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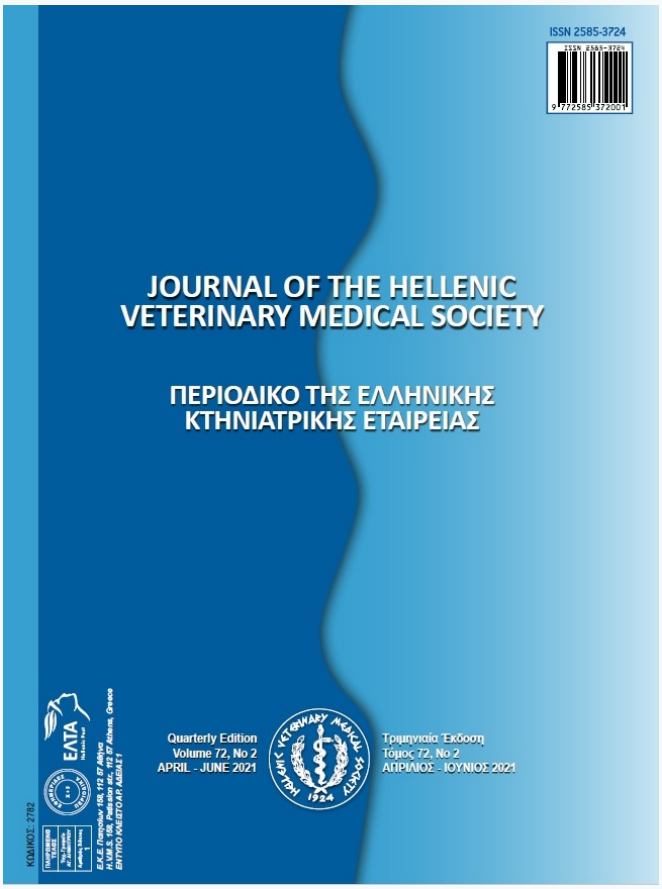

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Research article
Ερευνητικό άρθρο

Evaluation of blood omega-3 and omega-6 levels in healthy female dogs and female dogs with mammary tumours

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ABSTRACT: The study was designed to determine the blood levels of omega fatty acids, which have an important role in the etiology of mammary tumours, in healthy and female dogs with mammary tumour. The study was carried out in 9 female dogs with histologically confirmed mammary tumour and a control group with 9 healthy female dogs without clinical mammary neoplasia. 10 ml cephalic blood samples were collected by using a 21G x 1.5´´ blood collecting needle into anticoagulated tubes before the surgical removing of the mammary masses. Mastectomy was performed in all female dogs with mammary tumours and all the mammary specimens were sent to laboratory for histopathological examination. According to histopathological diagnosis results, all of the tumours were found to be malignant. Omega-3 levels were found to be higher in healthy female dogs ($p<0.001$) whereas omega-6 levels were higher in female dogs with mammary tumour ($p<0.001$). These observations support the notion tha thigh levels of omega-3 fatty acids might proove to have a protective role on mammary tumor formation in female dogs, while increased levels of omega-6 fatty acids may be related to an increased mammary tumor risk. This difference between omega-3 and omega-6 levels was found to be caused mainly by Eicosatrienoic acid. It is concluded that omega fatty acids mayplay an important role in the biological mechanism of mammary tumour in female dogs.

Keywords: female dog, mammary tumour, omega-3, omega-6.

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INTRODUCTION

Mammary tumours are important for canine health as they are encountered very commonly in female dogs. Reports indicate that among all mammalian species, female dogs present with the highest incidence of mammary tumours. Compared to other reproductive organs, the mammary glands are five times more prone to the development of tumours. Mammary tumours can be localized to a single gland or can be observed simultaneously in all mammary glands. In female dogs, approximately 40% of all mammary tumours occur in the inguinal mammary glands, and 60% occur in the thoracic and abdominal glands. Nearly 50% of all mammary tumours are of malignant character. Definitive diagnosis is based on the histopathological examination of an incisional or excisional biopsy (Bostock, 1986; Reddy et al., 2009).

Diet is suggested to have a major role in etiology of mammary neoplasia (MacLennan and Ma, 2010). Essential fatty acids (EFAs) are polyunsaturated fatty acids (PUFAs), which are not synthesized in the body, and therefore need to be ingested with food. Essential fatty acids are classified under two groups, namely, omega-3 (ω-3) fatty acids and omega-6 (ω-6) fatty acids (Gültiken and Vural, 2004; Zatsick and Mayket, 2007). The principal ω-3 fatty acids include linolenic acid (LNA), eicosapentaenoicacid (EPA), docosahexaenoicacid (DHA) andstearidonic acid (SDA) (Gebauer et al., 2005). Omega-3 fatty acids are found in vegetable oils (linseed oil, walnut oil), seeds, green-leafy vegetables, beans and nuts, and fatty fish. Furthermore, the principal ω-6 fatty acids include linoleic acid (LA), arachidonic acid (AA) and gamma-linoleic acid (GLA).Omega-6 fatty acids are found in maize oil, sunflower seed oil, soybean oil, cottonseed oil,safflower oil, peanut oil, and margarine, as well as in the liver, brain, and red meat and poultry meat (Lasekan and Ney, 1990; Greenly, 2002).

