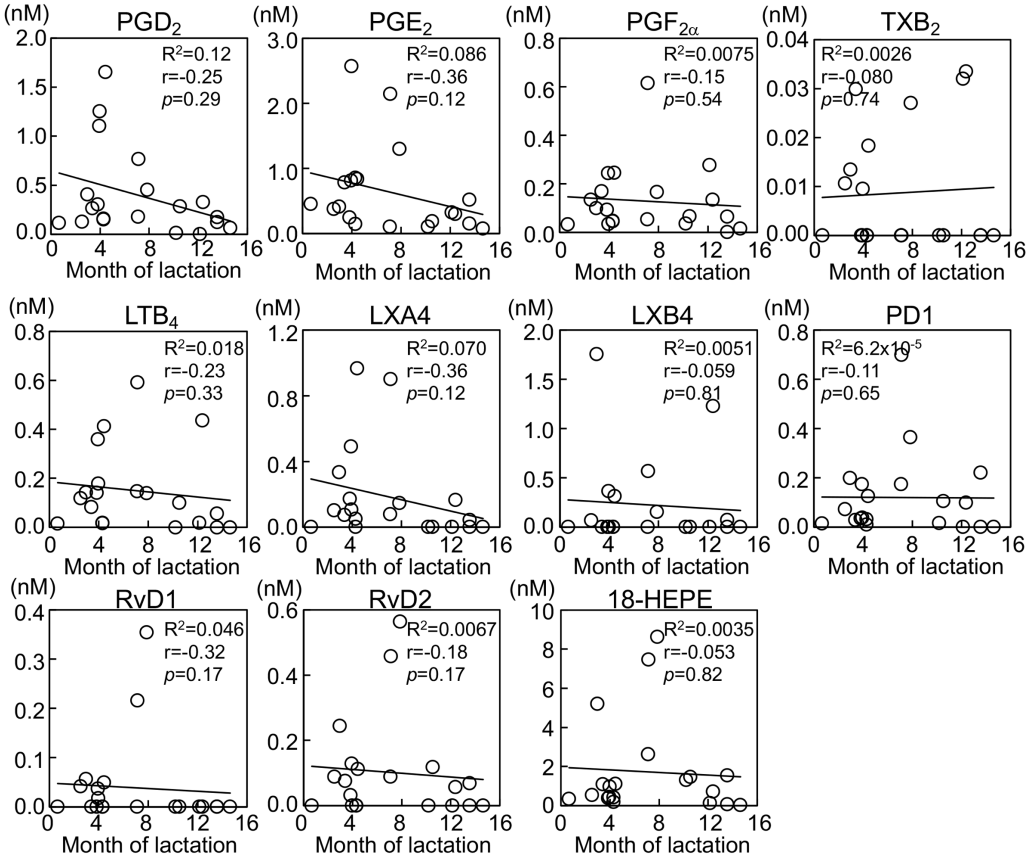

Fig. 7. Changes in levels of lipid mediators in normal human milk during the lactation period.
Data were analyzed by Spearman's rank correlation test. n=20 in each graph.

III-2. Changes in lipid profiles in mastitic milk

Given that lipid mediators play critical roles in inflammation, it would be expected that changes in lipid mediator profiles in milk would be related to exacerbation or resolution of mastitis, a common inflammatory disease in the mammary gland. Therefore, we next compared the lipid profiles in milk from patients with obstructive mastitis and healthy volunteers. Protein and TG concentrations did not differ significantly between milk samples from normal subjects and those from subjects with mastitis (Fig. 8A and B). Similarly, the ratios of long-chain FFA/TG, PUFA/total FFA, (EPA+DHA)/AA and SPM/pro-inflammatory lipid mediators, and the amounts of most AA and DHA metabolites were not significantly changed by mastitis (Fig. 8C−H).

