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The lipidomic analysis in the veterinary field: Study of the red blood cell membrane lipid profile in physiological and pathological conditions.

A dissertation presented by

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The lipidomic analysis in the veterinary field:

Study of the red blood cell membrane lipid profile in physiological and pathological conditions

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Declaration

I, Prasinou Paraskevi, declare that this thesis is an original report of my research, has been written by me and has not been submitted for any previous degree or professional qualification. The experimental laboratory work is entirely my own work; the collaborative contributions have been indicated clearly and acknowledged.

Our work on the erythrocyte membrane lipidome in healthy dogs (presented in chapter 2.2.1) has been published in Frontiers in Veterinary Science, section Animal Nutrition and Metabolism: *The erythrocyte membrane lipidome of healthy dogs: creating a benchmark of fatty acid distribution and interval values*, by Paraskevi Prasinou, Paolo Emidio Crisi, Chryssostomos Chatgilialoglu, Morena Di Tommaso, Anna Sansone, Alessandro Gramenzi, Benedetta Belà, Francesca De Santis, Andrea Boari, Carla Ferreri. Front. Vet. Sci. (2020) doi:10.3389/fvets.2020.00502

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ABSTRACT

The role of lipids in health and disease in humans has been recognized for many decades and over the last fifteen years there has been an intense effort to develop suitable methodologies to discover, identify, and quantitatively monitor lipids in biological systems. That led to a dramatical increase on the use lipidomics in search of new possible biomarkers or to better understand pathological mechanisms. The cell membrane lipidomics is based on homeostatic balance of the fatty acid metabolic pathways and can give us important information on the composition activity and the cause of oxidative stress in the cellular membranes. Erythrocyte membrane lipidomic analysis offers some new perspectives in nutraceuticals and personalized health interventions in order to help therapies at molecular level, to maintain the best homeostasis possible in a pathological condition. Several studies have shown modification of the phospholipid content of cell membranes and point to alterations of the enzymic activity on the fatty acid metabolic pathways in diabetic patients.

Using a protocol widely tested in human profiling, in the present study erythrocyte membrane lipidome was examined in healthy and diseased dogs. In particular, a cluster composed of 10 cis fatty acids, present in membrane glycerophospholipids and representative of structural and functional properties of cell membrane, was chosen and quantitatively analyzed. This cluster consists of: 2 saturated fatty acids (SFA: palmitic and stearic acids); 3 monounsaturated fatty acids (MUFA, palmitoleic, oleic and cis-vaccenic acids); 3 polyunsaturated fatty acids omega-6 (PUFA, linoleic, dihomo-gamma linolenic, arachidonic acids); 2 polyunsaturated fatty acids omega-3 (PUFA, eicosapentaenoic and docosahexaenoic acids).

For the purpose of this study, blood samples were collected from 68 clinically healthy dogs, including 30 males (6 neutered) and 38 females (12 sterilized), weighting from 2.6 to 43 kg, aged from 2 to 156 months (median 41). The interval values and distribution for each fatty acid of the cluster were determined, providing the first panel describing the healthy dog lipidomic membrane profile. We used these interval values to evaluate correlations between FAME types, families and lipid indexes with the dog characteristics and the results showed an interesting correlation to bodyweight increase. More specific, with the increase of the bodyweight, an increase in palmitic acid and in the total levels of SFA, while a decrease of the omega-6 and the total PUFA was observed.

The same intervals when then used to evaluate diseased dogs:

ι) 49 dogs affected with chronic enteropathy (CE), consisting of 17 females (7 spayed) and 32 males (1 neutered), with a median age of 47 months (range 4–144). Interestingly, the CE dogs had higher values of palmitic acid and lower levels of stearic acid compared to healthy dogs, with an overall reduction of total SFA. In addition, the CE dogs showed reduced content of LA and increased DGLA when compared to healthy dogs.

ii) 12 dogs affected with diabetes mellitus (DM), consisting of 7 females (5 spayed) and 5 males (1 neutered), with a median age of 131 months (range 94–181). The results obtained showed increased levels of palmitoleic and oleic acids as well as the total MUFA content on DM dogs when compared to the healthy dogs. These fatty acids cannot derive from the enzymatic transformation of palmitic acid by the delta-9 desaturase and their increased levels indicate an accelerated Δ9 desaturase activity on the DM dogs. The activation of this enzymatic transformation is known to be related to the insulin response and the carbohydrate management, which is connected to lipid biosynthesis through the pyruvate and acetyl-CoA pathways.

In the current study, we provided the first panel of erythrocyte membrane fatty acids in healthy dogs choosing a cohort representative for the main structural and functional roles of these hydrophobic molecules in the cell membrane compartment. and demonstrated a first utility of the interval values established for healthy dogs to evaluate the condition of these two pathological conditions. The results obtained from this study indicate that the erythrocyte membrane lipidome of animal samples may be successfully applied in veterinary medicine and provide important of the cell membrane status in physiological and pathological conditions and a better understanding to how membranes are influenced by dietary habits.

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- from the 11th of June 2018 until the 9th of October 2018,

- from the 11th of November 2018 until the and 6th of January 2019

- from the 8th of April 2019 until the 25th of June 2019,

- from the 27th of July 2019 until the and 9h of October 2019

and finally

- from the 11th of November 2019 until 2rd of April 2020.

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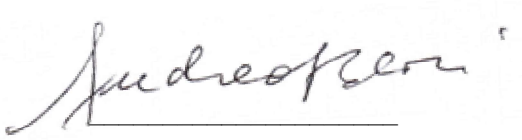

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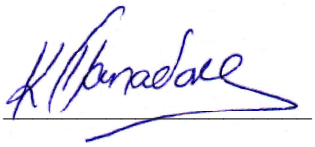

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- from the 7th of January 2019 until 7th of April 2019.

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I undersigned Chryssostomos Chatgilialoglu, in my quality of tutor at Lipinutragen s.r.l., Bologna, Italy, confirm that Paraskevi Prasinou has carried out her activity as scheduled in her career development plan, at our institution:

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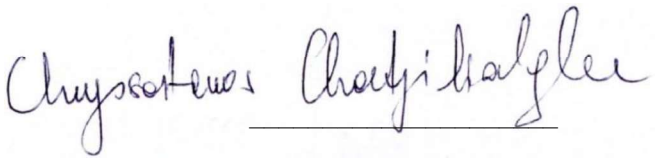

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List of Abbreviations

AA, ARA – Arachidonic Acid

ARE – Antibiotic-Responsive Enteropathy

BCS – Body Condition Score

CE – Chronic Enteropathy

DGLA – Dihomo-Gamma-Linolenic Acid

DHA – Docosahexaenoic Acid

DM – Diabetes Mellitus

EDTA – Ethylenediaminetetraacetic acid

EPA - Eicosapentaenoic Acid

FA – Fatty Acid

FAME – Fatty Acid Methyl Esters

FRE – Food-Responsive Enteropathy

GC – Gas Chromatography

HD – Healthy Dogs

IBD – Inflammatory Bowel Disease

IRE – Immunosuppressant-Responsive Enteropathy

LA – Linolenic Acid

LCFA – Long Chain Fatty Acid

MUFA – Monounsaturated Fatty Acids

PBS – Phosphate-Buffered Saline

PC – Phosphatidylcholine

PE – Phosphatidylethanolamine

PLE – Protein-Losing Enteropathy

PS – Phosphatidylserine

PUFA – Polyunsaturated Fatty Acids

RBC – Red Blood Cell

SFA – Saturated Fatty Acids

UC – Ulcerative Colitis

VTH – Veterinary Teaching Hospital

CHAPTER 1: Introduction

1.1 Lipids

Lipids are molecules essential for life with important biological roles within structural, functional and signaling activities. They are known to be highly dynamic since they are constantly changing along with physiological, pathological, and environmental conditions (van Meer et al. 2018). The structural disposition of protein channels and lipid components influencing fluidity and permeability, as well as the presence of specific fatty acid components in membrane phospholipids, involving receptor functioning and intra/inter cellular signaling, provide proper conditions for physiological operations (Giacometti et al.2017; Ferreri and Chatgilialoglu 2012). Fatty acids (Fig.1), as structural components of membranes and inflammation/anti-inflammatory mediators, have well-known protective and regulatory effect (Ferreri et al. 2017)

SFA - Saturated fatty acids	MUFA - Monounsaturated fatty acids	PUFA - Polyunsaturated fatty acids:	
		ω3	ω6
Palmitic Stearic	Palmitoleic Oleic Vaccenic	EPA DHA	Linoleic Diomo-gamma-linolenic Arachidonic
			

Figure 1. Structural demonstration of fatty acids according to the three fatty acid families (SFA, MUFA and PUFA)

Lipids are molecules essential for life with important biological roles within structural, functional and signaling activities. They are known to be highly dynamic since they are constantly changing along with physiological, pathological, and environmental conditions (van Meer et al. 2018). The cell membrane lipidomics is based on homeostatic balance of the fatty acid metabolic pathways (Fig.2) and can give us important information on the composition activity and the cause of oxidative stress in the cellular membranes (Giacometti et al. 2017; Berliner and Watson 2005; Tsimikas et al. 2006).