Essential fatty acids may contribute to formation of mammary neoplasia (MacLennan and Ma, 2010). In the extensive meta-analyze study of Fay et al. (1997), it was shown that ω-6 fatty acids promoted the tumour development substantially in the rodents. Rose et al. (1995) reported that diets containing linoleic acid supplemented with eicosapentaenoic acid or docosahexaenoic acid led to lesser tumour growth and lung metastasis compared to the diets with only linoleic acid in the athymic nude mice. In an *in vitro* study, arachidonic acid, a member of the ω-6 fatty acids, was shown to increase the proliferation of endothelial cells derived from human breast carcinomas and trigger formation of vessel-like structures(Pla et al., 2008). On the contrary, in the prepubertal rats, exposure to low-fat ω-3 PUFA diet caused reduction in the mammary tumorigenesis (Olivo and Hilakivi-Clarke, 2005). Exogenous supplementation of ω-3 fatty acid docosahexaenoic acid (DHA) in human breast cancer cells downregulates Her-2/neu expression and decrease tumour growth rate (Menendez et al., 2005). Sonnenschein et al. (1991) showed that high-fat diet had protective effects on the mammary tumour risk in dogs. But this study didn't have any detailed information on which type of fatty acid are present in the diet due to the scarce data about dog food preparations. Obesity or overweight might be associated with more aggressive tumours (Costa-Santos et al., 2019). This relationship between obesity/overweight is resulting from the activation of insulin/IGF-1 pathway, high level of pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and their influence on adipocytokines (Lim et al., 2015). The level of plasma insulin-like growth factor-I (IGF-I), known to have a regulatory role in tumour development, has been reported to decrease in mammary tissue with an increase in the ratio of omega-3 fatty acids to omega-6 fatty acids (Zhu et al., 2011).

Supplementation for long period than three months with fish oil, rich in omega-3 fatty acids, can control dyslipidemia in dogs. It is expressed that dyslipidemias are possible factors related with accelerated tumor genesis (Costa-Santos et al., 2019). Major changes occured in our diet since the Agricultural Revolution began 10.000 years ago (Simopoulos, 1999). Especially, type and amount of essential fatty acids in the diets changed dramatically (Eaton and Konner, 1985). In today's industrialized societies, diets include more ω-6 fatty acids and less ω-3 fatty acids (Eaton and Konner, 1985; Simopoulos, 1999). Due to the increased production of industrial food and the altered nutrient composition of animal feedstuffs, various food such as red meat, fish and poultry meat have become significantly poorer in ω-3. Therefore, the dietary intake of ω-3 fatty acids has fallen below that of ω-6 fatty acids (Candela et al., 2011).

It is indicated that food rich in ω-6 triggers the production and release of oestrogen, the increased levels of which are known to induce tumour growth, in adipose tissue in human (Nagata et al., 2007).Therefore, it is suggested that reducing the dietary intake of ω-6 fatty acids could decrease the prolactin-binding capacity of tumours and thereby, slow down tumour

growth in rats (Cave and Erickson-Lucas, 1982).Increasing the ratio of omega-3 fatty acids toω-6 fatty acids in the diet is considered an important tool in reducing the risk of mammary tumours and preventing their post-excision recurrence (Rose, 1997).

The ω-6 fatty acid most used by tumour cells is reported as AA (MacLennan and Ma, 2010). Linoleic acid, which is an ω-6 fatty acid, is converted to AA, a precursor of eicosanoids, when metabolized in the body. A high level of dietary intake of ω-6 fatty acids increases the level of AA in the body, and thereby, increases the production of pro-inflammatory eicosanoids such as prostaglandins, which are synthesized from AA (Gebauer et al., 2005). Malignant tumour cells are reported to synthesize a higher level of eicosanoids than benign tumour cells, and eicosanoids are known to be capable of accelerating tumour growth. As eicosanoids synthesized in the body are produced from EFAs, the eicosanoid level of the tumour and host are both affected by the type and level of EFAs in the diet (Cave, 1996).

The EFA least used by mammary tumour cells is EPA, anω-3 fatty acid, which is used in the treatment of certain types of cancers (Ward and Singh, 2005). In the body, by means of various desaturase isoforms, LA is converted to AA, and LNA is first converted to EPA and then to DHA. As ω-3 fatty acids and ω-6 fatty acids compete for desaturase enzymes, an increased consumption of LNA, EPA and DHA decreases the production ofAA (Cowing and Saker, 2001). The two main compounds required for eicosanoid synthesis are AA and EPA. Eicosanoids synthesized from AA (AA-derived eicosanoids) have properties opposite to those of EPA-derived eicosanoids. As AA and EPA compete for cyclooxygenases (COX) and lipoxygenases (LOX), they lead to the synthesis of eicosanoids with opposing properties. Generally, while AA-derived eicosanoids show a pro-inflammatory effect,EPA-derived eicosanoids have an anti-inflammatory effect (Cowing and Saker, 2001).

