The effect of EPA and DHA on gingivitis in healthy dogs.

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Abstract

Periodontal disease is a frequently occurring health problem in dogs. The first stage of periodontal disease is gingivitis. The hypothesis of this randomized, double-blinded clinical trial is that feeding a diet supplemented with the omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosapentaenoic (DHA) to healthy dogs with natural occurring gingivitis will reduce gingivitis scores. 22 Portuguese dogs and 22 Dutch dogs were fed a diet supplemented with either corn oil (0.00 g EPA and 0.00 g DHA, both per 1000 kcal ME) or fish oil (1.53 g EPA and 0.31 g DHA, both per 1000 kcal ME) for 5 months. After these 5 months gingivitis, plaque, calculus and plasma values of EPA, DHA and C-reactive protein (systemic marker of inflammation) were compared. After 5 months no significant differences in gingivitis scores between the 2 groups were found. The dogs in group B (corn oil) showed a significant rise in gingivitis scores after 5 months, group A did not show a significant rise in gingivitis scores. After 5 months all dogs had lower plaque scores. All dogs had higher EPA and DHA plasma values at the end of the study. Group A (fish oil) had significantly higher plasma levels of EPA and DHA than group B. The levels of C-reactive protein were significantly lower at the end of the study, but no differences between group A and B were found. In conclusion, supplementing EPA and DHA did lead to higher plasma levels of EPA and DHA, but it did not lower gingivitis scores in healthy dogs with natural occurring gingivitis.

1. Introduction

Periodontal disease is a frequently occurring health problem in dogs, a prevalence of up to 80% has been described.[1] Therefore, it is important for veterinary practitioners to address this disease. To prevent periodontal disease tooth brushing is the golden standard, however owner compliance is low.[2,3] Therefore, easier alternatives, like dietary adjustments, are sought after. It has been shown that the eicosapentaenoic acid (EPA) and docosapentaenoic (DHA) omega-3 (ω3) polyunsaturated fatty acids (PUFAs) modulate gingival inflammation in rats and periodontal disease in humans.[2-5] In dogs it has been shown that EPA and DHA help in other inflammatory diseases,[6,7] however their effect on gingival inflammation and the progression of periodontal disease have not been shown yet. The aim of this study is to investigate whether a diet supplemented with EPA and DHA can reduce gingivitis in healthy adult dogs.

1.1 Anatomy of canine teeth

Dogs have an anelodont dentition with brachydont type teeth. This means that the teeth grow for a short period of time and consist of a short crown with a relatively long root. Because different teeth have different functions, they differ morphologically. The number of roots also differs per tooth. Dogs have 42 permanent teeth, 10 in each maxillary quadrant and 11 in each mandibular quadrant. In the maxillary quadrants they have 3 incisors, 1 canine tooth, 4 premolars and 2 molars. In the mandibular quadrant they have 3 incisors, 1 canine tooth, 4 premolars and 3 molars.[8-12]

The tooth itself consists of a pulp cavity surrounded by dentine. The pulp cavity contains living pulp tissue, which contains the blood vessels, nerves, lymphatics and other cells in a collagenous matrix needed to keep the tooth alive. Dentine envelops the pulp cavity and takes up the largest part of the tooth. On the crown of the tooth, the part that is visible above the gingiva, the dentine is covered with enamel (fig. 1.). Enamel is highly mineralized and is a very hard material. On the root, beneath the gingiva, the dentine is covered by a layer of cementum which is a bone-like, mineralized connective tissue that is produced continuously. The part where the enamel and the cementum meet, the cemento-enamel junction, is usually located subgingival and is also the part where anatomically the crown ends and the root starts.[8-12]

The teeth are surrounded and supported by the periodontium, which consist of the alveolar bone, the gingiva, the periodontal ligament and the cementum. The alveolar bone, or alveolar process, surrounds the root of the tooth and contains the tooth sockets or alveoli. The alveolar bone is trabecular bone, covered by a lingual and labial cortical plate. The walls of the alveoli are covered with cortical bone as well, this is called the lamina dura. The periodontal ligament lies in the periodontal space, the space between the tooth and the alveolar bone, connecting the two through bundles of collagen fibrils (fig. 1.). The ends of the ligament are imbedded in the cementum and the alveolar bone, keeping the tooth in place. The collagen fibers are normally horizontally and obliquely oriented. The ligament also acts as a shock absorber by spreading the force around the entire surface area of the root. The oral mucosa that covers the alveolar process is called the gingiva. The gingiva can be divided into attached and free gingiva, the first is bound to the periosteum and the latter is not. The gingiva and alveolar mucosa (mucosa that covers the alveolar bone) meet at the mucogingival junction. The space between the tooth and the free gingiva is called the gingival sulcus (fig. 1.), which should be less than 3 mm in depth in dogs.[8-12]

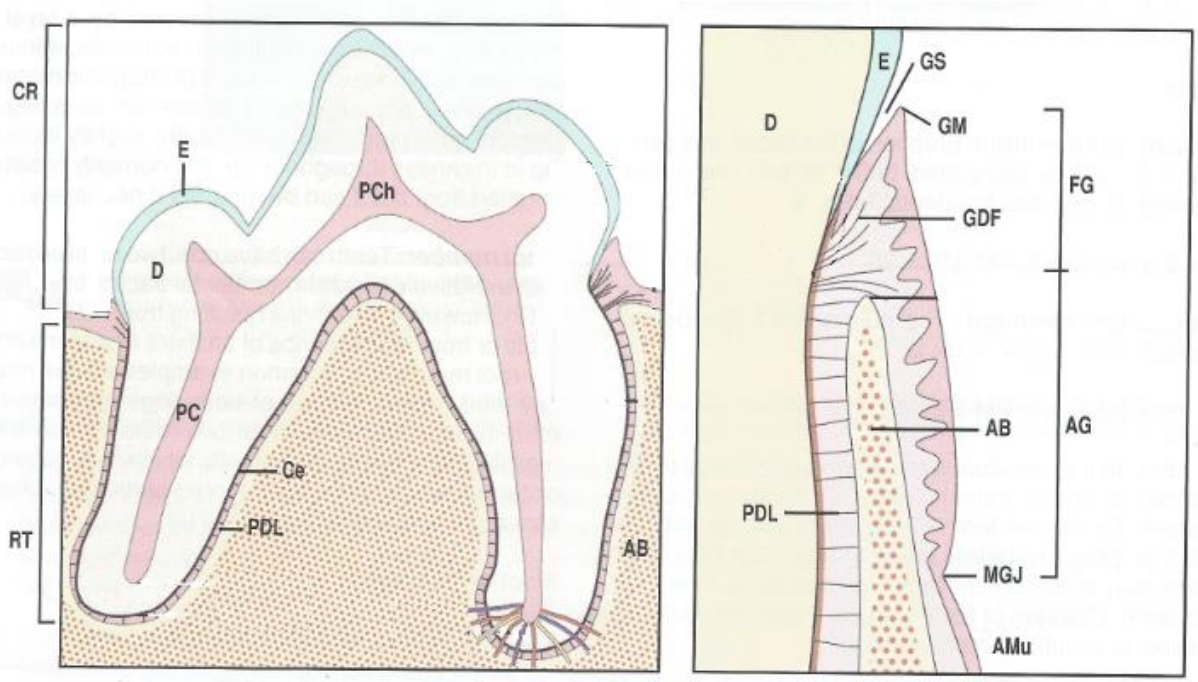

Fig. 1. Schematic presentation of canine tooth. AB = Alveolar bone; AG = Attached gingiva; Amu = Alveolar mucosa; Ce = Cementum; CR = Crown; D = Dentine; E = Enamel; FG = Free gingiva; GDF = Gingivodental fibers; GM = Gingival margin; GS = Gingival sulcus; MGJ = Mucogingival junction; PC = Pulp canal; PCh = Pulp chamber; PDL = Periodontal ligament space, RT = root. Modified after [10].