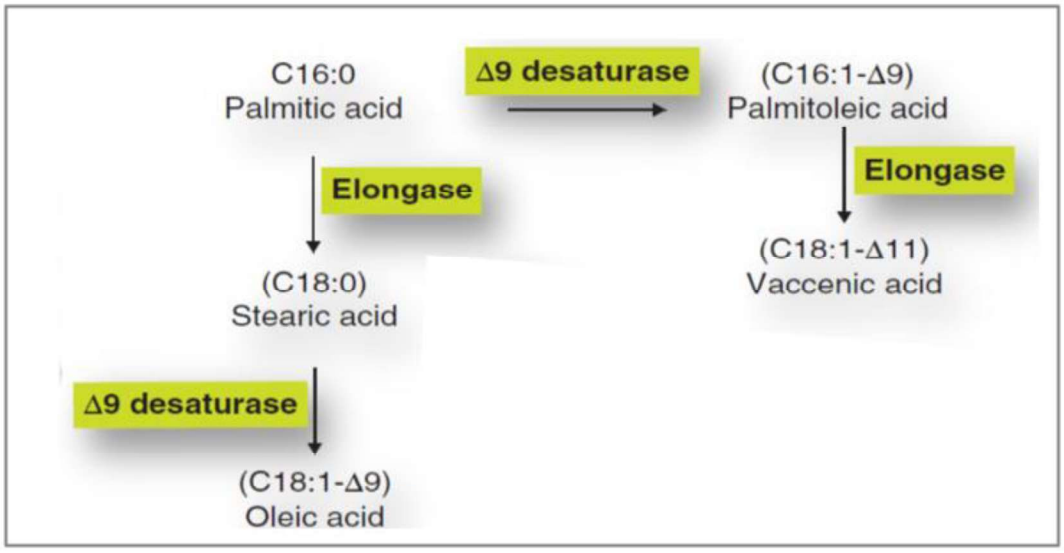


Figure 2. The main transformations of the SFA-MUFA pathway. The SFA (palmitic and stearic acids) transform into MUFA (palmitoleic, oleic and vaccenic acids) with the help of enzymatic activity (elongase and Δ9 desaturase).

1.2 Lipidomics

Lipidomics is the discipline that gathers lipids, not only considering lipid structures and their transformations, but also providing a "dynamic" interpretation of lipid diversity and functions in view of cellular, metabolic and environmental conditions influencing living organisms (Han 2016). The lipidomic research covers a wide range of topics, going from physiological to oxidative and pathological processes (Ni et al. 2019; Zhao et al. 2014). With the use of lipidomics a large set of data can be acquired, giving important information about the entire lipidome and its variations as a fingerprint of health status (Quehenberger et al. 2010; Wong et al. 2019).

The physiological importance of lipids is illustrated by the numerous diseases to which lipid abnormalities contribute including atherosclerosis, diabetes, obesity, and Alzheimer's disease (Watson 2006). Cholesterol has been early identified as an important risk factor of heart disease (Kuburovic et al. 2017; Bays et al. 2017).

Many diseases are characterized by dysregulated lipid metabolism and alterations of lipid profiles that can precede the onset of diseases, rather than being a consequence (Markgraf et al. 2016), allowing the development of specific disease biomarkers.

Lipidomic approaches were applied to study diseases ranging from cancer to metabolic diseases such as obesity and diabetes (Wang et al 2003; Señars et al. 2003; Berliner and Watson 2005; Holland and Summers 2008Sewell et al. 2012; Fan et al. 2015; Rojas-Gutierrez et al. 2017).

The role of lipids in health and disease in humans has been recognized for many decades and over the last fifteen years there has been an intense effort to develop suitable methodologies to discover, identify, and quantitatively monitor lipids in biological systems. That led to a dramatical increase on the use lipidomics in order to discover biomarkers or to better understand pathological mechanisms

The development of shotgun lipidomics accelerated the process of gathering information on thousands of lipid molecules in different organisms and conditions, going from yeast, virus and bacteria to murine models and humans (Jain et l. 2013; Klose et al. 2012; Pradas et al. 2018; Zhao et al. 2015). In particular, the membrane lipidome has a central place in a multidisciplinary context of biophysical, biological, pharmacological and clinical studies that demonstrates its importance in fundamental processes such as cell formation, regeneration and metabolic regulation (Casares et al. 2019; Ferreri et al. 2016; Zhu et al. 2015). Focusing on membrane glycerophospholipids, the analysis of the two fatty acid chains esterified to the glycerol moiety led to the discovery of positional and geometrical isomers and of the homeostatic balance created by these hydrophobic molecules, based on the fact that the fatty acid structures influence bilayer thickness, permeability, fluidity and membrane protein functions (Casares et al/ 2019; Ferreri et al. 2016; Zhu et al. 2015; Diaz et al. 2019;Ferreri and Chatgilialoglu. 2012) [10-14]. In Figure 3 the main fatty acid types are shown, that can be saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) (Prasinou et al. 2020).

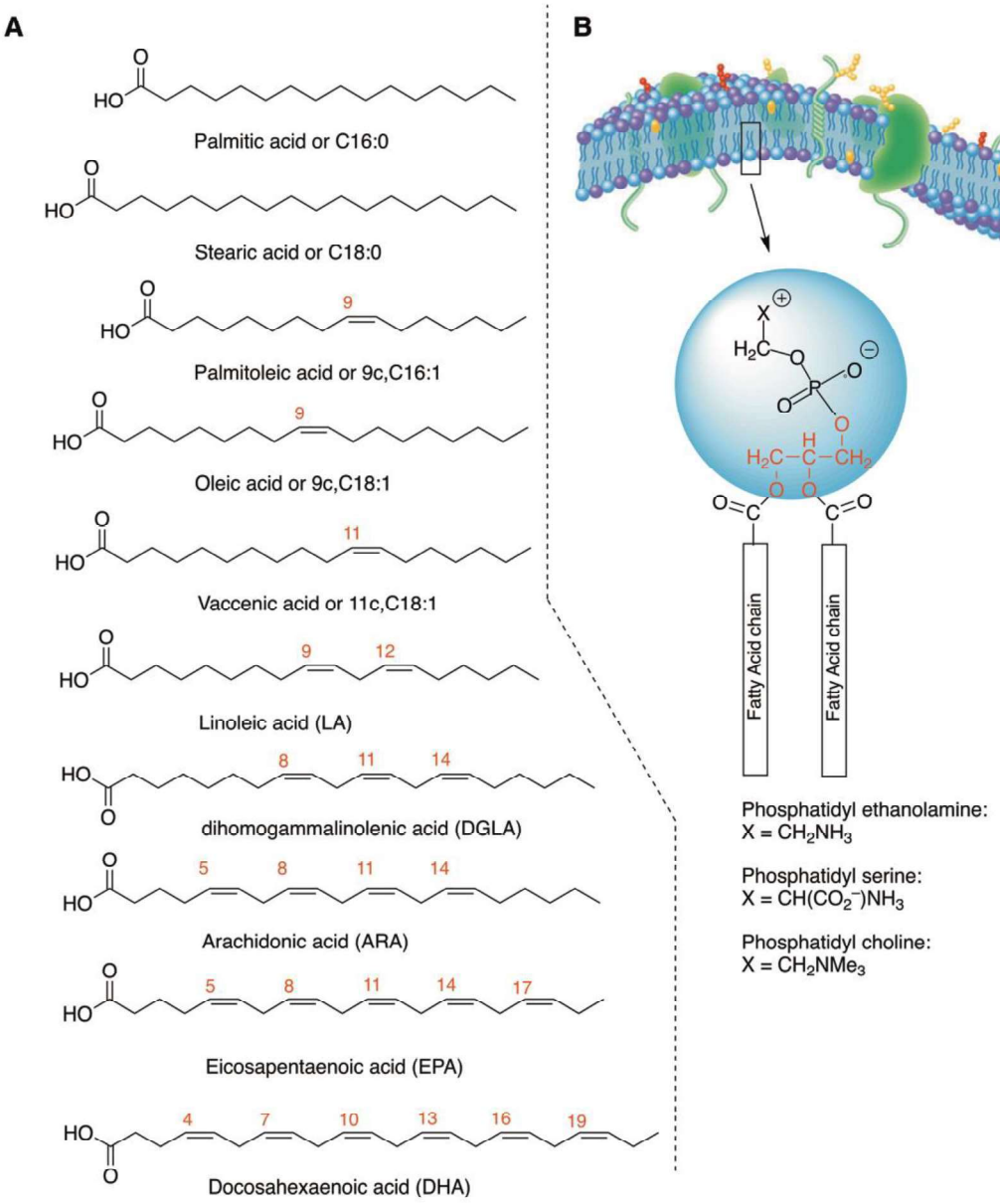


Figure 3. (A) The structure of fatty acids considered in the present work. Together with the common names, the abbreviations describe the position and geometry of the double bonds (e.g., 9c for palmitoleic acid), as well as the notation of the carbon chain length and total number of double bonds (e.g., C18:1). For polyunsaturated fatty acids (PUFA) in the parenthesis their acronyms are indicated. (B) Molecular structures of glycerophospholipids with the two fatty acid residues at the C-1 and C-2 positions of the glycerol moiety (in red), whereas in the C-3 position the polar head group is connected (examples of polar heads: ethanolamine, serine and choline).