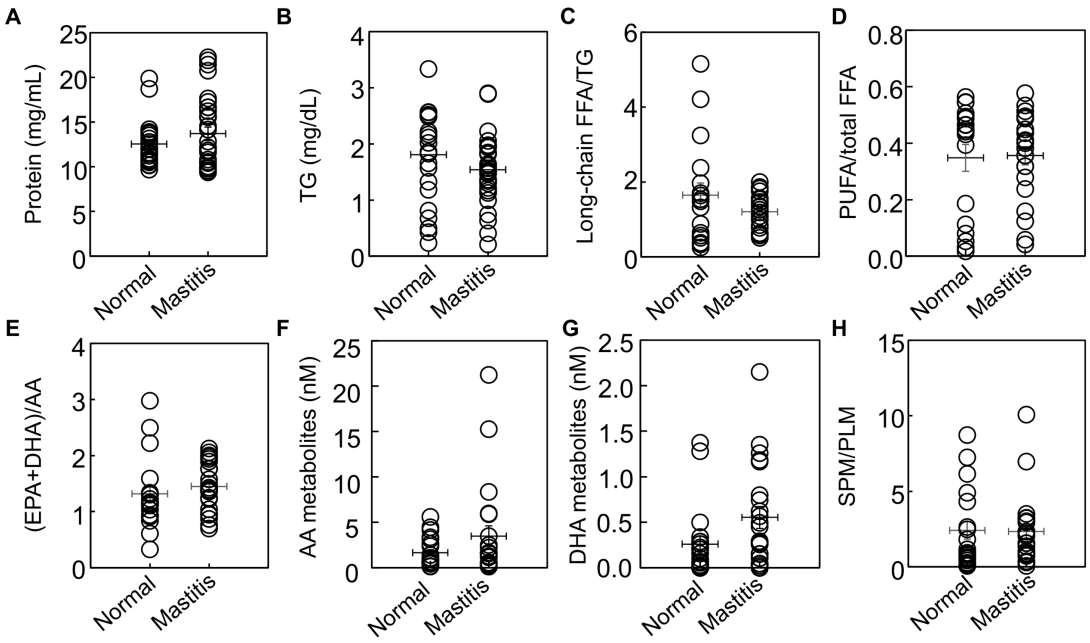

Fig. 8. Comparison of milk constituents between normal and mastitic human milks
Protein concentration (A), TG concentration (B), long-chain FFA/TG ratio (C), PUFA/total FFA ratio (D), (EPA+DHA)/AA ratio (E), AA metabolites (F), DHA metabolites (G), SPM/pro-inflammatory lipid mediator (PLM) ratio (H). AA metabolites mean a total of PGD_2, PGE_2, PGF_{2a}, TXB_2, 6keto-$PGF_{1\alpha}$, LTB_4, LTC_4, LTD_4, LTE_4, LXA_4 and LXB_4. DHA metabolites mean a total of PD1, RvD1 and RvD2. PLM means a total of PGD_2, PGE_2, PGF_{2a}, TXB_2, 6keto-$PGF_{1\alpha}$, LTB_4, LTC_4, LTD_4 and LTE_4. SPM means a total of LXA_4, LXB_4, 18-HEPE, RvE1, PD1, RvD1 and RvD2. Data are represented as the mean±SEM. Normal=23; mastitis=30 (A and B), normal=18; mastitis=22 (C-E), normal=20; mastitis=22 (F-H).

The amounts of individual FFAs did not show significant differences between normal and mastitic human milk (Fig. 9). Exceptionally, among the PUFA metabolites, pro-inflammatory TXB_2 and pro-resolving PD1 were significantly increased to approximately 3.7-fold (p<0.03) and 2.2-fold (p<0.05), respectively, in mastitic milk relative to normal milk (Fig. 10).