The study was designed to determine the blood levels of omega fatty acids, which may play an important role as modifiers of breast cancer risk, in healthy female dogs and female dogs with mammary tumors.

MATERIALS AND METHODS

Material

The study material comprised 18 female dogs of various breeds. Study group was created with German Shepherd (n:4), Terrier (n:3), Setter (n:2); and control group was composed by German Shepherd (n: 2), Terrier (n:3), Beagle (n:1), mix (n:3) breed. Nine of which were admitted to the veterinary clinic with signs of a mass structure (lump) in the mammary gland and were clinically diagnosed with mammary tumour, and the other 9 of which were healthy female dogs. Of the female dogs diagnosed with mammary tumour, four were eight years old, one was nine years old, and the remaining four were aged 10 years or older. The healthy female dogs were selected among animals older than 5 years of age. Reproductive history of the female dogs with mammary tumours and healthy female dogs include status of ovariohysterectomy, age at first estrus, number of full-term pregnancy, age at first pregnancy, hormonal treatment to prevent or inhibit estrus. Female dogs diagnosed with mammary tumors, 7 were not neutered and 2 were neutred previously and these animals had been neutered after the age of five. All healthy dogs for control group were selected from neutered and these animals had been neutered before the age of three. Any animals were not treated with the hormones to prevent or inhibit the estrus. All of the dogs are owned by the second owners. Therefore, no definite information about when the first estrus took place. None of the dogs in control group gave a birth except from one which had a dystocia in its first parturition. Only this dog had an ovariohysterectomy operation as an surgical treatment of dystocia. Of female dogs with mammary tumours, only two of them gave birth. The rest of them didn't have pregnancy. All animals were fed with homemade and dry commercial diet. Amount of commercial diet fed to the dogs are based on brand recommendation. Homemade meals are not main part of diet, generally given to the dogs as a reward per day. Body condition score (BCS) was performed according to nine-point BCS system (Laflamme, 1997). All animals were in the normal body weight (4-5/9).

Mammary tumors were found in inguinal mammary glands in 5 female dogs in which 3 female dogs had tumor on only one mammary gland while 2 female dogs had tumors on two mammary glands. Axillar mammary gland tumors were detected in 3 female dogs in which 2 female dogs had tumors on only one mammary gland while one female dog had tumors on two mammary glands. In one female dog multiple masses detected, one tumoral mass on axillary gland and two masses on inguinal gland.

Methods

Informed client consent was taken before the clinical examination of the dogs. Prior to clinical examination, the medical history including the location of the mammary masses, history of ovariohysterectomy, housing and general nutrition conditions of the female dogs was recorded on an inspection form. Ten-ml whole blood samples were collected by cephalic venepuncture by using 21G x 1.5´´ blood collecting needle from both the animals diagnosed with mammary tumour, prior to surgery, and the healthy animals, into anticoagulated tubes, and were stored during two months at -20 ◦C until being analyzed for fatty acids. The masses suspected of being mammary tumours were excised by mastectomy, and were transferred to the laboratory for histopathological examination after fixed with formalin. Any biopsy procedure was not performed before surgery to the tumoral mass.

Following atropine sulphate(Atropin 0.2%, Vetaş, Turkey)(0.45 mg/kg, sc) application for preanesthetic,general anesthesia was performed by xylazine hydrochloride (Basilazin %2, Bavet, Turkey) (2 mg/kg, im) and ketamine hydrochloride (Ketasol 10%, Richter Pharma AG, Austria)(10 mg/kg, im) combination. After general anesthesia routine surgical procedure was applied. Female dogs which had multiple masses on mammary gland were undergone unilateral total mastectomy (all tumor masses, mammary glands, inguinal and axillary lymph nodes were removed).Female dogs which had even single or more masses on inguinal or axillary gland were undergone unilateral partial mastectomy (primer tumoral mass, connected mammary glands and lymph nodes were removed).

Histopathological Method

The mammary masses, belonging to the 9 animals suspected of having mammary tumours, were firstly fixed in 10% formalin solution. The tissue samples were processed in an automated processor (Leica TP 1020), such that they were dehydrated through alcohol and xylolseries, embedded in paraffin and hardened on a cryoconsole (Hestion TEC 2800 Embedding Center), and sliced into5-µm-thick sections using a Leica RM 2135 rotary microtome. The sections were deparaffinised in xylol, passed through a series of graded alcohols (100%, 96%, 80%,70%), and stained with haematoxylin-eosin (H&E) (Luna, 1968). The sections were examined under a light microscope (Olympus BX35), and were photographed with a digital camera (Olympus SC 180).Tumour classification was based on the criteria described by Michael et al. (2017).