Application of fatty acid-based membrane lipidomics to human health is a hot topic of research, comparing healthy controls with disease subjects (Sansone et al. 2016; Amézaga et al. 2018; Gai et al. 2019; Harris et al. 2013). Lipidomic analysis of tissue highlights local

alteration, however the necessity of sequential biopsies may be unpractical in a first screening clinical setting, as it occurs in diseases such as cancer (Bardelli et al. 2017). Plasma was considered to follow a similar lipidomic pattern of that of the tissues (Arab 2003), however nowadays it is well known that the lipid content of plasma can be greatly affected by the recent dietary intake (Timlin and Parks 2005; Barrows and Parks 2006). Lipidomic analysis of the red blood cell seems to overcome these limitations: indeed, erythrocytes come in contact with all the bodily districts as they travel for the duration of their life (approximately 120 days), and they are informative of metabolic, nutritional and healthy conditions of long term dietary habits (Ebaugh et al. 1953); therefore it can be considered a representative cell of the general health status (Table 1). Secondly, blood sampling necessary for lipidomic analysis is a routinely performed technique, non-invasive with the possibility to obtain several samples over time. Furthermore, analyses carried out on plasma give information about lipid metabolism of a few weeks before withdrawal, whereas blood cell membrane fatty acids (e.g. RBC) account for more stable information obtained from metabolic transformations together with stabilized dietary contributions (Ferreri and Chatgilialoglu 2012).

FATTY ACID	Adipose tissue (%rel)	RBC (%rel)	LIVER (%rel)	RETINA (%rel)	BRAIN (%rel)
18:2, omega-6, LIN	10.5	9.3	17.5	1.4	0.6
20:4 , omega-6, AA	**0.3**	**15.2**	**7.7**	**9.6**	**7.7**
20:3, omega-6, DGLA	0.2	1.5	1.6	nd	1.2
20:5, omega-3, EPA	traces	0.7	0.4	0.1	traces
22:6, omega-3, DHA	**0.3**	**3.2**	**3.4**	**19.7**	**7.2**
SFA	27.2	43.1	42.0	48.2	45.9
MUFA	59.7	23.0	23.8	14.2	29.7
PUFA	13.1	33.3	32.0	37.2	23.4

Table 1. The red blood cell – RBC with all classes of lipids. Looking at all the different tissues it can be observed that the families of fatty acids have a similar contribution across tissues but different quantities of each fatty acid characterize the specific tissue.

Fatty acid analysis can be carried out starting from a non-invasive blood withdrawal, separating blood fractions that contain molecular information at different metabolic levels [(Harris et al. 2012; Furtado et al. 2019). In fact, while plasma informs on the daily fatty acid dietary intakes, cell membrane fatty acids result from more complex contributions of physiological and metabolic processes together with nutritional intakes. Erythrocytes are the most numerous cell

type in blood (Sender et al. 2016) and their membrane homeostasis derives from the distribution of the various fatty acid types, playing roles in several functions, such as for example oxygen distribution. Having lost DNA very early, RBC fatty acids cannot come from biosynthesis, but derive from exchange with lipoproteins and tissues. In this scenario, it is worth mentioning that eukaryotic cells need essential fatty acids (EFA), such as omega-3 and omega-6 PUFA, that must be taken daily from the diet in order to be processed enzymatically in the body and provide long chain PUFAs. The PUFA pathways, together with SFA and MUFA, contribute to the formation of the cellular lipid pool and subsequently to the specific fatty acid biodistribution in RBC membranes as well as in the various organs. The RBC fatty acid composition is informative of levels reached in tissues (fig, like muscle and liver, and also in tissues not withdrawable during life, like retina and brain (Makrides et al. 1994; Baur et al. 2000; Acar et al. 2012; Abbott et al. 2012).

Analysis of erythrocyte membrane lipidome represents a powerful diagnostic tool in humans for assessing the quantity and quality of fatty acids and for the follow-up of the membrane fatty acid remodeling that is associated with different physiological and pathological conditions (Ferreri et al. 2017; Acar et al. 2006).

1.3 Lipidomics in the Veterinary field

The analysis of erythrocytes in animals is also reported in veterinary, monitoring different health conditions and supplementations (Ferreri and Chatgilialoglu 2015; Stoecle et al. 2011; Mehta et al. 1991). However, a systematic study of the membrane fatty acids of domestic animals (dogs and cats) to evaluate the healthy membrane balance for different animal species and pathologies has not yet been established.

1.3.1 Chronic Enteropathy in the Veterinary field

Canine chronic enteropathies (CE) are chronic, inflammatory processes that affect the gastrointestinal tract of dogs and, according to the response to subsequentially therapeutic trial, are represented by food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (ARE) and immunosuppressant-responsive enteropathy (IRE). Dogs not responding to treatment are categorized as having non-responsive enteropathy (NRE) (Washabau et al. 2010; Dandrieux 2016).

In addition to this classification, dogs with loss of protein across the gut are grouped as protein-losing enteropathy (PLE), highlighting the more guarded prognosis of this particular form of CE compared to dogs with normal serum albumin concentration (Craven et al 2005; Allenspach et al. 2007).

The dysfunction in the interaction between the mucosal immune system, the intestinal microbiota and dietary components are considered the trigger for the aberrant inflammatory response, however knowledge on the underlying mechanisms of chronic enteropathy are still in its infancy (Hall 1994; German et al. 2003; Washabau et al. 2010; Suchodolski et al. 2012). Lipids are critical to cell membrane structure and fluidity, mucus production and barrier integrity; furthermore, fatty acids are incorporated into phospholipids of the membrane bilayer and they are recognized as key components of multiple signal transduction cascades, including those associated with the activation and resolution of inflammation (Serhan et al. 2008).

In the recent years along with the development of the molecular medicine, membrane lipidome and fatty acid-based functional lipidomics became important tools to examine the membrane compartment, evidencing a relationship between health and lipidome composition (Hu et al. 2009; Ferreri and Chatgilialoglu 2012; Ferreri et al. 2017) and lipid alterations have been observed in several canine conditions including idiopathic hyperlipidemia (Xenoulis et al. 2007), diabetes mellitus (Seage et al. 2018), renal disease (Behling-Kelly 2014; Smith et al. 2017), parvoviral or systemic infections (Yilmaz and Senturk 2007; Gültekin et al. 2017).

Lipidomic analysis of tissue highlights local alteration, however the necessity of sequential biopsies may be unpractical in a first screening clinical setting, as it occurs in diseases such as cancer (Bardelli et al. 2017). Plasma was considered to follow a similar lipidomic pattern of that of the tissues (Arab 2003), however nowadays it is well known that the lipid content of plasma can be greatly affected by the recent dietary intake (Timlin and Parks 2005; Barrows and Parks 2006). Lipidomic analysis of the red blood cell seems to overcome these limitations: indeed, erythrocytes come in contact with all the bodily districts during their life of 120 days, and they are informative of metabolic, nutritional and healthy conditions of long term dietary habits (Ebaugh et al. 1953); secondly, blood sampling necessary for lipidomic analysis is a routinely performed technique, non-invasive with the possibility to obtain several samples over time.

Increasing evidence, in both humans and rodents, suggest that inflammation alters lipid utilization in the intestine. Indeed, ulcerative colitis (UC) and active and inactive Crohn disease patients had a significantly higher proportion of tissue saturated fatty acids (SFA) and long-

chain ω-3 and ω-6 polyunsaturated fatty acids (PUFA) with a concomitant decline in MUFA (Pacheco et al. 1987; Nishida et al. 1987; Buhner et al 1994; Fernandez-Banares et al. 1997). Membrane phospholipids of circulating erythrocytes in IBD patients had higher concentrations of linoleic acid and total ω-6 PUFA, without difference in ω-3 levels compared to control group (Ueda et al. 2008). Recently, significant variances were identified in the plasmatic phospholipid profiles of dogs with inflammatory bowel disease and food-responsive diarrhea (Kalenyak et al. 2019). At molecular level, it is also known that SFAs induce inflammatory signalling by stimulating toll-like receptors TLR2 and TLR4, whereas PUFA and, in particular the omega-3 docosahexaenoic acid (DHA) is able to contrast such cascade (Hwang et al. 2016). Mechanisms for the activation of Toll-like receptor 2/4 by saturated fatty acids and inhibition by docosahexaenoic acid. (*Eur. J. Pharmacol. 785*, 24–35). On the other hand, long chain fatty acids (LCFA) have different roles in the regulation of the intestinal barrier and functions, therefore the fatty acid profile of the individuals has become more and more important for the deep understanding of the molecular asset that influences pathological conditions (Ma et al. 2019).

1.4 Aims and Objectives

In human studies, there has been a contribution to research in membrane fatty acid-based lipidomics making the arbitrary choice of analyzing a specific fatty acid cohort of erythrocyte membrane glycerophospholipids, made of 10 cis fatty acids representative of SFA, MUFA and PUFA families (Ferreri et al. 2016). Their interval values in healthy cohorts were reported in membrane fatty acids by several studies (Heude et al. 2003; Giacometti et al. 2017; Pallot et al. 2019) and in one of the most complete meta-analysis appeared in the literature (Hodson et al. 2008). Taking into account that novel fatty acid pathways and transformations are continuously studied (Sansone et al. 2013) it is important to make the strategic choice of a 10 fatty acids cohort considering their fundamental biochemical/biological roles and the consensus reached about their levels in cell membranes.