1.2 Periodontal disease

Periodontal disease refers to a group of conditions that affect the health of the periodontium, commonly it is used to describe gingivitis and periodontitis.[14] Gingivitis is the first stage of the disease process and is characterised by an inflammation confined to the gingiva. Clinically gingivitis is seen as reddening and swelling of gingiva up to ulceration and spontaneous bleeding as it progresses in severity. [11] Gingivitis is reversible, however if the inflammation persists, the disease could progress to periodontitis. Periodontitis refers to an inflammation of other periodontal tissues: the periodontal ligament, the cementum and the alveolar bone. The inflammation can lead to the destruction of the periodontal tissues, which can result in gingiva recession, pocket formation, loss of alveolar bone and ultimately loss of the involved teeth. Periodontitis is more severe than gingivitis and is not reversible, advanced treatment and plaque control is needed to prevent further progression of the disease.[1,11-13] Gingivitis does not always advance to periodontitis, this only happens when the host is susceptible.[11,13] The susceptibility of hosts varies among individuals and can depend on age, stress, immunological competence, health status, nutritional status and probably many other factors not yet known or understood.[11,14,15]

1.2.1 Pathogenesis

Periodontitis is primarily caused by bacterial colonization. Dental plaque plays an important part in this process. A mouth is a warm, moist and nutrient rich environment, ideal for microorganisms to grow. Oral fluids cover the tooth surfaces and deposit a thin glycoprotein layer upon evaporation. This layer, called the pellicle, provides a surface for the bacteria colonizing the mouth to adhere to. The bacteria then form a biofilm, commonly called dental plaque.[1,11-13] Plaque forms within 24 hours and when it is not removed it can become thicker and more complex. Enzymes and antibodies in the salivary fluid work against the bacteria, however when the plaque accumulates the enzymes and antibodies cannot reach the microbial inhabitants in the deeper layers. [1,11,13] Calciumcarbonate and

calciumphosphate salts in the salivary fluid mineralize the plaque, forming calculus. The mineralisation needs to take place for 2 to 3 days to form calculus that is hard enough to resist being removed easily. Once it is mineralised, the calculus can only be removed by professional dental cleaning. It is believed that calculus is not pathogenic and that the bacteria in plaque are the real cause of periodontal disease, however the rough surface of the calculus can irritate the gingiva and provides additional places for the bacteria to adhere to, enhancing further plaque development.[13,15]

The formation of plaque usually starts above the gingiva and can progress to the areas beneath the gingiva. The same is true for calculus. Supragingival plaque is primarily composed of gram-positive facultative aerobic bacteria, such as *Streptococcus* spp. and *Actinomyces* spp.. Subgingival plaque is formed in the gingival sulcus and is composed of gram-negative motile anaerobic bacteria. [1,8,12] Plaque and calculus can contain up to 100 billion bacteria per gram, and as the plaque matures the bacterial population shifts from being gram-positive facultative aerobic bacteria to gram-negative motile anaerobic bacteria.[1,10,16] This shift in bacterial flora can initiate gingivitis and, in susceptible animals, to periodontitis. The number of bacteria, especially the number of gram negative rods and anaerobic species, is increased during gingivitis. Some bacterial species are associated with periodontal disease and are called periodontopathogens.[1,8,11,13] These bacteria are usually obligate anaerobic bacteria, such as *Porphyromas* spp. and spirochetes, which are found in periodontal pockets.[1,8,11,13] In chronic periodontal disease anaerobic species account for up to 90% of the flora.[1,18]

The bacterial infection causes inflammation of the gingiva, which attracts inflammatory cells, mainly neutrophils. When the inflammation progresses, the integrity of the connective tissue is broken down by collagenases produced by the bacteria and proteases from the neutrophils. In a later stage, when the inflammation has become more chronic, other inflammatory cells like macrophages, plasma cells and lymphocytes infiltrate the gingival tissues. These cells also release cytokines and destructive enzymes, which enhance the inflammation and will lead to further damage to the tissues. As more tissue is damaged, the gingival sulcus widens and pockets develop. The epithelium that lines the pocket becomes thin and ulcerated in some areas and thick in other areas. A shift in inflammatory cells from lymphocytes to plasma cells occurs, which leads to an increased production of immunoglobulins. This event is associated with the progression to irreversible periodontal disease. In this stage gingival fibers lose their attachment to the cementum and cementoblasts will die. Cytokines such as interleukins, tumour necrosis factor and prostaglandins, as well as enzymes such as matrix metalloproteinases, are produced by host cells and inflammatory cells. These cytokines inhibit collagen production and stimulate osteoclasts,

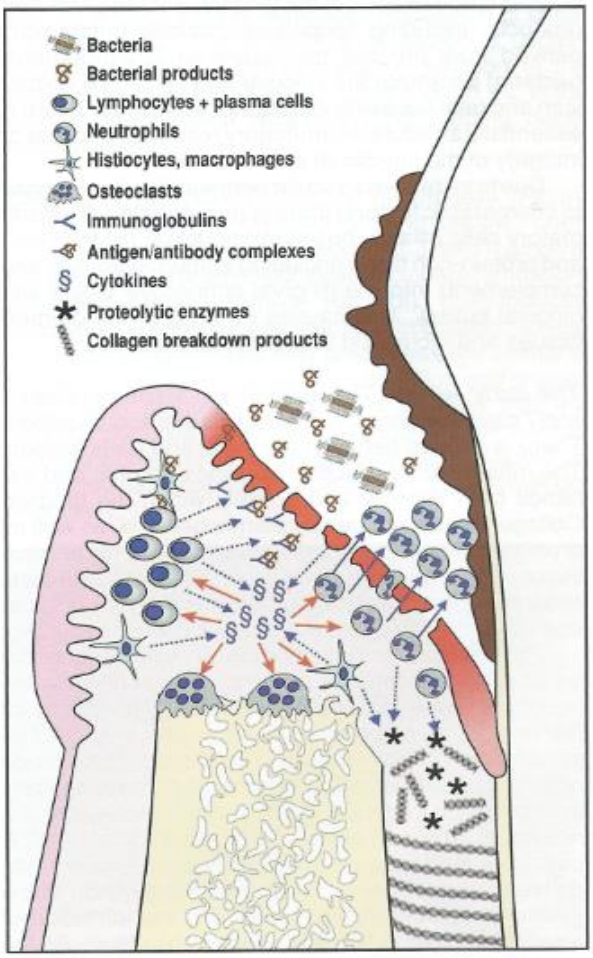

Fig. 2. Histopathogenesis of periodontal disease. Modified after [10].