Fatty Acid Analysis

Ten-ml blood samples taken from all of the animals into anticoagulated tubes containing sodium EDTA were transferred to the laboratory under cold chain. The tubes were added 50 ml of diethyl ether, mixed on a shaker for 4 h, and centrifuged at 2000 rpm for 5 min. Blood fat was extracted from the supernatant. Fatty acid methyl esters were produced modified methods of Kocak et al. (2016). For this purpose, 4 ml of 2% methanolic NaOH was added to each sample (approximately 50-70 µl blood fat) for saponification at 95 °C for 2-3 min. Subsequently, 5 ml of 14% methanolic BF_3 was added, and the samples were maintained at 95 °C for 5 min. Next, the samples were added 2 ml of n-heptane, maintained at 95 °C for 1 min, added 4 ml of saturated NaCl, and centrifuged at 1000 rpm. Later, 1.5 ml-portions of the resulting supernatant were transferred into vials and analyzed by gas chromatography.

Fatty acid methyl esters were analyzed by gas chromatography (Trace 1300, Thermo Scientific, USA) using a flame ionization detector (FID). The FID temperature was adjusted to 260 ^{0}C. Cyanopropylpolysilphenylene-siloxane (length: 60 m, diameter: 0.25 mm × 0.25 µm, film thickness: 0.25 µm) (TR-FAME, Thermo Scientific, USA) was used in the column. The continuous flow rate of the carrier gas, helium, was set as 1.5 ml/min. The dry airflow rate was set as 300 ml/min, and the flow rate of hydrogen was programmed as 30 ml/min. The injection volume was 1 µl and the injection was performed in split form. The injector temperature was 250 ^{0}C. The oven temperature was initially set to 100 ^{0}C for 3 min, and was increased up to 240 ^{0}C at a rate of 4 ^{0}C/min. The unknown peaks in the chromatogram were identified by comparison with the standard FAME Mix (Chem-Lab, Belgium) of known composition, to determine the fatty acids.

Statitstical Analysis

The values obtained in fatty acid analyses were evaluated with Student's t-test using the Statistical Package for Social Sciences (SPSS, version 14.0) software.

RESULTS

The fatty acid composition of blood is shown in Table 1. Sum and ratios of blood fatty acids are shown in Table 2.

Table 1. Fatty acid composition of blood (Means ± SE)

Fatty acids (%)	Groups		P
	Control (n=9)	Tumour (n=9)	
Capriylic acid	0,013±0,001	0,133±0,071	0,184
Capric acid	0,079±0,002	0,165±0,040	0,075
Undecylic acid	0,012±0,001	0,019±0,004	0,326
Lauric acid	0,062±0,014	0,116±0,019	0,047
Tridecylic acid	0,012±0,001	0,047±0,007	0,001
Myristic acid	0,468±0,051	0,583±0,040	0,089
Myristoleic acid	0,057±0,009	0,086±0,017	0,200
Pentadecylic acid	0,156±0,018	0,106±0,014	0,045
Pentadecanoic acid	0,047±0,018	0,036±0,017	0,669
Palmitic acid	14,892±0,957	15,395±0,845	0,699
Palmitoleic acid	1,732±0,313	1,629±0,211	0,783
Heptadecanoic acid	0,367±0,031	0,284±0,048	0,189
Stearic acid	7,395±0,527	11,269±0,812	0,003
Oleic acid	19,734±1,22	18,711±1,013	0,525
Linoleic acid ω 6	26,469±1,412	25,260±1,457	0,566
Ɣ- Linolenic acid ω 6	0,750±0,127	0,490±0,069	0,025
Linolenic acid ω 3	0,209±0,067	0,102±0,015	0,109
Arachidic acid	0,647±0,103	0,362±0,057	0,022
Paullinic acid	0,266±0,034	0,311±0,071	0,604
Eicosadienoic acid ω 6	0,364±0,203	0,152±0,041	0,272
Eicosatrienoic acid ω 6	0,225±0,102	10,443±0,861	<0,001
Eicosatrienoic acid ω 3	11,980±0,973	0,230±0,043	<0,001
Arachidonic acid ω 6	0,692±0,410	0,151±0,090	0,173
Eicosapentaenoic acid ω 3	0,395±0,124	0,371±0,076	0,866
Heneicosanoic acid	0,280±0,046	0,226±0,031	0,337
Erucic acid	12,408±1,034	11,606±0,552	0,480
Docosadienoic acid ω 6	0,176±0,085	0,338±0,156	0,478
Docosahexaenoic acid ω 3	0,371±0,050	0,382±0,085	0,917
Lignoceric acid	0,798±0,037	0,710±0,121	0,544
Nervonic acid	0,064±0,009	0,391±0,143	0,059

Significant level was accepted as P<0,05

Table 2. Sum and ratios of blood fatty acids (Means±SE)

Fatty acid (%)	Groups		P
	Control (n=9)	Tumour (n=9)	
ΣSFA	23,892±1,272	29,136±1,042	0,005
ΣMUFA	34,678±1,174	33,053±1,167	0,347
ΣPUFA	41,428±1,235	37,809±1,305	0,066
ΣUFA	76,107±1,272	70,863±1,042	0,005
Σ PUFA/ ΣSFA	1,772±0,114	1,320±0,078	0,004
ΣUFA/ ΣSFA	3,254±0,185	2,472±0,124	0,002
Σ ω 6	28,471±1,449	36,722±1,298	0,001
Σ ω 3	12,748±0,962	0,984±0,146	<0,001
Σ ω 6/ Σ ω 3	2,344±0,226	42,296±4,166	<0,001