By these premises, we considered whether analogous data are reported in domestic animals, finding only a few studies on plasma or other tissues (brain, sperm) and on correlations between dietary uptake and lipidomic profiles of dogs (Snigdha et al. 2011; Hall et al. 2012; Risso et al. 2016; Sieber-Ruckstuhl et al. 2019; Boretti et al. 2020). Limited studies presented a small number of samples (Mehta et al. 1991; Fuhrmann et al. 2006), focusing for example on PUFA

(omega-6 and omega-3), and not deepening the different fatty acid families which are instead important for molecular contribution to membrane homeostasis (Stoeckel et al. 2011).
In addition, analyses conducted on plasma provide information essentially related to the daily dietary intake, while the lipidomic analysis conducted on the erythrocyte membrane provides information derived from endogenous metabolic processes balanced by the effects of the diet (Ferreri et al. 2016).
Although fatty acids are recognized to be crucial for all animals (Siobhan et al. 2017) an effort to build up the methodology for fatty acid-based membrane lipidomics in the veterinary field is missing.
In the current study we propose a systematic methodology for erythrocyte fatty acid-based membrane lipidomics in healthy dogs, extending our previous studies of human healthy and disease conditions (Ferreri et al. 2016).
The erythrocyte membrane profile is nowadays a well-assessed tool for the evaluation of both stabilized dietary habits and metabolic processes and this is an important information to gather especially in case of intestinal diseases, where the nutritional strategy can be relevant for local and systemic effects. Indeed, it is well known that malnutrition is associated with intestinal diseases (Sugihara et al. 2019) therefore it is often suggested a “balanced diet” that in many cases needs to be personalized.
In the present work we evaluated the composition of fatty acids in erythrocyte membrane phospholipids in a group of clinically healthy dogs (n = 68), focusing on the interval ranges of the 10 cis fatty acid cohort previously used for humans. We are also interested in teh correlations with dog characteristics such as age and bodyweight. The general aim of this work is to create a benchmark of fatty acid interval values in the membrane lipidome of healthy animals, useful to start a systematic approach for the examination of metabolic and nutritional status in healthy and diseased dogs.

Lipidomics is an important aspect of personalized medicine in relation to nutrition and metabolism and it is aiming to individuate molecular profiles and indicate personalized nutra-strategies, useful to keep a healthy balance or to synergize with pharmacological treatments (Brenner 2016). It is therefore of high interest to be able to apply this methology in order to investigate the analysis of erythrocyte membrane lipidome for assessing the quantity and quality of fatty acids in the erythrocyte membrane lipidome of dogs with pathological conditions such as CE and DM.

CHAPTER 2: LIPIDOMIC ANALYSIS

2.1 MATERIALS AND METHODS

2.1.1 Chemicals

Table 2. The materials used on this project with the corresponding suppliers

Materials	Company
n-Hexane 95%	TITOLCHIMICA, Pontecchio Polesine (Ro) Italy
Methyl alcohol HPLC	TITOLCHIMICA Pontecchio Polesine (Ro) Italy
Chloroform extra pure 99.5%	TITOLCHIMICA Pontecchio Polesine (Ro) Italy
PBS pH 7,4 RS	Carlo Erba, Milan (Italy)
Polar Lipid Mixture (quantitative)	MATREYA LLC State College, PA, USA
non-Polar Lipid Mixture B (quantitative)	MATREYA LLC State College, PA,USA
Phosphatidylserine	MATREYA LLC State College, PA, USA
L-α-Phosphatidylcholine	Merck, Darmstadt, Germany
ALUGRAM Xtra sheets 200x200mm	Carlo Erba, Milan Italy
Potassium hydroxide, pellets RPE - For analysis	Carlo Erba, Milan Italy
Sodium sulfate anhydrous RS - For anhydrification	Carlo Erba, Milan Italy
C16:0 – palmitic acid methyl ester	Merck, Darmstadt Germany
C16:1 – palmitoleic acid methyl ester	Merck, Darmstadt Germany
C18:0 – stearic acid methyl ester	Supelco, Bellefonte, PA, USA
9c, C18:1 – oleic acid methyl ester	Merck, Darmstadt, Germany
11c, C18:1 – vaccenic acid methyl ester	Supelco, Bellefonte, PA ,USA
LA omega-6 – C18:2 – linoleic acid methyl ester	Merck, Darmstadt Germany
DGLA omega-6 – C20:3 dihomogammalinolenic acid methyl ester	Merck, Darmstadt Germany
ARA omega-6 C20:4 – arachidonic acid methyl ester	Merck, Darmstadt Germany
EPA omega-3 – C20:5 – eicosapentaenoic acid methyl ester	Supelco, Bellefonte, PA, USA
DHA omega-3 – C22:6 – docosahexaenoic acid methyl ester	Merck, Darmstadt Germany
Supelco 27 component FAME mix	Supelco, Bellefonte, PA, USA

Materials were used as received.

2.1.2 Inclusion criteria and samples collection

Blood samples from healthy and diseased dogs were collected in EDTA tubes from the medical staff of the VTH of Teramo and of other Veterinary Hospitals. The samples were stored at 4oC, for a maximum of 15 days.

All the subjects have been selected based on physical examination and haemato-biochemical laboratory workup.

2.1.2.1 Ethical Statement

The project has been approved by the Health Ministry and the Committee on Animal Research and Ethics of the Universities of Chieti-Pescara, Teramo and Experimental Zooprophylactic Institute of AeM (CEISA), Protocol UNICHD12 n.1168 (Chieti, March 19, 2018). In addition, a written informed consent by the animal's owners is being acquired.

2.1.2.2 Healthy Dogs

Inclusion criteria for the study were: healthy dogs without any clinical or pathological evidence of disease accordingly to unremarkable history, physical examination and results of CBC, serum biochemistry and voided urine analysis (Table 3) within the reference range for each dog.

Table 3. Complete blood count, serum chemistry and urine parameters evaluated to determine the healthy status of enrolled healthy dogs.

Variable (Unit of measure)	Manufacturers
Red Blood Cells (cells/mm³)	ADVIA 2120; Siemens Healthcare Diagnostics
Hematocrit (%)	
Hemoglobin (g/dL)	
Mean Cell Volume (fL)	
Mean Platelet Volume (fL)	
Mean Corpuscular Hemoglobin Concentration (g/dL)	
Mean Corpuscular Hemoglobin (pgr)	
Red Blood Cell Distribution Width (%)	
White Blood Cells (cells/mm³)	
Neutrophils (cells/mm³)	
Lymphocytes (cells/mm³)	
Monocytes (cells/mm³)	
Eosinophils (cells/mm³)	
Platelets (cells/mm³)	
Basophils (cells/mm³)	
Microscopic evaluation blood smear	
Creatinine (mg/dl)	OLYMPUS AU 400; Olympus-Beckman Coulter
Urea (mg/dl)	
Total calcium (mg/dl)	
Ionized calcium (mmol/l)	
Phosphate (mg/dl)	
Sodium (mEq/l)	
Chloride (mEq/l)	
Potassium (mEq/l)	
Magnesium (mg/dl)	

Variable (Unit of measure)	Manufacturers
Total proteins (g/dl)	OLYMPUS AU 400; Olympus-Beckman Coulter
Albumin (g/dl)	
Albumin-to-globulin ratio	
Glucose (mg/dl)	
Fructosamine (mg/dl)	
Aspartate transaminase (U/l)	
Alanine transaminase (U/l)	
Gamma(γ)-glutamyl transferase (U/l)	
Alkaline phosphatase (U/l)	
Total bilirubin (mg/dl)	
Total cholesterol (mg/dl)	
Triglycerides (mg/dl)	
Lipase (U/l)	
Amylase (U/l)	
c-reactive protein (mg/dl)	
Iron (µg/dL)	
Total Iron Binding Capacity (µg/dL)	
Latent Iron Binding Capacity (µg/dL)	
Iron Saturation (%)	
Urinary Specific Gravity	Refractometer; American Optical
Urine dipstick examination	Combur10Test; Roche Diagnostic
Microscopic evaluation urine sediment	

Furthermore, recruited dogs were on commercial diet and did not receive supplements and medications in the previous 4 months (except regular preventive treatments for ecto- and endoparasites and prophylactic vaccination), as ascertained by an interview with the owners.

2.1.2.3 Dogs diagnosed with Chronic Enteropathy

Dogs with primary chronic gastrointestinal signs presented to Veterinary Teaching Hospitals of the Universities of Teramo and Bologna and to the Veterinary Hospital “I Portoni Rossi”, Italy, were prospectively enrolled in the study.

Dogs were eligible for the study if they had at least 3-week history of clinical signs consistent with CE, including vomiting, diarrhea, borborygmus, hyporexia, abdominal pain and/or weight loss.

The Canine Chronic Enteropathy Clinical Activity Index (CCECAI) (Allenspach. et al 2007) and the body condition score (BCS) were obtained for each patient and every dog underwent a 5-day course of fenbendazole 50 mg/Kg once a day regardless to the faecal tests results. Moreover, all dogs underwent complete blood count, serum chemistry panel, evaluation of

basal cortisol, c-reactive protein (CRP), canine Trypsin-like Immunoreactivity (cTLI), folate, cobalamin and abdominal ultrasound.