which results in alveolar bone loss.[10,13] Ongoing loss of alveolar bone will lead to loss of attachment of the tooth resulting in mobility. When mobility is present, the tooth will be pushed against the bone during chewing, which causes squeezing of blood vessels resulting in further bone loss. The resorption of alveolar bone will eventually lead to loss of the tooth and possibly fractures of the bone.[13] With the tooth gone there is no longer a bacterial burden and the remaining tissues can recover. However, during the inflammation process the bacteria have had the opportunity to enter capillaries and cause bacteraemia. In healthy patients the bacteraemia is usually rapidly cleared, however the bacteraemia together with the release of inflammatory mediators in the systemic circulation can also cause distant organ abnormalities.[10,13,19] Periodontal disease has been associated with liver- and kidney disease as well as cardiovascular diseases, such as endocarditis.[16-18] It has been shown that C-reactive protein, which is a systemic marker of inflammation, reaches elevated levels during (severe) periodontitis, which implies that periodontitis can have systemic effects.[10,19]

As mentioned above, the main etiologic factor of periodontitis is the presence of pathogenic bacteria. There are other factors that have an influence on the initiation and progression of the disease. One factor, calculus, has already been mentioned above. Calculus provides a surface for bacteria to adhere to and can irritate the gingival tissues. Other predisposing factors are tooth crowding, tooth morphology and supernumerary teeth. When the spaces between the teeth are small, plaque cannot be easily removed, leading to faster accumulation of plaque. Breathing through the mouth is also a risk factor because it dries the mucosal surface. Decreased saliva flow, which can be caused by a salivary gland dysfunction, is a predisposing factor because the saliva helps to clear unattached bacteria. Furthermore, the composition of the saliva can have an effect as well. If the saliva contains less antimicrobials, such as enzymes and antibodies, then the bacteria can grow faster. Systemic factors that have an influence include underlying diseases, immunopathologies and nutritional imbalances.[10] In humans Diabetes mellitus is a known risk factor for periodontal disease.[8,15,19]

1.2.2 Therapy of periodontal disease

Gingivitis is caused by bacteria in plaque, treatment of gingivitis is therefore aimed at removing the plaque. After this it is necessary to maintain gingival health to prevent progression to periodontitis. Often this can be done easily by home care and periodic assessment and treatment by a professional. Periodontitis is more severe than gingivitis and the treatment therefore is more difficult, however the core of periodontal therapy is also to control bacterial plaque. The goal of the therapy is to stall progression of the disease and prevent further tissue damage and spreading of the disease to unaffected tissues.[8,20]

There are two types of treatment for periodontitis: non-surgical periodontal therapy and surgical periodontal therapy. The choice between surgical or non-surgical treatment depends on the severity of periodontitis and on if the owner is willing to provide home care on the long term. The treatment of periodontal disease always starts with non-surgical therapy.[8,20]

1.2.2.1Non-surgical therapy

The first step is to perform a thorough oral examination including periodontal probing. Probing is a method to assess the loss of periodontal attachment. The probe can be used to measure gingival sulcus depth, normally < 3mm, pocket depth, gingival recession and furcation. Other elements to pay attention to during the examination are, missing teeth, attachment loss, mobility, bleeding on probing and lesions. The findings of the examination should always be recorded, preferably on a dental chart. The dental chart can be used to formulate a treatment plan, but also as a comparison for future examinations.[8,20]

A recommended second step is a full-mouth radiographic examination. The radiographs help to determine the level of disease and can reveal pathologies that may not be visible during the oral examination. The dental radiographs can also be used as a comparison in the future. Signs of periodontitis that can be seen on a dental radiograph are alveolar bone loss, loss of lamina dura, resorption of the alveolar margin and widening of the periodontal ligament space.[8,20]

The third step is to make a treatment plan based on the found abnormalities and the extent and severity of the disease. Other factors have to be taken into account while making a treatment plan. One of these factors is the willingness and ability of the owner to provide home care, if the owners cannot provide home care more aggressive periodontal treatment could be necessary. A second factor is the value of the affected teeth, do they have to be preserved or can they be extracted.[8,20]
The next step is rinse the oral cavity with 0.12-0.2% chlorhexindine gluconate solution. This will decrease the bacterial load, thereby decreasing the degree of bacteraemia and the number of aerosolized bacteria during the treatment.[8,20-22]

The following step is removing supra- and subgingival plaque and calculus via hand scaling and (ultra)sonic scaling. Subgingival cleaning can be done manually by using a curette. Using a mechanical scaler is also a possibility, as long as the power setting is reduced and the water cooling is adequate. After scaling the tooth surfaces should be polished by using a rubber cup on a slow-speed hand piece and polishing paste. Polishing smoothens the surface of the teeth, thereby reducing plaque attachment.[8,20]

A possible next step is to remove debris, such as polishing paste and calculus, from the gingival sulcus by performing a sulcular lavage. However, some studies say that the flow of gingival fluid and bleeding from the sulcus is sufficient to remove this debris, making a sulcular lavage unnecessary.
At this point the non-surgical treatment stops. After this, owners should start providing home care the prevent recurrence of periodontal disease.[8,20] The options available to help prevent periodontal disease will be discussed later.

1.2.2.2 Surgical periodontal therapy

Further treatment is necessary if cleaning and polishing are not sufficient to control the periodontal disease. Furthermore, more aggressive treatment is also required in the case of deep pockets (>4mm), furcation level II and III (access >1/3 of the element width), moderate alveolar bone loss and inaccessible areas. If the tooth is too diseased or if the owners do not wish to retain the tooth or if long term home care cannot be provided, the best option is to extract the element. If the goal is to retain the elements, periodontal surgery can be performed. There are three main surgical procedures: procedures to restore tissue attachment, respective procedures and grafting and regeneration procedures. An example of a procedure to restore tissue attachment is the open flap debridement. For this procedure an incision is made in the sulcus from the free gingival margin to the bone. The root surface becomes visible and root debridement can be performed. Examples of respective procedures are: gingivectomy, apically repositioned flap and osseous resection. Gingivectomy can be used to correct gingival deformities and pseudo-pockets caused by gingival hyperplasia. The apically repositioned flap is a technique whereby the whole mucogingival unit is displaced apically to eliminate pockets. Osseous resection is a procedure which is used for reshaping the alveolar bone to achieve a physiological contour. Grafting and guided tissue regeneration techniques aim at correcting loss of soft tissue and alveolar bone. An example of this is harvesting a gingival graft from an area with sufficient healthy attached gingiva to replace a lost attached gingiva in a different area.[8,20]

1.2.3 Home care

Home care refers to the actions owners can do at home to prevent periodontal disease. The aim is to prevent accumulation of plaque, to prevent accumulation of calculus, to prevent accumulation of pathogenic bacteria and reduce their effects and to suppress the inflammatory reaction in the periodontium.[11,23]

1.2.3.1 Preventing plaque accumulation

The gold standard for preventing accumulation of plaque is frequent tooth brushing. Brushing the teeth disrupts the plaque layer, thereby slowing down progression of the disease. Although there are toothpastes available for use in pets, they are not necessary. However, most of the veterinary toothpastes are flavoured and the taste may improve the acceptance by dogs and can be a positive reinforcement. Furthermore, the abrasive material and active compounds often found in these pastes, could improve the results of tooth brushing. The minimum frequency that is often recommended is 3 times a week, although daily brushing is the most ideal frequency.[23-25] However, owner compliance rates to brushing teeth are low.[2,3]