Significant level was accepted as P<0,05
SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids

Histopathological Findings

Histopathological examination demonstrated that each of the nine mammary masses were malignant tumours. Four of the cases were diagnosed with mixed carcinoma (Fig. 1). In these cases, histopathologically, the malignant epithelial fusion, observed in the form of irregular tubules, was supported by a fibrovascular stroma. This mass contained sporadic non-atypical cartilaginous formations and mesenchymal tissue composed of benign fusiform myoepithelial cells.

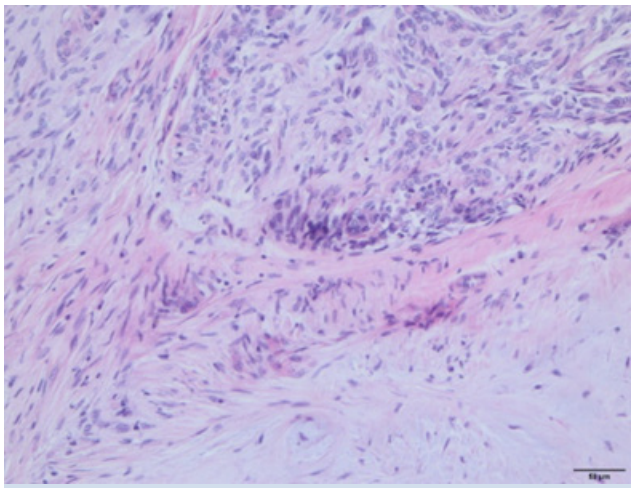

Fig.1. Mixed carcinoma

Three of the cases were diagnosed with tubular carcinoma (Fig. 2). These malignant tumours contained tubular and gland-like structures. The tubules were lined by a single layer or two layers of nuclear pleomorphic cells with an eosinophilic cytoplasm and hyperchromatic nucleus. The intertubular spaces contained sporadic aggregates of fibroblasts and inflammatory cells, including plasma cells, macrophages and lymphocytes.

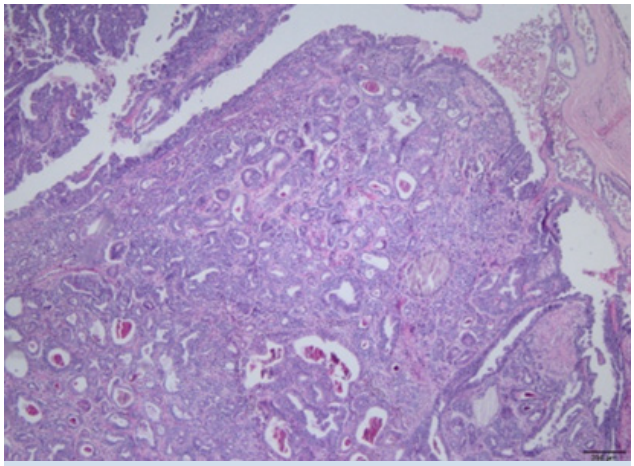

Fig.2. Tubular carcinoma

One case was diagnosed with malignant mesenchymal tumour (fibrosarcoma) (Fig. 3).This malignant tumour originated from the interstitial stroma of the mammary gland and was characterized by the proliferation of interwoven fusiform cells. The neoplastic cells had indistinct cell borders, a small amount of eosinophilic fibrillary cytoplasm, and an oval elongated nucleus.

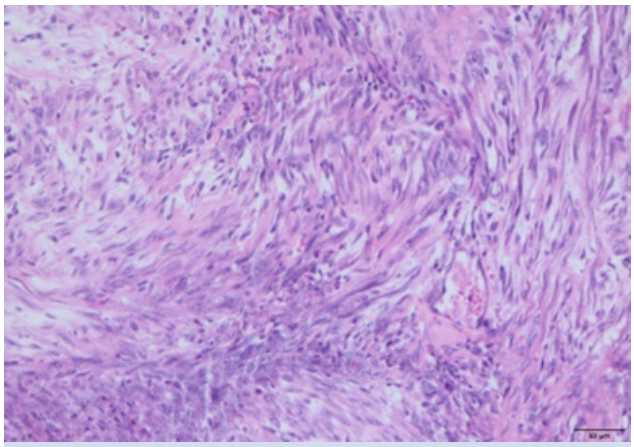

Fig.3. Malignant mesenchymal tumour (fibrosarcoma)

One case was diagnosed with carcinoma and malignant myoepithelioma (Fig. 4).The tumour contained both epithelial and myoepithelial components of malignant character. The cells, which varied from cuboidal to columnar in shape, formed irregular tubules. Furthermore, the interstitium contained atypical cells with an oval-fusiform vesicular nucleus and eosinophilic cytoplasm. These cells were surrounded by a small amount of basophilic fibrillary material (myxoid matrix), which was also observed within the cytoplasm of some of the cells.