Based on the retrospective diagnosis achieved considering the clinical response to sequential therapeutic trials, dogs affected by chronic enteropathy (CE) were further divided into 4 subgroups (Washabau et al. 2010; Dandrieux 2016). The FRE group included patients with complete remission of the gastrointestinal symptoms within 3 weeks of dietetic trial with a new exclusive diet (hydrolysed or restricted antigen diet); the ARE group included patients with partial or no response to dietetic trial but complete remission of the gastrointestinal symptoms within 3 weeks of antibiotic trial with tylosin at dose of 15 mg/Kg twice a day; the IRE group included patients that neither responded to diet nor to antibiotics and that went to complete remission on antinflammatory/immunosuppressive drugs such as prednisolone or budesonide. Regardless to the diagnosis (i.e. FRE, ARE, IRE), those patients with low albumin concentration (< 2 mg/dl) due to a severe loss of serum proteins into the intestine were further classified into the PLE sub-group. For IRE, NRE and PLE dogs a histopathological analysis of multiple gastrointestinal endoscopic biopsies was available.

Exclusion criteria were the presence of extra-gastrointestinal disease or exocrine pancreatic insufficiency and recent (< 1 month) anti-inflammatory/immunosuppressive and/or antibiotics treatment and dietary ω-3 supplementation in last 4 months.

2.1.2.4 Dogs diagnosed with Diabetes Mellitus

All the diseased animals were selected based on diagnostic international guidelines. Dogs were diagnosed with DM based on consistent clinical signs, and persistent fasting hyperglycaemia and glycosuria.

Dogs with signs of presence of Diabetic Ketoacidosis or other concurrent relevant disorders or/and dogs that received supplementation with ω3 or medications in the last 4 months were excluded from the study.

2.1.3 Lipidomic analysis of the blood samples

The steps of the protocol procedure are depicted in the following figure (4).

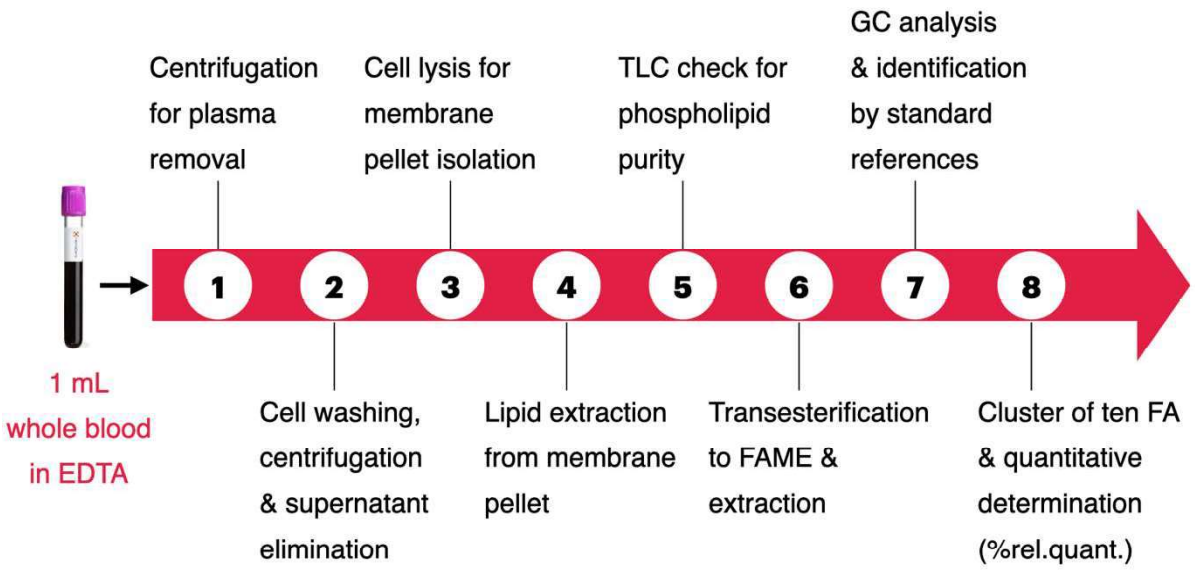


Figure 4. The 8 steps of the fatty acid-based erythrocyte membrane lipidomic analysis.

2.1.3.1 Isolation of phospholipids from erythrocyte membranes

Step 1: separation of red blood cells from plasma, effected on 1 mL of fresh EDTA-treated whole blood sample, by two consecutive centrifugations (3000 g × 5 min, each) followed by plasma removal. Step 2: cell washings (2 times) with phosphate buffer (0.5 mL) followed by centrifugation (3000 g × 5 min, each) and elimination of supernatant. Step 3: lysis of erythrocytes by twice mixing cells with distilled water (1 mL), followed by centrifugation (15000 g × 15 min), in order to eliminate the aqueous layers and to obtain the erythrocyte membrane pellet. Step 4: the pellet was added by a mix of 2:1 chloroform:methanol (2 mL) and partitioned with pure water (1 mL) according to the Folch's procedure (Folch J. et al., 1957), for the lipid extraction. The organic layer was separated and evaporated under vacuum to dryness.

2.1.3.2 Thin Layer Chromatography - TLC

Step 5: This procedure determines the efficacy of the lipid extraction using chloroform/methanol/water 65:25:4 to determine the purity of the phospholipid fraction (Fuchs B. e al., 2011).

2.1.3.3 Transesterification procedure

Step 6: The phospholipid extract was transesterified at room temperature for 10 min with 0.5 M KOH/MeOH to obtain the fatty acid methyl esters (FAME), derived from the fatty acid residues present in membrane glycerophospholipids. This chemical procedure avoids oxidative and degradation reactions and gives the fatty acid composition which represents that of the membrane, as ascertained using appropriate MUFA and PUFA internal standards. FAME were extracted by partition between *n*-hexane (2 mL) and water (0.5 mL), followed by evaporation of the organic phase under vacuum to dryness. Steps 7 and 8: analysis using gas chromatography (GC) as described below.

2.1.3.4 Gas Chromatography (GC)

Step 7 of the protocol (fig 5): GC analysis and identification by standard references.

2.1.3.4.1 Calibration procedure

Firstly, GC analysis of the commercially available reference standard materials for the 10 fatty acids of the cluster was performed as follows:

For this study we chose to study a cluster of 10 fatty acids, which also corresponds to chromatographic peak areas >97%. This cluster consists of: 2 saturated fatty acids (SFA: palmitic and stearic acids); 3 monounsaturated fatty acids (MUFA, palmitoleic, oleic and cis-vaccenic acids); 3 polyunsaturated fatty acids omega-6 (PUFA, linoleic, dihomo-gamma linolenic, arachidonic acids); 2 polyunsaturated fatty acids omega-3 (PUFA, eicosapentaenoic and docosahexaenoic acids) (table 4).

Table 4. The 10 fatty acids that were chosen for this study. Common name, name IUPAC and their chemical formula is demonstrated,

FATTY ACIDS		COMMON NAME	NAME IUPAC	FORMULA
1	C16:0	palmitic	Hexadecanoic	$CH_3(CH_2)_{14}COOH$
2	9c,C16:1	palmitoleic	(9Z)-Hexadec-9-enoic	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
3	C18:0	stearic	Octadecanoic	$CH_3(CH_2)_{15}CH$
4	9c,C18:1	oleic	(9Z)-Octadec-9-enoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
5	11c,C18:1	Cis-vaccenic	(E)-Octadec-11-enoic	$CH_3(CH_2)_4CH=CH(CH_2)_7COOH$
6	9c,12c,C18:2	Linolenic (LA)	(9Z,12Z)-9,12-Octadecadienoic	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
7	8c, 11c,14c,C20:3	Dihomo-gamma linolenic (DGLA)	8,11,14-eicosatrienoic	$C_{20}H_{34}O_2$
8	5c,8c,11c,14c,C20:4	Arachidonic (AA)	5,8,11,14-eicositetraenoic	$CH_3(CH_2)_4CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_3COOH$
9	EPA	EPA	(5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-eicosapentaenoic	$CH_3(CH_2)_{14}COOH$
10	DHA	DHA	4,7,10,13,16-docosatetraenoic	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_2COOH$

The quantitation of the fatty acids was carried out by calibration procedures, for which the following protocol has been followed:

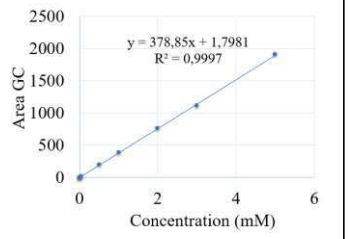
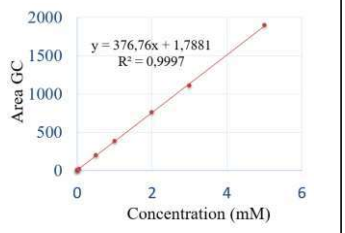
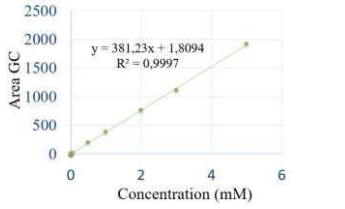
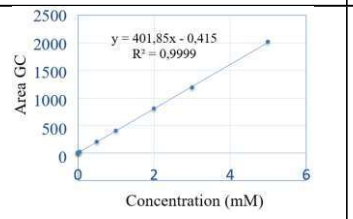
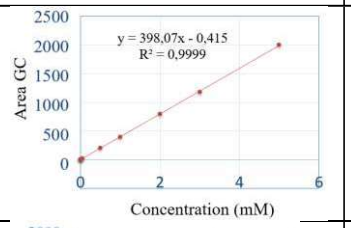
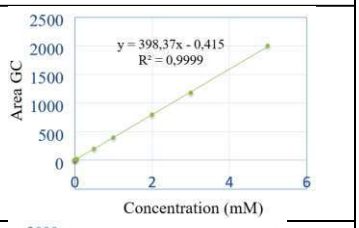
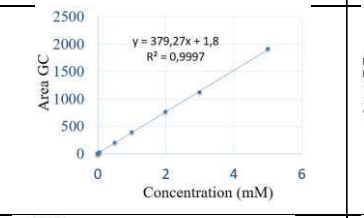
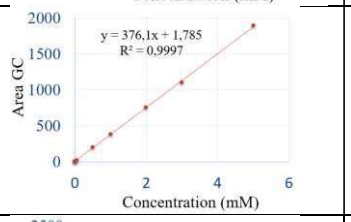
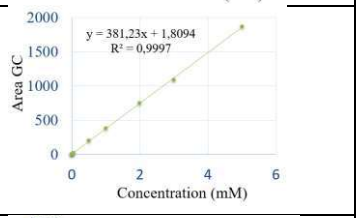
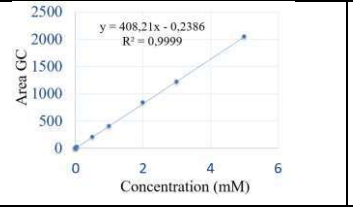
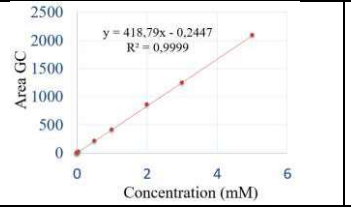
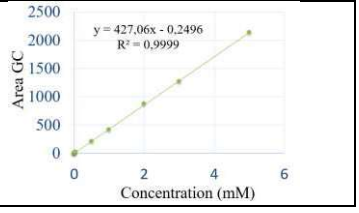
initially a n-hexane (HPLC grade, Titolchimica) 5mM solution of stearic acid methyl ester (2 mg in 1340 µL) was prepared and 1µl was directly injected to the Agilent 7890B GC system equipped with a flame ionization detector and a DB-23 (50%-Cyanopropyl)-methylpolysiloxane capillary column (60 m, 0.25 mm i.d., 0.25 µm film thickness). The following oven conditions were established to be kept for all the analyses: the initial temperature was 165 °C, held for 3 min, followed by an increase of 1 °C/min up to 195 °C, held for 40 min, followed by a second increase of 10 °C/min up to 240 °C, held for 10 min. The carrier gas was hydrogen, held at a constant pressure of 16.482 psi. The injections were repeated in triplicates.

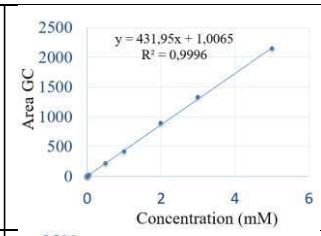
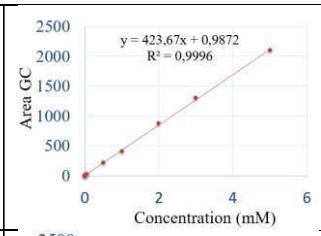
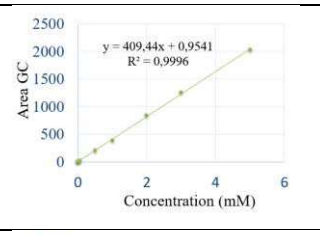
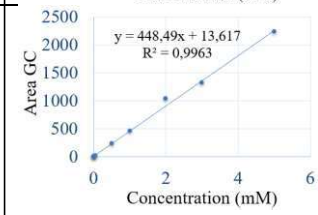
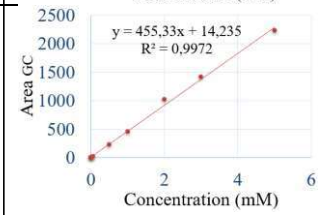
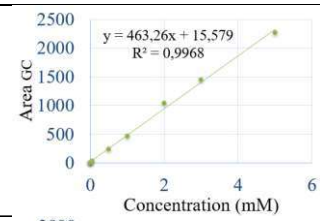
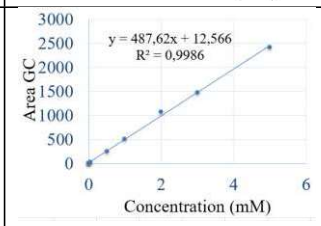
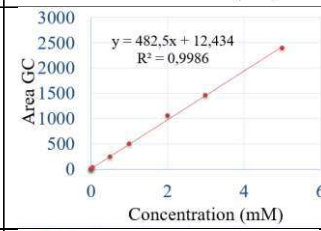
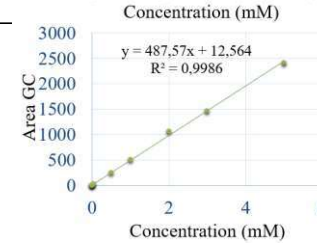
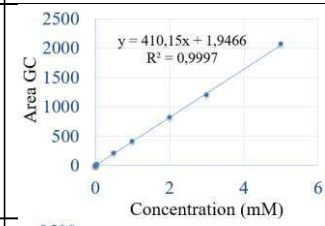
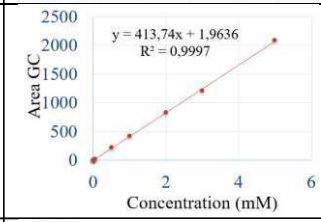
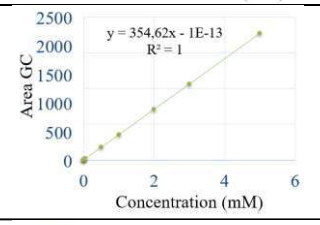
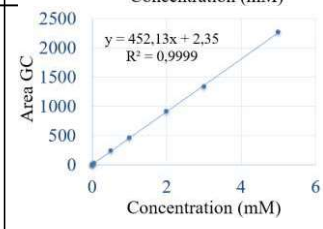
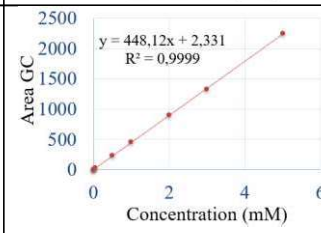
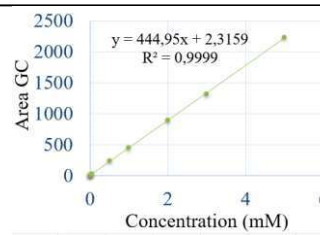
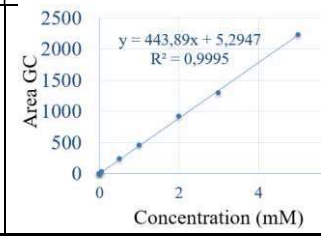
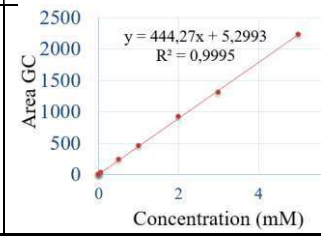
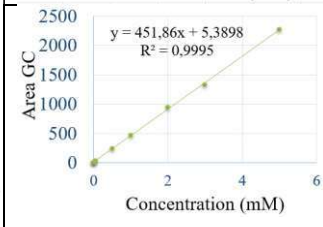
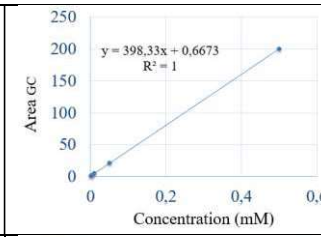
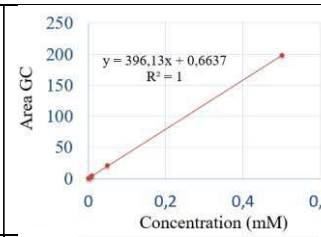
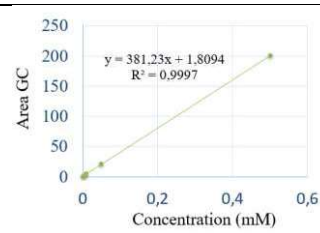
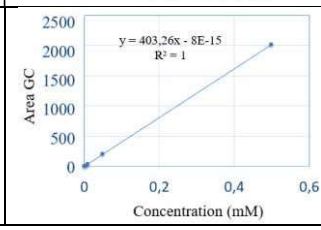
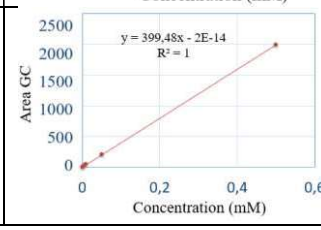
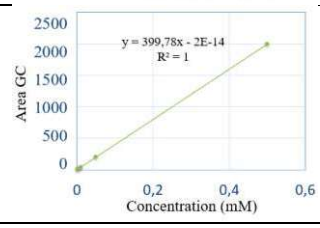
The second round of injections for calibration was then performed with 0.5 mM solution of the same fatty acid methyl ester (taking 100µL of the initial solution and diluting with 900µL of n-hexane), injecting 1 µL as previously described for triplicates.