Other ways of preventing accumulation of plaque are to provide chew toys, treats and diets that mechanically remove the plaque during the action of chewing. Having access to chewing materials, such as rawhide, is associated with less plaque and calculus accumulation, gingivitis and periodontitis.[31] Numerous studies have shown that a daily dental chew treat aids in reducing plaque and calculus accumulation and may play a role in maintaining gingival health.[29-34] Dry foods are often recommended rather than moist foods, because dry food would provide some form of mechanical cleansing, whereas moist food are said to promote plaque accumulation. However, consuming dry food is not always associated with better oral health compared to consuming moist food.[31] Most dry foods crumble at initial tooth contact and have therefore little beneficial effect on oral health. Increasing the mechanical stability of the kibble, and thus allowing the teeth to completely enter the kibble before it breaks apart, can greatly improve tooth cleansing during feeding. Dental foods aim for this by enhancing kibble size and altering the texture, in order to promote chewing and maximize tooth contact.[26] Several studies have demonstrated that dental food can benefit oral health by reducing plaque and calculus accumulation.[35-37] Chewing and mechanical cleansing can also be promoted by providing chew toys. However, not all toys have a beneficial effect and some can even be dangerous. Examples of potentially harmful toys are string and rope chew toys. These toys often claim to have a flossing effect, however pieces of string can cause health problems when ingested. Furthermore, when owners pull on these ropes to play with their dog, the force can cause avulsion or fracturing of the teeth.[26]

A method of reducing attachment of plaque is to apply a dental sealant. A dental sealant is a high viscosity, hydrophilic solution that can be applied along the gingival margins. It forms a bond with the tooth enamel and acts as a barrier. It reduces plaque and calculus formation in the gingival sulcus and thereby aids the prevention of periodontal disease. The sealant can be applied by a veterinarian after a cleaning procedure and can be reapplied every week by the owners.[26,38,39]

1.2.3.2 Prevention of mineralization of plaque

Calculus forms when calcium salts in the saliva mineralize the dental plaque. To inhibit this process, polyphosphates can be given. Polyphosphates, such as sodium hexametaphosphate and sodium tripolyphosphate, bind to calcium in the saliva thereby making it unavailable for mineralisation. Polyphosphates do not dissolve calculus however, they only slow calculus mineralisation.[36] Polyphosphates can used as a coating on treats, food and dental chews. Several studies show that calculus formation in dogs that receive polyphosphate coated food or treats daily, is lower than in dogs not receiving this food or treats.[37,40-42]

1.2.3.3 Prevention of accumulation of pathogenic bacteria and reducing their effects

Antibiotics can prevent the accumulation of pathogenic bacteria. Clindamycin is frequently used for management of oral disease in dogs and cats and has been shown to be effective in reducing plaque accumulation and gingivitis.[10,13,26] Nevertheless, antibiotics should only be used lightly. An important risk of using antibiotics is resistance development. Antibiotics should therefore only be used when necessary during periodontal treatment, and certainly not as a prevention method.[10,26]

A different chemical agent that acts against bacteria is chlorhexidine. Chlorhexidine disrupts bacterial cell wall lipoproteins and penetrates the cells where it causes precipitation of cytoplasm.[43-48] Chlorhexidine can provide sustained antimicrobial activity because it binds to the pellicle layer on the teeth and is then slowly released.[47,48] An advantage of chlorhexidine is that bacteria cannot develop a resistance against it.[43,44] Studies have shown its effectiveness against dental plaque and gingivitis.[49-51] Unfortunately, long term use of chlorhexidine is associated with increased calculus accumulation, staining of the teeth and tongue and altered taste.[46,52,53] There are veterinary products available that contain chlorhexidine, including rawhide chews, dental gels, oral rinses and bioadhesive tablets.[26]

Soluble zinc salts, like zinc ascorbate and zinc gluconate are thought to have antimicrobial activity. Therefore they may aid in controlling plaque accumulation. Although this effect has not been demonstrated in dogs yet, in cats a decrease in plaque accumulation, gingivitis and anaerobic periodontal pathogens after receiving a zinc ascorbate gel was found.[54]

A study has been conducted to evaluate the possibilities of vaccination against periodontal pathogens. In this study a vaccine against *Porphyromonas gulae* was developed and tested on mice. The mice displayed high antibody titers and had reduced alveolar bone loss when challenged.[55] Although these results sound promising, it must be remembered that periodontal disease is complex and caused by multiple pathogens.[10]

1.2.3.4 Suppression of the inflammatory reaction of the periodontium

The inflammatory reaction in the periodontium causes destruction of the tissues. To inhibit this reaction anti-inflammatory drugs can be given. Nonsteroidal anti-inflammatory drugs (NSAIDs) have been used with success to reduce periodontitis in dogs.[56] Unfortunately, long term use of NSAIDs could have adverse effects[57] and therefore this strategy is not recommended.

Another possible measure to inhibit the inflammatory response is the use of omega-3 polyunsaturated fatty acids, in particular eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA). Studies have shown that EPA and DHA can have a beneficial effect in inflammatory diseases in dogs.[58,59] In humans and rats an anti-inflammatory effect on the gingiva has been found as well.[4-6] The effects of EPA and DHA on gingival inflammation and periodontal disease progression have not been reported yet.

1.3 Omega-3 fatty acids

Omega-3 fatty acids are polyunsaturated fatty acids that have a carboxylic acid on one end, a methyl group on the other end and have multiple double bonds. Counting the methyl carbon as carbon number one, the first double bond is located between carbon number 3 and 4, which is characteristic for the omega-3 polyunsaturated fatty acids (PUFAs). Eicosapentaenoic acid (EPA) is a PUFA with 20 carbon atoms and 5 double bonds, docosahexaenoic acid (DHA) is a PUFA with 22 carbon atoms and 6 double bonds.[60,61] EPA and DHA can be synthesised from other fatty acids derived from plants, however it seems this metabolic pathway is not very efficient.[62,63] High quantities of EPA and DHA

can be found in fish and other seafood, especially in oily fish, which can contain up to 1.5 to 3.0 grams of PUFAs per meal.[60]

There are a few mechanisms through which EPA and DHA could inhibit inflammatory response. These mechanisms include: incorporation into cell membrane phospholipids, changing lipid mediators, changing protein mediators, altering gene expression and influencing T cell reactivity.[60,61]

1.3.1 Incorporation into cell membrane phospholipids

The base of the anti-inflammatory influence of EPA and DHA is considered to be their incorporation into cell membrane phospholipids.[60,64] Usually the concentration of EPA and DHA in phospholipid cell membranes is low, compared to other fatty acids such as arachidonic acid (ARA).[65] ARA is a polyunsaturated omega-6 fatty acid, which consist of 20 carbon atoms and contains 4 double bonds, the first one located at carbon atom number 6. It can be synthesized from simpler fatty acids derived from plants.[60] Increased dietary intake of EPA and DHA results in increased amounts of these fatty acids in the phospholipid membranes at the expense of other fatty acids like ARA.[66-69] Since the phospholipids are substrates for lipid mediators, these changes in fatty acid composition may have an influence on the inflammatory response.

1.3.2 Lipid mediators

Lipid mediators are chemical inflammatory mediators that are derived from fatty acids. These mediators include eicosanoids, endocannabinoids and platelet activating factor.