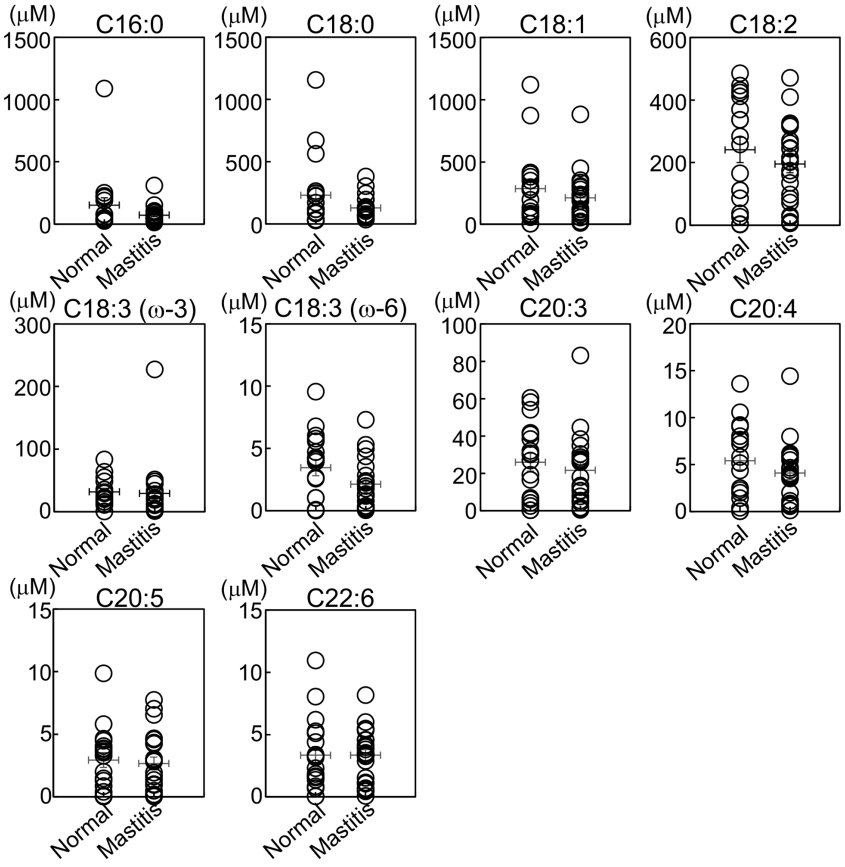

Fig. 9. Comparison of long-chain FFAs between normal and mastitic human milks
Data are represented as the mean ± SEM. Normal n=18; mastitis n=22 in each graph.