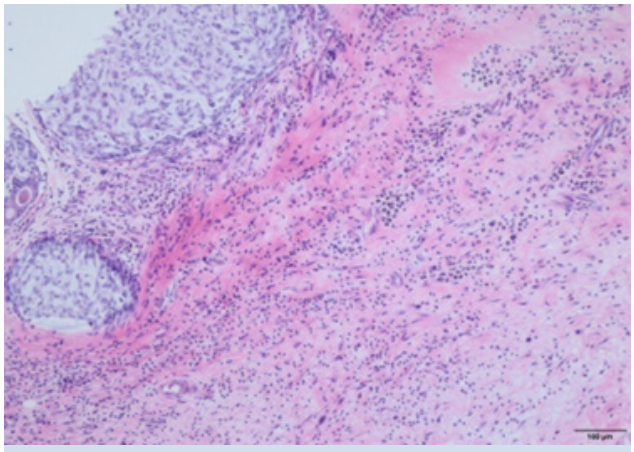

Fig.4. Carcinoma and malignant myoepithelioma

DISCUSSION

It has been reported that the serum fatty acid profile of an individual is a reflection of the fatty acid composition of his/her diet (Cohen et al., 1993), and that the ratio of ω-3 fatty acids to ω-6 fatty acids in the serum is an indicator of the level of dietary intake of these fatty acids (Yang et al., 2014). Thus, the measurement of serum LA levels is suggested as a reliable method of determining the dietary intake level of LA (Dougherty et al., 1987). Söderhjelm (1962) reported that linoleic acid supplementation caused substantial

changes in the level of the fatty acid in the serum in dogs. In a study examining the effect of dietary intake of fatty acid on serum fatty acid levels, it was shown that fatty acid supplementation can be used to change serum and cutaneous fatty acid composition of dogs. Sunflower (78% 18:2n6) supplementation to Beagle puppies increased serum linoleic acid (18:2n6) levels after three weeks. In the control group in which no supplementation of fatty acid are made, there is no significant change in serum fatty acid profile throughout the study period (12 weeks) (Campbell and Dorn, 1992). Therefore, it may be inferred that serum linoleic acid levels is a reflection of dietary fatty acid intake. In the present study, serum fatty acid levels were determined in both healthy female dogs and female dogs with mammary tumour (Table1).

In a study carried out in mice, Rose et al. (1995) determined that primary tumour growth was delayed and lung metastasis was less in animals given feed that was supplemented with ω-3 fatty acids(EPA and DHA), compared to animals given feed containing 8% of ω-6 fatty acids (LA). The researchers attributed this result to the inhibitory effect ofω-3 fatty acids on tumour eicosanoid biosynthesis.

There is a limitation in our study concerning diet. We couldn't exact amount of diet, preparation or variety of homemade food. However, due to the local eating habits, it is possible that dogs had been fed with red meats. Alenza et al. (1998) stated that homemade meals and high intake of red meat are associated with the risk for mammary tumour in female dogs. This contrasts with our results. In our study, both groups are fed with mainly homemade food.

Given the impact of diet on the development of mammary tumours in humans, diet-related factors have been well scrutinized. Multiple factors, including among others, advanced age, neutering, undergoing ovariohysterectomy after 2-5 years of age, and progesterone treatment, all increase the risk of mammary tumour development (Benavente et al., 2016). Simon et al. (2006) has reported that mammary tumour incidence is highest in female dogs aged 8 years and older. In the present study, four of these female dogs were 8 years old, one was 9 years old, and the remaining four were aged 10 years or older. Of the female dogs with mammary tumour, only two were reported to have undergone ovariohysterectomy, and these animals had been neutered after the age of five. Similarly, the healthy female dogs were also determined to be fed mainly on homemade food, and those that were older than 5 years of age were sampled for blood.

It is reported that the most common mammary tumours in female dogs are mixed tumours (67%), and adenomas and adenocarcinomas (32%) (Gültiken and Vural, 2004). Moulton (1990) suggested that almost half of all mammary tumours are of the mixed type, and 37% are adenocarcinomas. Furthermore, Fidler and Brodey (1967) indicated that of all mammary tumours, 50-65% are mixed tumours, 25-40% are carcinomas, and the remaining are either hyperplasic structures, adenomas or myoepitheliomas**.** In the present study, all of the mammary masses were confirmed to be malignant tumours. Four were diagnosed as mixed carcinomas (Fig.1), 3 as tubular carcinomas (Fig.2), 1 as fibrosarcoma (Fig.3), and 1 as carcinoma and malign myoepithelioma (Fig.4).