Eicosanoids are a group of mediators, including prostaglandins (PGs), thromboxanes (TXs), leukotrienes (LTs) and lipoxins (LXs), which are oxidized derivatives of fatty acids in the phospholipid membrane that contain 20 carbon acids. The major substrate for eicosanoid synthesis is usually ARA. Under the influence of inflammatory stimuli, ARA is released from the phospholipid membrane and then acts as a substrate for various enzymes, such as cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 enzymes. COX enzymes produce PGs and TXs, LOX enzymes produce LTs and LXs, cytochrome P450 enzymes produce hydroxyeicosatetraenoic and epoxyeicosatrienoic acids. The metabolism of ARA leads to 2-series PGs and TXs and 4-series LTs and LXs.[60,61] Especially the 2-series PGs and 4-series LTs are well-known inflammatory mediators and regulators.[60,70] The incorporation of EPA and DHA in the phospholipid membrane causes a reduction in the amount of ARA available as substrate, resulting in a decrease in ARA-derived mediators.[71] EPA itself is also a substrate for COX, LOX and cytochrome P450 enzymes, since it contains 20 carbon atoms as well.[60] Although the metabolism of EPA is analogous to that of ARA, it gives rise to different mediators: 3-series PGs and TXs and 5-series LTs (fig. 3).[60,69] These mediators have a different structure and are less potent, since the eicosanoid receptors usually have a lower affinity for them.[72]

The metabolism of phospholipids results in the production of endocannabinoids. Again, ARA is generally the substrate for this metabolism, although the endocannabinoids can also have EPA or DHA in their structure. The EPA and DHA derived endocannabinoids, docosahexaenoyl ethanolamide and eicosepentaenoyl ethanolamide, bind to CB1 and CB2 receptors and are thought to have anti-inflammatory properties.[60,73]

Other mediators that can be derived from EPA and DHA are resolvins, protectins and maresins. These mediators are anti-inflammatory and inflammation resolving. Resolvin E1 (derived from EPA), resolving D1 (derived from DHA) and protectin D1 (derived from DHA) prevent the infiltration of neutrophils into inflammatory sites by inhibiting transendothelial migration. Furthermore, the production of cytokine IL-1β is inhibited by resolvin D1 and protectin D1 and the production of cytokine TNF-α is inhibited by protectin D1. The resolvins, protectins and maresins are synthesised

from EPA and DHA using the COX and LOX pathways (fig. 3). In the presence of aspirin, different epimers with different effects are produced.[60,74,75]

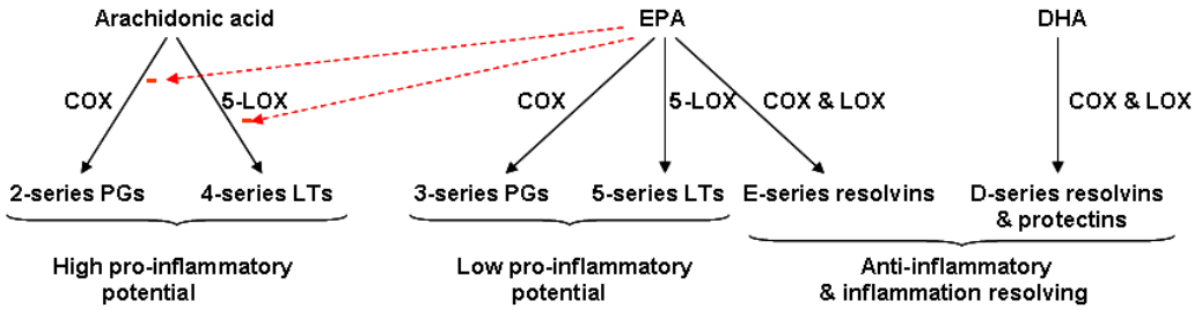

Fig. 3. Overview of synthesis and actions of lipid mediators produced from ARA, EPA and DHA. Modified from [76]

Platelet activating factor (PAF) is a mediator of many leukocyte functions and inflammation. It is continuously produced by many cells including platelets, endothelial cells, neutrophils, monocytes, macrophages and basophils, but in the presence of inflammatory stimuli its production is upregulated. Studies have been conducted to examine the effect of EPA and DHA on the production of PAF, with inconsistent results. Some studies found a decrease in the production of PAF when feeding EPA or DHA or both, but not by all cells.[60,77-79]

1.3.3 Protein mediators

There are various proteins that are involved in inflammatory reactions as well. Cytokines, such as tumor necrosis factor (TNF), interleukins (ILs), interferons, chemokines and lymphokines, are small proteins that are associated with inflammation, affecting the activity of inflammatory and other cells. Cytokines are produced by and released by a variety of cells, including monocytes, macrophages, T- and B lymphocytes, mast cells, endothelial cells, fibroblasts and adipocytes.[60] Various studies have researched the effects of EPA an DHA on the production of pro-inflammatory cytokines like TNF, IL-1β, IL-6 and IL-8. These studies demonstrated that EPA and DHA decreased circulating cytokines and inhibited the endotoxin stimulated production of these cytokines in various cells.[80-85]

Other proteins that are important in inflammatory processes are adhesion molecules. Adhesion molecules can be found on the surfaces of a variety of cells, including leukocytes and endothelial cells. Leukocytes can use these molecules to adhere to the blood vessels walls and leave the bloodstream to migrate to inflammatory sites. When an animal is in a state of inflammation, the expression of adhesion molecules is increased.[60,85] EPA and especially DHA seem to lower the expression of adhesion molecules on endothelial cells and leukocytes. Furthermore, a functional effect of EPA and DHA has been found in some studies. In these studies EPA and DHA decreased the adhesion between leukocytes and endothelial cells.[85-89]

There are in vitro studies that show that EPA and DHA reduced mRNA or protein levels of matrix metalloproteinases in various cell types.[90-93] Matrix metalloproteinases (MMPs) are enzymes that are associated with tissue damage in inflammatory sites, since they degrade extracellular matrix proteins. The production of the MMPs is regulated by, among other factors, cytokines and eicosanoids.[60] A study in dogs showed lowered levels of certain MMPs in knee synovium after feeding fish oil.[94]

1.3.4 Gene expression

Some effects of EPA and DHA seem to involve an altering of gene expression. A possible mechanism to explain these effects is that EPA and DHA have an effect on nuclear factor kappa B (NFκB), which is a transcription factor that is involved in upregulating genes that encode for inflammatory proteins such as cytokines, adhesion molecules and the COX enzymes.[60,95] The signalling cascade that activates NFκB can be triggered by inflammatory stimuli, such as endotoxin binding to a toll-like receptor (TLR). The activation of NFκB involves phosphorylation, and it seems that EPA and DHA can decrease NFκB phosphorylation. EPA and DHA may also be able to prevent saturated fatty acids like lauric acid from interacting with TLR-4, which would activate NFκB.[60,96] EPA and DHA may also inhibit NFκB activation by activating peroxisome proliferator activated receptor-γ (PPAR-γ). PPAR-γ is an anti-inflammatory transcription factor that interferes with NFκB translocation to the nucleus.[60,97] Another possible mechanism involves G-protein coupled cell membrane receptor called GPR120. GPR120 is expressed on macrophages and is involved with anti-inflammatory signalling. DHA and EPA are able to bind to GPR120 and enhance its anti-inflammatory signalling, which inhibits NFκB.[60,98]

1.3.5 T cell reactivity

T cells are leukocytes that act as effector and regulatory cells. There are different types of T cells expressing different kinds of glycoproteins. T cells expressing the glycoprotein CD4 on their surface can, under the influence of immunologic stimuli, develop into different phenotypes, such as T-helper 1 (Th1) and T-helper 2 (Th2).[60] It has been reported that EPA and DHA reduce T cell proliferation and reduced production of cytokines by Th1 and Th2 cells.[65,99-101] There are many suggested mechanisms via which EPA and DHA can influence T cells, but recent research suggest that raft disruption is involved.[60]

1.4 Hypothesis

Since it has been shown that EPA and DHA have anti-inflammatory properties, the hypothesis is that feeding healthy dogs a diet supplemented with EPA and DHA will reduce gingivitis, compared to dogs given a control diet without supplementing EPA and DHA. It is also expected that supplementing EPA and DHA will result in higher levels of these fatty acids in blood samples.