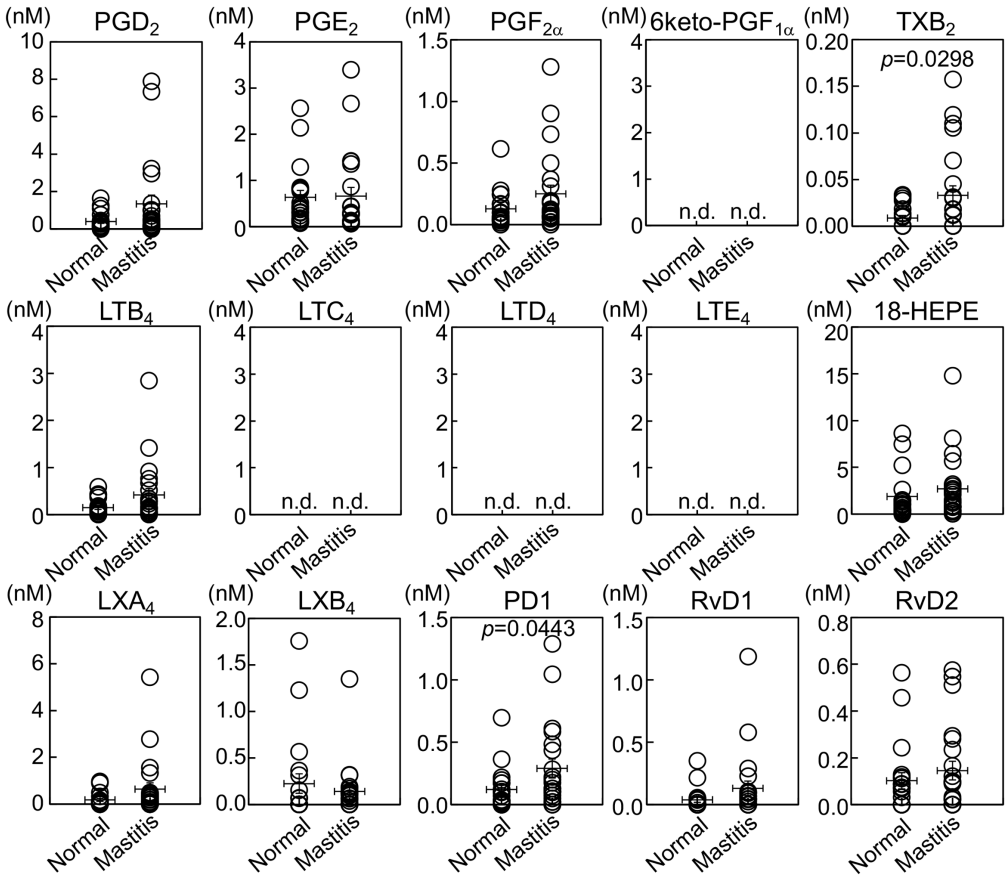

Fig. 10. Comparison of milk lipid mediators between normal and mastitic human milk.
Values are shown as means ± SEM. Data were analyzed by Welch's test (TXB_2) and Wilcoxon-Mann-Whitney rank sum test (PD1). Normal n=20; mastitis n=22 in each graph. n.d.: not detected.

VI. Discussion

We performed a comprehensive analysis of lipid constituents in human milk in comparison with those in cow milk, a major source of infant formula, and revealed distinctive features of the lipid profile including major FFAs and their metabolites in human milk. In contrast to cow milk, human milk was characterized by a high content of long-chain FFAs (Fig. 3D), particularly PUFAs (Fig. 3E), and a high ratio of ω-3 PUFA (EPA+DHA)/ω-6 PUFA (AA) (Fig. 3F). Secretion of pancreatic TG lipase from the pancreas and bile acid from the liver is very low in early infancy, although excretion and activity of gastric lipases including lingual lipase in infants are similar to those in adults[21]. Although 10–30% of TGs are hydrolyzed by gastric lipase in the stomach[21], this lipase cleaves TG molecular species with short- to medium-chain fatty acids in preference to those with long-chain fatty acids[22]. Thus, human milk, which contains higher levels of FFAs, is a desirable nutritional source for infants with immature digestive ability. It is well known that unsaturated fatty acid is a ligand of peroxisome proliferator-activated receptor α (PPARα). It is demonstrated that activated hepatic PPARα induces hepatic and plasma fibroblast growth factor (FGF) 21, which increased the expression of genes involved in thermogenesis within brown fat in infants[23]. In addition, prolonged breastfeeding activates brown adipose tissue thermogenesis, facilitates browning of white adipose tissue and controls the feeding behavior, eventually protecting against diet-induced obesity in adulthood[24]. Thus, the intake of breast milk with a large amount of PUFAs in infancy may contribute to increasing energy metabolic activity not only in infants but in adults.

Furthermore, human milk contains a higher content of PUFAs, especially ω-3 PUFAs and their metabolites, than does cow milk (Fig. 3G and Fig. 5A–F), indicating that human milk may have more biological activities driven by lipid mediators. The

difference in the content of PUFAs and the derived lipid mediators in human milk and cow milk may be due to differences in the diet or the expression and activation of the enzymes involved in their production. PUFAs constructed to the *sn*-2 position of phospholipids of biological membranes are released as FFAs enzymatically by phospholipase A_2 (PLA_2) super family. More than 50 PLA_2s or related enzymes are encoded in the mammalian genome, and are classified into several families on the basis of their structures, evolutionary relationships, and functions[25]. Sequentially the released PUFAs are metabolized to various lipid mediators by the other specific enzymes. It is required to investigate the related enzymes that characterized human milk with such profile of PUFAs and lipid mediators.

The temporal changes in lipid profiles during lactation showed that the levels of ω-3 and ω-6 PUFAs were higher in the early lactation period and gradually decreased thereafter, while those of SFAs did not change significantly throughout the period (Fig. 6). Compared with human plasma, in which the concentration of ω-6 AA is 1.7-fold higher than that of ω-3 PUFAs (EPA + DHA)[26], the concentration of EPA + DHA in human milk was 1.3-fold higher than that of AA. Moreover, we detected 18-HEPE in human milk at a concentration of 1.72 ± 0.551 nM, whereas its concentration in human whole blood is estimated to be only 0.3 nM[27]. Thus, enrichment of ω-3 PUFAs and their specific metabolites appears to be a signature lipid profile in human milk, confirming that proactive breast-feeding in the early lactation period would be more efficient for supply of bioactive lipids to infants than provision of cow milk.