The inhibitory effect of ω-3 fatty acids on mammary tumours is explained by these EFAs supressing the generation of arachidonic acid-derived prostanoids (prostaglandin E2), which are responsible for inflammatory response, cell growth, apoptosis, angiogenesis and metastasis. It is reported that ω-3 fatty acids increase apoptosis and decrease the rate of mitosis in the mammary gland. It is also hypothesised that ω-3 fatty acids alter membrane stability in tumour cells (Habermann et al., 2010). Huerta-Yepezet et al. (2016) reported that omega-3 fatty acids show an antiangiogenic effect by inhibiting the synthesis of several angiogenic mediators, including vascular endothelial growthfactor (VEGF), platelet-derived growth factor (PDGF) and prostaglandin E2 (PGE2). Fabian et al. (2015) indicated that, EPA and DHA, which increase with arachidonic acid and belong to the family of ω-3 fatty acids, reduce the risk of mammary tumour development by decreasing the level of pro-inflammatory lipid derivatives, inhibiting the synthesis of nuclear factor-κB-induced cytokines, and reducing growth factor receptor signals due to cell membrane changes. In the present study, it was determined that serum ω-3 fatty acid levels were higher in the healthy female dogs, compared to those diagnosed with mammary tumour ($p<0.001$) (Table2). Besides, omega-6 level were found higher in female dogs with mammary tumour. The difference between ω-3 and ω-6 levels on mammary tumors was found to be caused mainly by Eicosatrienoic acid (Table 2).

Tumours consist of different cell types with stem-cell character having capability of self-renewal, differentiation and high-tumorigenic activity. Also, these cells are responsible for lower sensivity to chemotherapy and radiotherapy. These cells are term as cancer stem cells (CSCs) or tumor-initiating cells (TICs)

which are a contributing factor in initiation, recurrence and metastasis of tumours (Clarke et al., 2006; Nguyen et al., 2012). In canine mammary tumours, CSCs can be detected by using a sphere-forming assay. CSCs derived from canine mammary cancer lines are of high tumorigenic activity in immune deficient mice. CSCs also have ability to resist to anticancer drugs. So, CSCs show stem-cell like features (Michishita et al., 2011; Pang et al., 2011). In canine mammary adenocarsinoma cell culture study, DHA levels in sphere-forming cells (CSCs) were found to be low and variable (Michishita et al., 2011). It was stated that EPA and DHA inhibit the ability of self-renewal and decrease the survival of CSC via apoptosis (Erickson and Hubbard, 2010; Yang et al., 2013).

The main components for the production of eicosanoids are AA and EPA which is produced by metabolizing omega-6 and omega-3 fatty acids respectively. However AA and EPA, leads to the production of eicosanoids which has opposite effects. Typically, AA leads to the emergence of eicosanoids that stimulate mammary tumors, while EPA causes the emergence of eicosanoids which has preventive effects on mammary tumors (Rose, 1997; Cowing and Saker, 2001; Gebauer et al., 2005). It has been reported that ω-6 fatty acids increase the synthesis of AA, and thereby, accelerate cell division, supress the immune system, and enhance the growth, development and spread of tumours (Abou-El-Ela et al., 1989). It is suggested that a diet rich in ω-6 fatty acids disrupts the balance between cell proliferation and apoptosis in the mammary gland, and induces cell proliferation (Solanas et al., 2010). Moreover, it is indicated that eicosanoids produced from ω-6 fatty acids are involved in tumour angiogenesis, and thereby, facilitate the growth and metastasis of tumours (Rose, 1997). In a study carried out in mice (Lasekan et al., 1990), 16 weeks of dietary supplementation with safflower oil, known to be rich in ω-6,was observed to increase the incidence of mammary tumours and 74% of the cases were diagnosed with adenocarcinoma.

Based on the significant decrease they observed in the mammary tumour incidence of mice given EPA- or DHA-supplemented feed with an ω-3:ω-6 fatty acid ratio of 1:1.8, Noguchi et al. (1997) suggested that increasing the ω-3:ω-6 ratio of the diet could provide protection against the development of mammary tumours. In another study conducted by Rose et al. (1996), dietary ω-3 supplementation was observed to inhibit the local recurrence and metastasis of mammary tumours in mice, the mammary tumours of which were surgically excised. Literature reports suggest that rather than the level of ω-3, the ratio of ω-3 to ω-6 is important in relation to mammary tumour development, and a ratio ranging from 1:1 to 1:2 would provide protection against the growth and development of mammary tumours. In another study, Abou-El-Ela et al. (1989) demonstrated that food with anω-3: ω-6 ratio of 1:2 reduced the formation of mammary tumours induced by 7-12 dimethylbenzanthracene (DMBA). On the other hand, in the opposite situation, in the event of an increased ω-6:ω-3 ratio, it is reported that tumour incidence increases due to a decrease in the level of DHA among membrane phospholipids (Pettersen, 2012). In a research conducted by Yang et al. (2014) on 8,331 mammary tumour cases among 274,135 participants, the association of the ω-3:ω-6 fatty acid ratio with the risk of mammary tumour development was investigated, and it was determined that an increase of 1/10 in the ω-3: ω-6 ratio reduced mammary tumour risk by 6%. In their study in mice, Fabian et al. (2015) ascertained that increasing the total ω-6:ω-3 ratio in feed to >1 decreased the incidence and multiplicity of mammary tumours by 20-35%. In the present study, it was determined that the blood ω-6:ω-3 ratio was lower in the healthy female dogs, compared to those diagnosed with mammary tumour ($p<0.001$) (Table 2). This result supports the correlation suggested for the blood ω-6:ω-3 ratio and mammary tumour incidence in several literature reports (Abou-El-Ela et al., 1989;Rose et al., 1996; Noguchi et al., 1997; Pettersen, 2012; Yang et al., 2014; Fabian et al., 2015).