2. Material and methods

2.1 Animals

For this study a maximum of 44 dogs could be selected. The inclusion criteria for the dogs were: (1) adult male or female dog between 1 and 7 years of age, (2) some degree of natural occurring gingivitis, (3) owners willing to cooperate and consent to all the procedures. Exclusion criteria were: (1) Severe periodontal disease, (2) any concurrent disease, (3) access to the exterior environment, (4) the use of any other diet or supplement during the study, (5) the use of any hygienic measures during the study. 32 Client-owned and 12 clinic-owned dogs of different genders (24 females, 12 castrated females, 2 males, 6 castrated males) and breeds (18 crossbreed/undetermined breed, 11 Beagles, 4 Dachshunds, 2 Stabyhouns, 2 Labrador retrievers, 1 English cocker spaniel, 1 Jack Russell, 1 Chihuahua, 1 Pug, 1 Poodle, 1 Boxer, 1 Epagneul Breton) aged between 1 and 7 years were selected in the Netherlands and Portugal. The dogs were randomly assigned to either group A or group B.

2.2 Diet

All dogs were being fed the same control diet (Science Plan VetEssentials Canine Adult) supplemented with either corn oil (0.00 g EPA and 0.00 g DHA, both per 1000 kcal ME) or fish oil (1.53 g EPA and 0.31 g DHA, both per 1000 kcal ME). To prevent rancidity, 10mg/mL α-tocopherol (Vitamin E) has been added to the oils. The oils, in the study named oil A or oil B have no differences in visual appearance or smell and are delivered in identical vials. The oils were labeled at the manufacturer and delivered with a locked key, which will be broken after the results of the trial are delivered to Hill's. The daily amount of food and oil were calculated for each dog and indicated to their owners. The oils could be added to the diet by using a dosage syringe.

2.3 Study protocol

This study has been conducted as a randomized double-blind trial. The clinical time line has been identical for each dog and started at day 0 (phase 1) with: an oral condition examination and periodontal disease scoring, blood sampling for base line reference, a complete veterinary periodontal treatment and random assignment of the dogs to either group A or group B. During the 5 months of the trial period the dogs were fed the control diet supplemented with oil A or oil B depending on the group they have been assigned to. The trial period ended on the last day of the 5^{th} month (phase 2) with: an oral examination with periodontal disease scoring, blood sampling for determination of inflammatory markers, blood cell counts and fatty acid profile and the cholesteryl ester fraction. At the start and end of the study, the owners had to complete a questionnaire regarding the health of the dogs, eating habits and living conditions. Before the start of the study, the owners also had to give their permission for all procedures. All dogs that participated in the study came in for medical care, thus they were not exclusively anesthetised for this study. This study was approved by the animal experimental committee and permission to use clinic-owned dogs was given.

2.4 Oral examination

The oral exam started with inspecting if all elements were present, missing elements were recorded on the dental chart (appendix 1). After this, the buccal/labial and palatal/lingual of every tooth was scored for calculus, plaque and gingivitis, using the criteria in table 1.

Table 1. Calculus, plaque, and gingivitis criteria.

Calculus:	Plaque:	Gingivitis:
0 - No calculus	0 - Plaque absence	0 - Normal gingiva
1 - Calculus coverage <25%	1 - Plaque coverage <25%	1 - Mild inflammation, slight redness
2 - Calculus coverage 25-50%	2 - Plaque coverage 25-50%	2 - Moderate inflammation and redness, no bleeding on probing
3 - Calculus coverage 50-75%	3 - Plaque coverage 50-75%	3 - Moderate inflammation with severe redness, bleeding on probing
4 - Calculus coverage >75%	4 - Plaque coverage >75%	4 - Severe inflammation and redness, edema, ulceration and spontaneous bleeding

A dental probe was used to score the gingivitis. The plaque was made visible with a disclosing agent or a plaque light. Other abnormalities, such as mobility, furcation or probing attachment loss were also scored and recorded using the criteria in table 2.

Table 2. Mobility, furcation and probing attachment loss criteria.

Mobility:	Furcation:	Probing attachment loss:
0 - static	I - access till 1/3 of the element width	Periodontal probing depth - A (mm)
1 - horizontal mobility 0.2-1 mm	II - access till > 1/3 of the element width	Gingival recession - B (mm)
2 - horizontal mobility > 1 mm	III - access to all of the element width	Probing attachment loss - C (A+B, mm)
3 - + vertical mobility		

After all scores were recorded on the dental chart, a complete veterinary periodontal treatment was given. Calculus and plaque were removed using a periodontal scaler and ultrasound scaler. If necessary, teeth with abnormalities were extracted. The treatment was finished with polishing the teeth using polishing paste and a slow moving hand piece with a rubber cup.
Before the start of the study, a workshop was held at Utrecht University to synchronise all procedures.

2.5 Blood sample collection and analysis

To check the health of the dogs before and during the study, blood samples were taken during the first (day 0) and second (last day of the 5th month) examination of the dogs. The blood samples were taken from the jugular vein and stored in heparin, EDTA and serum tubes. The heparin blood was send away to the academic veterinary diagnostic laboratory for measuring urea, creatinine, glucose, alkaline phosphatase, bile acids, total protein and albumin. Half of the EDTA blood was send there as well, for a complete blood cell count using an automated cell counter. The other half of the EDTA blood and the serum blood were centrifuged immediately and stored at - 20°C for determination of the fatty acid profile and the levels of C-reactive protein. The fatty acid analysis was performed according to Retra et al. (2008) using high-pressure liquid chromatography/mass spectrometry.[102]

2.6 Statistical analysis

All data were tested for normality using the Shapiro-Wilk test. The teeth were divided in 12 parts and the average scores were calculated. The parts were: (1) buccal side of the upper right premolars and molars, (2) labial side of the upper canines and incisors, (3) buccal side of the upper left premolars and molars, (4) buccal side of the lower left premolars and molars (5) labial side of the lower canines and incisors, (6) buccal side of the lower right premolars and molars, (7) palatal side of the upper right premolars and molars, (8) palatal side of the upper canines and incisors, (9) palatal side of the

upper left premolars and molars, (10) lingual side of the lower left premolars and molars, (11) lingual side of the lower canines and incisors, (12) lingual side of the lower right premolars and molars. The sum of averages scores of calculus, plaque and gingivitis were calculated (range 0-48) and an ANOVA test was performed to test for differences between group A and group B between the two test moments. The blood values were also tested for normality with a Shapiro-Wilk test. An ANOVA test was performed on normal distributed data, and not normally distributed data were analysed by using a Wilcoxon rank sum test. The level of significance was set at $\alpha = 0.05$. All statistical analyses were carried out using the JMP program. The power analysis equation was used to determine sample size. With the variables values $\alpha = 0.05$, $\beta = 0.90$ and $\delta = 0.5$, the result was $n \geq 18$ dogs per group. To insure a minimum of 18 dogs per group, 44 dogs entered the study.