Weiss *et al.*[28] have reported that the concentrations of C18:3 (ω-6), AA and DHA are decreased over the first four weeks of lactation, and Mohammad *et al.*[29] have demonstrated reduction of some of SFAs and PUFAs including AA and DHA, but not

EPA, from days 1 to 7 of lactation. Several studies have reported that the levels of DHA and EPA are increased, while that of AA remains unchanged, in human milk due to dietary intake of PUFAs during pregnancy[26,30]. On the other hand, it has been demonstrated that maternal intake of dietary AA, EPA and DHA during lactation increases the content of each in milk[26,30,31]. Thus, there has been no consensus as to whether the quality and quantity of dietary lipids would affect their composition or balance in human milk. Further investigations involving dietary surveys or interventions during pregnancy and lactation in combination with comprehensive lipidomic analysis will be required to fully clarify the relationship between maternal intake of lipids and their incorporation into breast milk.

A previous lipidomic analysis of milk from maternal mice fed linseed oil, which contains abundant α-linolenic acid, found an increased level of ω-3 PUFA metabolites, which inhibited skin allergy in the suckling pups[32]. Our present study showed that the level of ω-3 EPA-derived 18-HEPE was highest among the lipid mediators detected thus far, indicating 18-HEPE, in human milk may influence health promotion and disease prevention in infants. 18-HEPE has potent pro-resolving activity and protects against melanoma metastasis[33] and pressure overload-induced maladaptive cardiac remodeling[34]. The abundant presence of 18-HEPE in human milk leads us to speculate that it may have some beneficial effects on infants, and further investigation will be required to clarify its physiological importance. Based on the developmental origins of health and disease (DOHaD) theory[35,36], it has been thought that the nutritional environment during early infancy, i.e. the quality of the ingested milk, has significant effects on the health of the offspring not only during the developmental stage, but also during adulthood. In this

context, the amount and balance of PUFAs and their metabolites in human milk might affect the quality of life of the next generation.

Given that lipid mediators play critical roles in the immune system, changes in milk lipid mediators would be related to exacerbation or resolution of mastitis, an inflammatory condition of the mammary gland. This study demonstrated that the levels of AA-derived TXB_2 and DHA-derived PD1 were increased in human mastitic milk relative to normal milk. In contrast, Arnardottir *et al.* have reported that the levels of RvE1, LTB_4, PGD_2, PGF_{2a} and TXB_2 are higher in human mastitic milk than in normal milk[37]. The inconsistency between the two studies might be attributable to differences in race and dietary habits, the severity or duration of the disease, and also causal differences: suppurative mastitis, which is caused by bacterial infection, and obstructive mastitis, which is caused by clogged milk ducts. In our study, most of the mastitic patients had obstructive mastitis without suppurative symptoms. TXA_2, a pro-inflammatory AA metabolite that is unstable and rapidly converted to TXB_2 in biological fluid, is produced by platelets[38], monocytes[39], and endothelial cells[40], and plays important roles in platelet activation and aggregation, smooth muscle contraction, and inflammation[38,41]. The increase of TXA_2 in mastitic milk might result from induction of inflammation by galactostasis, where it might act locally within the mammary gland to promote obstruction of the duct, leading to exacerbation of mastitis. In contrast, pro-resolving PD1 is produced in eosinophils at an early phase of inflammation, limiting neutrophil infiltration and enhancing macrophage phagocytosis, and thereby accelerating resolution of the inflammation. Therefore, the increase of PD1 might reflect a counter-regulatory mechanism acting against inflammation in the mammary gland. Our finding suggests that AA-derived TXB_2 and DHA-derived PD1 may be promising novel biomarkers for early

detection of human obstructive mastitis. It seems that the obstructive mastitic milk has no baneful influence on the infant, because TXB_2 detected in mastitic milk is an inactive form of TXA_2, one of the pro-inflammatory mediators, and neither long-chain FFA profiles nor other molecular species of lipid mediators were altered in mastitic milk.

In conclusion, we have demonstrated that human milk is characterized by a high concentration of FFAs, especially PUFAs, a high ratio of ω-3 (EPA+DHA)/ω-6 AA, and a high proportion of SPMs, particularly EPA-derived 18-HEPE. These results support the significance of breast-feeding, in contrast to the usage of artificially produced milk formula or cow milk. Moreover, we have shown that elevated levels of TXB_2 and PD1 may represent novel biomarkers for obstructive mastitis.

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