The same protocol was carried out using dilutions of 0.05mM, 0.005mM and 0.0005mM of stearic acid methyl ester.

In all the injections a calibration curve was created using the software of the GC equipment (Agilent 7890B GC system).

Using the concentration of 0.0005mM for methyl stearate, the corresponding peak area was detectable but not quantifiable, indicating this concentration as the limit of detection (LOD) of the specific GC system (<0.5nM). The same protocol has been followed for all the fatty acids of the cohort. Calibration curves were obtained for the quantitative analysis of each peak of the chromatogram and are shown below in Figure 5.

Reference FAMEs (0.5-5mM)				
SFA	**C16:0**			
	C18:0			
MUFA	**9c,C16:1**			
	9c,C18:1			

	11c,C18 :1			
PUFA omega-3	EPA			
	DHA			
PUFA omega-6	LA			
	DGLA			
	ARA			
Reference FAMEs (0.001-0.5mM)				
SFA	C16:0			
	C18:0			

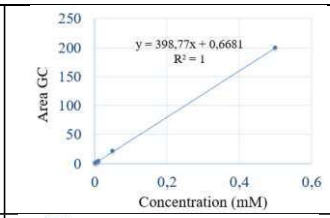
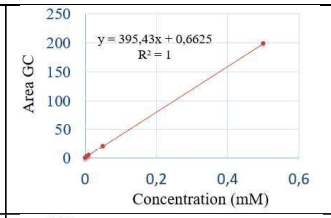
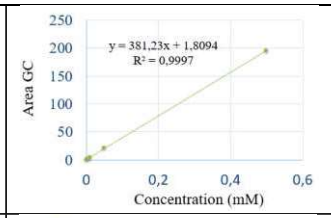
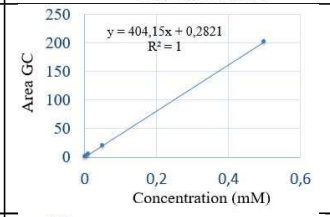
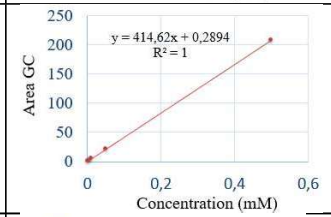
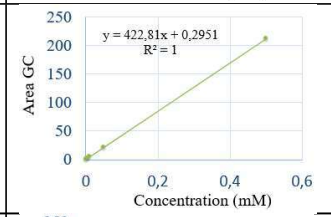
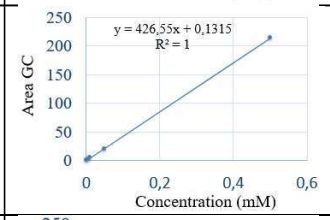
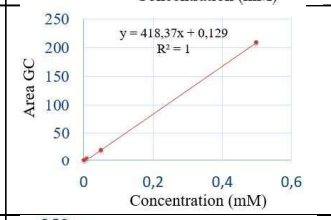
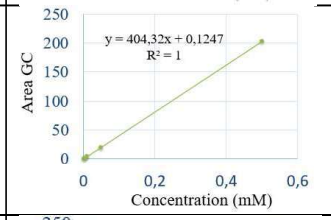
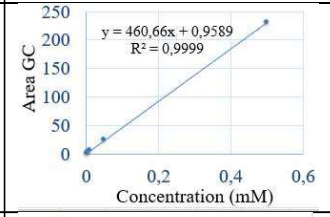
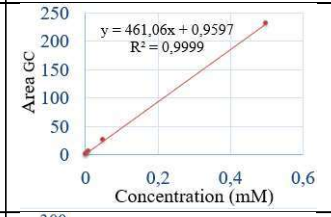
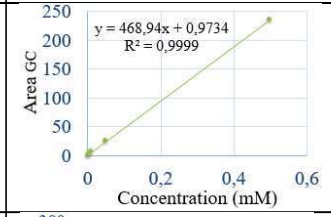
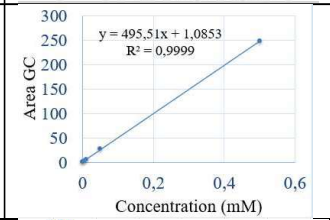
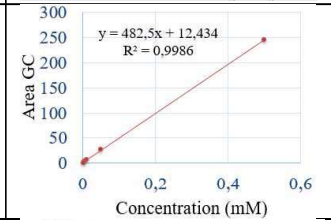
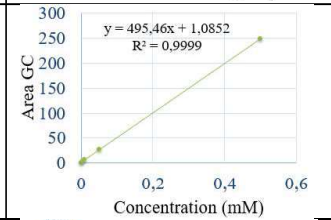
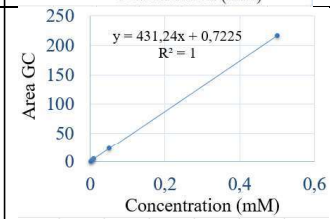
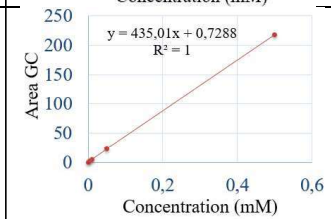
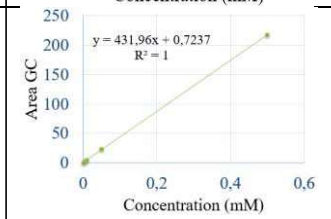
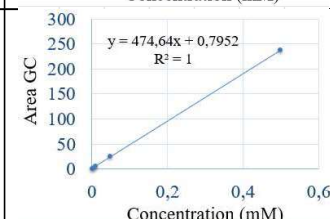
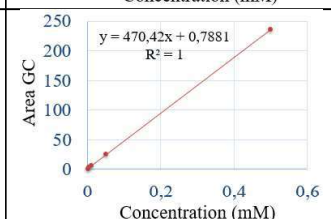
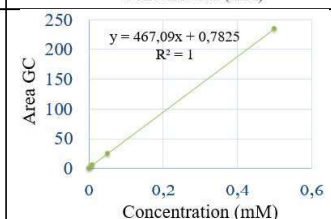
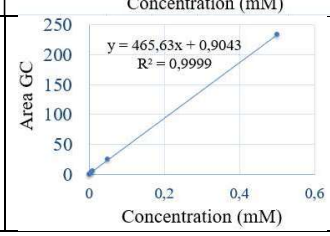
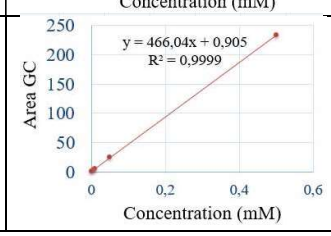
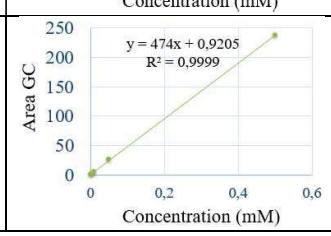
MUFA	**9c,C16:1**			
	9c,C18:1			
	11c,C18:1			
PUFA omega-3	**EPA**			
	DHA			
PUFA omega-6	**LA**			
	DGLA			
	ARA			

Figure 5. Calibration curves of the 10 fatty acids at high (0.5-5mM) and low (0.001-0.5mM) concentration ranges, chosen as representatives of the SFA, MUFA and PUFA families present in the erythrocyte membrane phospholipids.

2.1.3.4.2 GC Analysis of FAME

The FAME mixture obtained from the erythrocyte membrane pellet was dissolved in 20μL of n-hexane and 1μL was directly injected to the Agilent 7890B GC (Agilent, Milan) system equipped with a flame ionization detector and a (50%-cyanopropyl)-methylpolysiloxane capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness) (DB-23, Agilent; Milan). The initial oven temperature was 165 °C, held for 3 min, followed by an increase of 1 °C/min up to 195 °C, held for 40 min, followed by a second increase of 10 °C/min up to 240 °C, held for 10 min (figure 6).

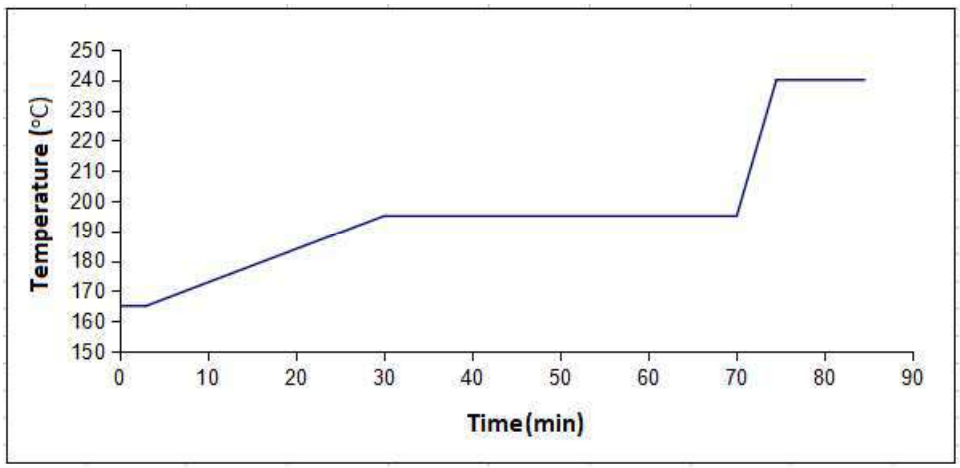


Figure 6. The temperature program of the gas chromatograph. Total program duration: 87 minutes. 165°C for 3 minutes. Increase to 195°C for 40 minutes (rate 1°C/min). Increase to 240°C for 10 minutes (rate 10°C/min). Increase to 250°C for 10 minutes.