3. Results

One dog was lost to follow up, so 43 dogs (22 in group A and 21 in group B) completed the study. All dogs remained healthy during the study.

The sum of all average scores for calculus, plaque and gingivitis were distributed normally. The starting (day 0) average scores for calculus, plaque and gingivitis were not significantly different between group A and group B (table 3). The Portuguese dogs had significantly lower gingivitis scores ($p<0.0001$) than the Dutch dogs in phase one. The calculus and plaque scores were not significantly different (table 4).

No significant differences in calculus, plaque or gingivitis scores have been found between group A and B at the end of phase 2 (table 3). The calculus and gingivitis scores of the Portuguese and Dutch dogs did not differ significantly in phase 2, the plaque scores were significantly lower in the Dutch dogs ($p<0.0001$) (table 4).

Between phase 1 and 2, no significant differences were found for the calculus and gingivitis scores of group A. The plaque scores of group A were significantly lower in phase 2 compared to phase 1 ($p=0.0029$). In group B no significant difference has been found between the calculus scores of phase 1 and phase 2. The gingivitis scores of group B were significantly higher in phase 2 compared to phase 1 ($p=0.0479$) and the plaque scores were significantly lower in phase 2 ($p<0.0001$) (table 3).

The Dutch group A and B did not differ significantly in phase 1 or phase 2. Between phase 1 and 2 the Dutch dogs showed a significant reduction in plaque scores (<0.0001). The Portuguese group A and B did not differ significantly in phase 1 or phase 2. A significant rise in gingivitis scores has been found between phase 1 and 2 in Portugal ($p<0.0001$), the plaque scores were significantly lower in phase 2 compared to phase 1 ($p=0.0042$) (table 4). The Portuguese dogs in group B showed a significant rise in calculus scores ($p=0.0323$), but the dogs in group A did not.

No significant differences have been found between the client-owned dogs and the clinic-owned dogs in phase 1. In phase 2, the clinic-owned dogs of group B had significantly higher gingivitis scores than the client-owned dogs of group B ($p=0.0088$). This difference has not been found in group A in phase 2.

Table 3. Sum of scores of calculus, gingivitis and plaque.

	Phase 1		Phase 2	
Oil	A	B	A	B
Calculus	12.5 ± 4.5	10.5 ± 5.4	10.3 ± 3.8	11.3 ± 3.3
Gingivitis	17.0 ± 4.2	16.1 ± 4.9†	19.4 ± 4.1	18.9 ± 4.1†
Plaque	23.2 ± 5.4*	24.8 ± 5.9**	18.3 ± 4.6*	17.8 ± 4.6**

The shown values are mean ± SD.

* Scores between phase 1 and phase 2 significantly different ($p<0.05$)

** Scores between phase 1 and phase 2 significantly different ($p<0.05$)

† Scores between phase 1 and phase 2 significantly different ($p<0.05$)

Table 4. Sum of scores of calculus, gingivitis and plaque.

	Phase 1				Phase 2			
Country	The Netherlands		Portugal		The Netherlands		Portugal	
Oil	A	B	A	B	A	B	A	B
Calculus	13.1 ± 4.4	12.6 ± 5.9	11.9 ± 4.8	8.5 ± 4.2‡	9.9 ± 4.9	10.6 ± 3.7	10.7 ± 2.6	12.0 ± 2.8‡
Gingivitis	19.3 ± 3.3*	19.3 ± 4.1*	15.0 ± 4.0*†	12.9 ± 3.1*†	19.1 ± 5.0	20.1 ± 4.1	19.7 ± 3.2†	17.7 ± 3.9†
Plaque	22.6 ± 5.4†	24.5 ± 7.0†	23.7 ± 5.6†	25.1 ± 4.8†	15.1 ± 4.3**†	15.5 ± 5.0**†	21.1 ± 2.7**†	20.0 ± 2.9**†

The shown values are mean ± SD.

* Scores between countries (group A+B) in phase 1 significantly different (p<0.05)

** Scores between (group A+B) in phase 2 significantly different (p<0.05)

† Country scores (group A+B) between phase 1 and 2 significantly different (p<0.05)

‡ Group B scores between phase 1 and 2 significantly different (p<0.05)

The values of EPA and DHA in the blood samples were not normally distributed. Using the Wilcoxon rank sum test, the values of EPA were found to be significantly lower in group A compared to group B at the start of the study (p=0.0276). The values of DHA did not differ significantly. A significant rise of EPA and DHA values was observed in both group A and group B between phase 1 and 2. At the end of phase 2, the values for EPA and DHA were significantly higher in group A compared to group B (EPA p=0.0003, DHA p=0.0043) (table 5.). A significant difference between the dogs in Portugal and the dogs in the Netherlands has been observed for EPA and DHA in phase 1 (EPA p=0.0077, DHA p=0.0035), but not in phase 2. A significant difference has been found between the Dutch and Portuguese dogs of group B in phase one for EPA and DHA (EPA p=0.0013, DHA p=0.0028), but not in phase 2 (table 6.). No significant difference has been found between the Dutch and Portuguese dogs of group A in phase 1 or 2.

Table 5. Plasma EPA and DHA values.

	Phase 1		Phase 2	
Oil	A	B	A	B
EPA	0.14**† (0.03-1.08)	0.33**‡ (0.07-1.96)	2.63*† (0.06-10.79)	0.57*‡ (0.17-3.25)
DHA	0.37† (0.07-2.90)	0.52‡ (0.11-2.36)	3.09*† (0.14-8.89)	1.15*‡ (0.45-5.16)
Total	0.51† (0.11-3.83)	0.90‡ (0.19-4.32)	5.82*† (0.20-19.35)	1.80*‡ (0.62-7.77)

The shown values are median and range values in nmol/sample (200µL).

** Group values in phase 1 significantly different (p<0.05)

* Group values in phase 2 significantly different (p<0.05)

† Group values between phase 1 and 2 significantly different (p<0.05)

‡ Group values between phase 1 and 2 significantly different (p<0.05)

Table 6. Plasma EPA and DHA values.

	Phase 1				Phase 2			
Country	The Netherlands		Portugal		The Netherlands		Portugal	
Oil	A	B	A	B	A	B	A	B
EPA	0.14† (0.05-0.35)	0.15*† (0.07-0.40)	0.23† (0.03-1.08)	0.49*† (0.9-1.96)	2.39 (0.06-6.71)	0.76 (0.19-3.25)	3.33 (0.84-10.78)	0.54 (0.17-1.10)
DHA	0.21† (0.12-0.57)	0.28*† (0.11-0.84)	0.51† (0.07-2.90)	0.81*† (0.39-2.36)	3.04 (0.14-7.24)	1.33 (0.59-5.16)	3.13 (1.14-8.88)	0.96 (0.45-2.09)
Total	0.35† (0.21-0.84)	0.47*† (0.19-1.21)	1.27† (0.11-3.83)	1.27*† (0.68-4.32)	5.65 (0.20-13.95)	2.09 (0.78-7.78)	6.69 (1.98-19.34)	1.39 (0.62-3.19)

The shown values are median and range values in nmol/sample (200µL).