Mammary tumours generate a high level of ω-6 metabolites, such as prostaglandin E2, which are known to place immune functions under risk and induce tumour growth. On the other hand, ω-3 metabolites have been shown to prevent tumour growth. Increased levels ofω-3 are reported to have a potential anticarcinogenic effect exerted through the inhibition of the generation ofω-6 metabolites (Simonsen et al., 1998). Simonsen et al. (1998) reported that in mammary tumour cell cultures, increasing the ratio of ω-3 fatty acids to ω-6 fatty acids decreased the generation of prostaglandin E2 by the tumours cells, and thus, showed a negative correlation with mammary tumour development. In another study carried out in mice, Sasakiet al. (1998) determined that the PGE2 concentration in the tumour tissue was significantly lower in the group with a blood ω-3: ω-6 ratio of 1.03, compared to the group with an ω-3: ω-6 ratio of 0.01. Simopoulos (1991) determined thatω-3 fatty acids reduced the metastasis of mammary tumours to the lungs in mice. The results of Simo-

nopoulos (1991) agree with our results stating that the lower ω-3 fatty acids in tumour groups.

In an investigation on the impact of diet on mammary tumour development in mice, Wirfalt et al. (2002) determined that high dietary intake levels ofω-6 fatty acids increased the incidence of mammary tumours. Rose et al. (1991) assigned 58 female mice to two equal groups, and provided one group with feed that was added 23% of maize oil rich in ω-6 fatty acidsand the other group with feed that was added 5% of maize oil for a period of 15 weeks. At the end of the first week of the study, the researchers injected tumour cells into the mammary tissue of the mice, and at the end of the study period, the necropsies performed on the animals demonstrated that in the group given feed containing 23% of maize oil, 27 of the animals had developed tumours, whilst in the group given feed containing 5% of maize oil, 21 of the animals had developed tumours. Furthermore, in the group fed on a high-oil diet, out of the 27 animals with mammary tumour, 18 presented with lung metastasis, whilst in the group fed on a low-oil diet, out of the 21 animals with mammary tumour, 8 presented with lung metastasis. In result, the researchers determined that the incidence of mammary tumours was high in the mice fed on a diet rich in ω-6 fatty acids, and thereby, concluded that this type of a diet could also increase lung metastasis. In another study carried out in mice, Rose et al. (1995) determined that primary tumour growth was delayed and lung metastasis was less in animals given feed that was supplemented with ω-3 fatty acids(EPA and DHA), compared to animals given feed containing 8% of ω-6 fatty acids (LA). The researchers attributed this result to the inhibitory effect ofω-3 fatty acids on tumour eicosanoid biosynthesis.

Sonnenschein et al. (1991) showed that high-fat diet had protective feature on the mammary tumour risk in dogs. But this study have some shortcomings, as no detailed information on which type of fatty acid are involved in the diet, are presented due to the scarce data about dog food preparations. Costa-Santos et al. (2019) reported that a relationship between dyslipidemia and tumor aggressiveness. Dyslipidemia is a substantial factor in the tumour formation (Aravani et al., 2018). Higher triglyceride, VLDL, albumin, globulin and lactate levels were observed in the aggressive carcinomas in overweight/obese dogs. Fish-oil supplementation didn't change the abovementioned parameters, but glucose, total protein, globulin levels increased, leading to alter metabolic parameters in cancer patients (Costa-Santos et al., 2019). In this study, the detection of higher blood ω-6 levels ($p < 0.001$) in female dogs with mammary tumors and higher serum ω-3 levels ($p < 0.001$) in healthy female dogs support the opinion that fatty acid levels may be associated with mammary tumor risk.

CONCLUSIONS

In result, ω-3 was determined at higher levels in the blood of the healthy female dogs whereas ω-6 was detected at higher levels in the female dogs diagnosed with mammary tumour.This suggest that omega fatty acids may be an important factor in the biological mechanism of canine mammary tumour development. We consider that the obvious differences between blood level ofω-3 and ω-6 might be caused by Eicosatrienoic acids. Further research should be done to understand the exact mechanism of how ω-3 and ω-6 contribute to development of female dog mammary tumours.

CONFLICT OF INTERESTS STATEMENT

The authors declare that there is no conflict of interests regarding the publication of this article.

ANIMAL RIGHTS STATEMENT

All the experimental procedures followed in this study were approved by the Animal Care and Use Committee of Hatay Mustafa Kemal University, Hatay, Turkey via letter no. 2017/5-1.

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