* Group B values between countries significantly different ($p<0.05$)

†Country values (group A+B) significantly different ($p<0.05$)

In phase 1, the CRP levels could not be measured in 5 dogs because they were lower than the measure limit of 10mg/L. In phase 2 the CRP levels of 39 dogs were lower than 10mg/L. In phase 1, no significant difference has been found between group A and B or between the Dutch and Portuguese dogs. In phase 2 the scores did also not differ significantly between group A and B or The Dutch and Portuguese dogs. The CRP scores were significantly lower in phase 2 compared to phase 1 ($p<0.0001$).

4. Discussion

The aim of this study was to investigate the effects of an EPA- and DHA-rich diet on gingivitis in healthy dogs. The hypothesis was that dogs that were fed a diet with supplemented EPA and DHA would have reduced gingivitis scores compared to dogs that were fed the control diet without supplemented EPA and DHA. In human gingivitis studies a tendency towards reduced gingivitis was found when the human patients received fish oil.[5,6] The effects of omega-3 fatty acids on gingiva have also been studied in rats. One study demonstrated that rats that received fish oil had lower pro-inflammatory cytokine gene expression and reduced alveolar bone resorption.[103] No such results were found in this study. No significant difference between group A and B for plaque, calculus or gingivitis has been found. A possible explanation for this is that, although the fatty acid analysis showed significant elevations of plasma EPA and DHA in phase 2, the EPA and DHA levels were not high enough to inhibit the inflammation. It could also be possible that dogs do not react the same way to EPA and DHA as humans do. However, it has been shown that omega-3 fatty acids have anti-inflammatory effect in dogs in other diseases.[8,9] Another possibility is that there was a difference between group A and B, but that it was smaller than expected. In that case, the statistical power was not high enough to mark these differences as significant. If future studies use more dogs per group, they could possibly find statistical differences that could not be found in this study.

A significant difference in plaque and gingivitis scores has been found between the dogs in Portugal and the dogs in the Netherlands in phase 1. It is a possibility that, even though all investigators participated in a workshop to ensure synchrony, these variations are caused by differences in interpretation by the investigators in Portugal and the Netherlands. Another possibility is that the diet they were receiving and the living conditions before the study had an influence on the gingivitis scores. The observations in phase 2 support this possibility, as no differences in gingivitis scores between the Portuguese and Dutch dogs were found after they had received the same control diet. However, the plaque scores were still significantly different in phase 2. Plaque can form within 24 hours,[1] it is possible that the time period between eating and scoring the plaque values were different in Portugal and the Netherlands. As the control diet removes dental plaque, and dental plaque forms quickly, the elapsed time between eating and scoring the plaque could greatly influence the scores. As such, the elapsed time between feeding and scoring could have been longer in Portugal, which would explain the higher plaque scores.

An overall reduction in plaque scores was observed between the first and the second phase. The reason for this could be that all dogs were fed Science Plan VetEssentials Canine Adult during the study. A feature of this diet is the texture of the kibble that promotes mechanical cleansing. The plaque could be removed by the kibble, which could lead to lower plaque scores.
The bacteria in plaque are an important etiologic agent in the development of gingivitis, therefore the expectation was that the reduction in plaque scores would lead to lower gingivitis scores. However, this effect has not been observed. On the contrary, a rise in gingivitis scores has been observed in (the Portuguese) group B. Although not all, many owners mentioned that they regularly gave their dogs rewards and chewing material such as dental sticks. In this study, treats and dental sticks were not allowed. Studies have shown that dental treats help reduce plaque and gingivitis in dogs by promoting mechanical and chemical cleansing.[32] It is a possibility that the kibble, even with special texture, is not as effective in cleaning the teeth as the dental treats. In that case, the rise in gingivitis scores could be explained by the absence of the dental treats. The same is true for brushing the teeth. The owners were not allowed to brush the teeth of their dogs during the study. Although it is not known whether the owners used to brush the teeth of the dogs or not, if they did brush the teeth before the study but not during the study, the lack of tooth brushing could be a possible explanation for the observed gingivitis scores. Another explanation is that the dogs were more susceptible for gingivitis during the study compared to before. It has been shown that stress and

other factors influence the susceptibility for gingivitis and periodontal disease in humans.[17] It is possible that dogs are also more susceptible for gingivitis and periodontal disease when they experience stress. It could be that changing to the control diet caused stress in the animals, leading to higher susceptibility for gingivitis. The gingivitis scores in group A were also higher, but this change was not significant. A possible explanation for this is that the higher EPA and DHA plasma levels slightly inhibited the gingivitis.

At the start of the study, the plasma EPA and DHA levels were significantly higher in the Portuguese dogs compared to the Dutch dogs. This could possibly be explained by different diets. If the Portuguese dogs received more dietary EPA and DHA than the Dutch dogs, this would lead to higher plasma levels. In phase 2, after the dogs received the same control diet, the dogs did not differ significantly anymore, which is consistent with the theory that the earlier difference was caused by diet differences. It is not known if the Portuguese dogs received different diets leading up to the study, to confirm this a analysis of the diets should be performed. The higher EPA and DHA plasma levels could also help explain why the Portuguese dogs had lower gingivitis scores in phase 1. In group A as well as group B a rise in plasma EPA and DHA levels has been observed. A possible explanation for this is that the control diet contained higher EPA and DHA levels than the diets the dogs used to eat. The observed difference between group A and group B in phase 2 could be explained if oil A would be the fish oil. In that case, the higher plasma levels of EPA and DHA would be the result of higher dietary EPA and DHA intake. This would be consistent with the hypothesis that supplementing EPA and DHA would lead to higher plasma levels of these fatty acids.

12 Clinic-owned dogs participated in this study. Because the living conditions could be different than the living conditions of the client-owned dogs and the genetic diversity could be less, the values of the clinic-owned dogs were compared to those of the client-owned dogs. No differences between the groups were found in phase 1. However, in phase 2 the clinic-owned dogs in group B had significantly higher gingivitis scores. It is possible that genetics could have influenced the scores. The clinic-owned dogs were mostly beagles and it could be that this breed or this population was more susceptible. The dogs in group A did not differ significantly, but again, this could possibly be explained by the higher plasma EPA and DHA levels.

The C-reactive protein is a systemic marker of inflammation, which can reach elevated levels during severe periodontitis.[19] Many of the dogs, mainly in phase 2, had C-reactive protein levels that could not be measured because they were lower than the measure limit of 10mg/L. Group A and B did not show any significant differences in phase 1 or 2. It is likely that the inflammation in the dogs was not severe enough to cause elevation of the CRP levels. A significant reduction in CRP between phase 1 and 2 has been observed in all dogs. This observation can possibly be explained by the higher EPA and DHA levels found in phase 2. An in vitro study has shown that EPA and DHA can reduce CRP expression in hepatocytes.[104] In humans, high plasma levels of EPA and DHA have been associated with lower CRP levels.[105,106]

In conclusion, this study showed that supplementing EPA and DHA to healthy dogs results in higher plasma levels of EPA and DHA. However, only small beneficial effects of EPA and DHA on gingivitis have been found. Future studies with a larger sample sizes are needed to investigate the possibilities of EPA and DHA in preventing periodontal disease.

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Appendix: Dental chart