The carrier gas was hydrogen, held at a constant pressure of 16.482 psi. FAMEs were identified by comparison with the retention times of standard references. In agreement with previously reported procedures, satisfactorily separating all the 10 fatty acids, without superimposition of other peaks [(Sansone A. et al., 2016) (Amézaga J. et al., 2018) (Giacometti G. et al., 2017) (Sansone A. et al., 2013)]. A representative chromatogram is demonstrated below on Figure 7.

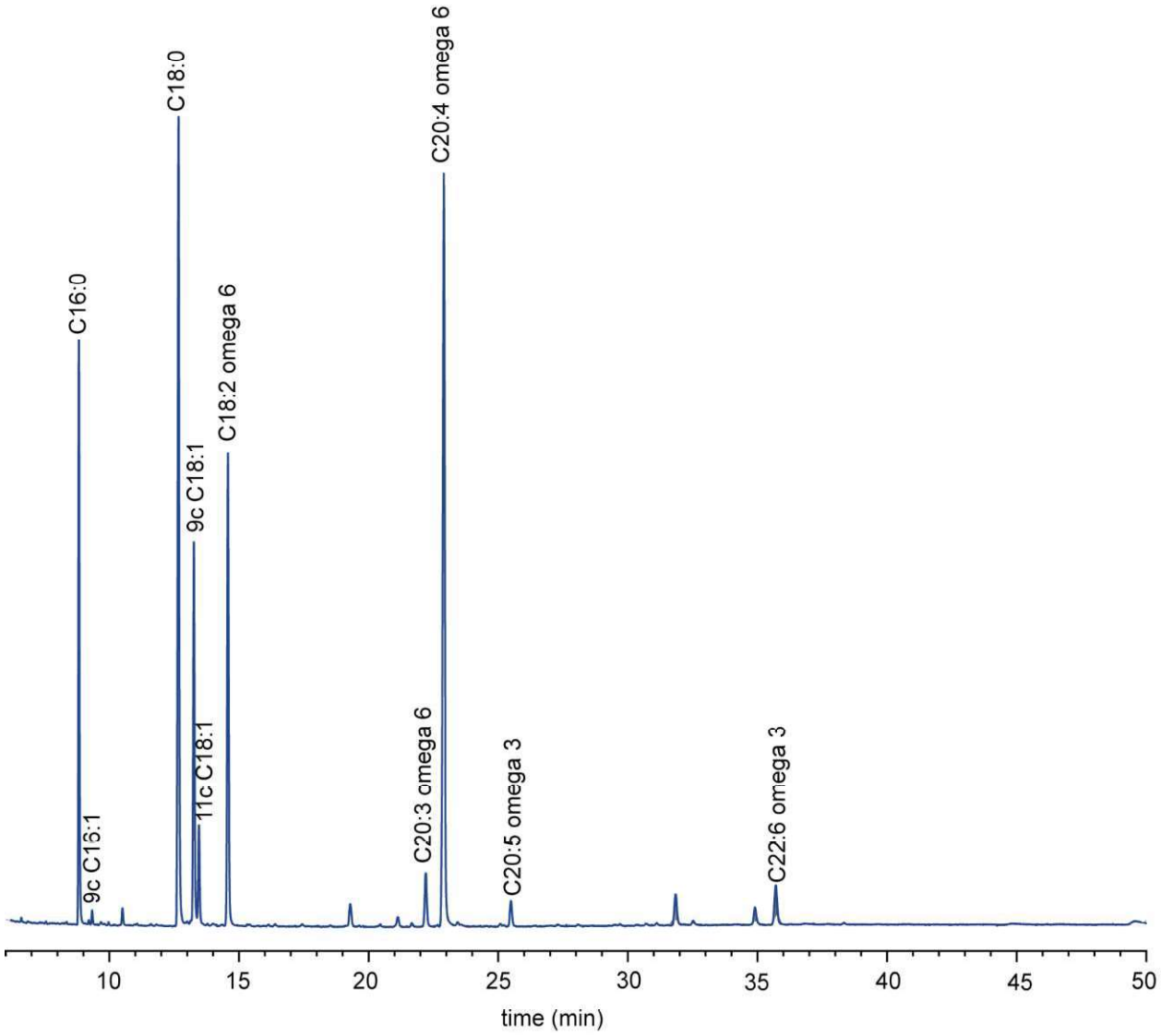


Figure 7. Representative GC chromatogram of the FAME obtained from dog erythrocyte membrane phospholipids after work-up, as described in the main text. The 10 fatty acids chosen for the cluster are satisfactorily separated and recognized by appropriate standard references. The sum of their areas corresponds to >97% of the total peak areas.

The group of 10 fatty acids corresponds to chromatographic peak areas >97%, and consisted of: 2 saturated fatty acids [SFA: palmitic (C16:0) and stearic acids (C18:0)]; 3 monounsaturated fatty acids [MUFA, palmitoleic (C16:1), oleic (9c,C18:1) and cis-vaccenic (11c,C18:1) acids]; 3 polyunsaturated fatty acids omega-6 [PUFA, linoleic (LA, C18:2), dihomogammalinolenic (DGLA;C20:3) and arachidonic (ARA, C20:4)]; 2 polyunsaturated fatty acids omega-3 [PUFA, eicosapentaenoic (EPA, C20:5) and docosahexaenoic (DHA, C22:6) acids]. Step 8: Using the calibration curves, the quantitative values for each peak were obtained as μg/mL allowing to calculate also the total fatty acid contents (total SFA, total

MUFA, total PUFA), ratios between the families (SFA/MUFA, omega-6/omega-3) and three indexes (unsaturation index, peroxidation index, PUFA balance). The obtained data for each fatty acid is expressed as % of its quantity (% quant. rel.) respect to the sum of the 10 fatty acid quantities taken as 100%.

2.1.3.5 Statistical Methods

Statistical analysis was performed using GraphPad Prism 6.01 software (GraphPad Software, Inc., San Diego, CA). All data were evaluated using a standard descriptive statistic and reported as mean ± standard deviation (sd) or as median and range (minimum-maximum), based on their distribution. Normality was checked graphically or using the D'Agostino Pearson test. A comparison between 2 groups was done using the unpaired *t* test or the Mann-Whitney test. According to the distribution of data, Pearson or Spearman correlations were used to evaluate the relationship between fatty acids percentages or fatty acids indexes with bodyweight and age. The threshold for the statistical significance (*p* value) was set up at 0.05. The distribution graphs were produced using Past 3.14 software.

2.2 RESULTS

2.2.1 The erythrocyte membrane lipidome in healthy dogs

Blood samples were collected from 68 clinically healthy dogs, including 30 males (6 neutered) and 38 females (12 sterilized), weighting from 2.6 to 43 kg, aged from 2 to 156 months (median 41). In the following table (5) the characteristics (breed, sex, age, and bodyweigh) of the cohort of HD are presented.

Table 5. Characteristics of the recruited healthy dogs (n=68). Breed, sex, age and bodyweight are reported. M: male; F: female; Mn: neutered male; Fs: spayed female.

n	Breed	Sex	Age (months)	Bodyweight (kg)
1	Boxer	M	36	30
2	Boxer	F	34	24,5
3	Mixed-breed	Fs	48	8
4	Boxer	F	42	22
5	Mixed-breed	F	63	8
6	Dobermann	F	40	35
7	Mixed-breed	F	75	8
8	Romagna Water Dog	M	38	14
9	Italian Mastiff	M	2	8
10	Romagna Water Dog	F	52	13
11	Mixed-breed	F	12	22
12	Mixed-breed	F	12	21,8
13	Alaskan Malamute	M	29	25
14	Boxer	M	36	35
15	Mixed-breed	Fs	96	29,5
16	Mixed-breed	M	98	17,5
17	Mixed-breed	M	6	17,5
18	Mixed-breed	Fs	20	24,6
19	Mixed-breed	Mn	26	8
20	Golden Retriever	M	112	25,8
21	Italian Mastiff	M	5	29
22	Mixed-breed	Mn	90	14
23	Mixed-breed	M	36	30,1
24	Mixed-breed	Mn	12	6,7
25	Mixed-breed	Fs	60	20
26	Mixed-breed	F	48	16
27	Mixed-breed	Mn	36	22
28	Mixed-breed	M	36	24
29	Border Collie	M	65	30

n	Breed	Sex	Age (months)	Bodyweight (kg)
30	Mixed-breed	Fs	48	28
31	Breton	Mn	48	17
32	Rottweiler	F	9	35
33	Miniature poodle	F	48	2,6
34	Maremma Scenthound	F	24	12
35	Golden Retriever	Fs	156	29
36	Maremma Scenthound	Fs	31	16
37	Border Collie	F	9	10
38	Cocker Spaniel	F	80	15
39	Labrador	Mn	91	30
40	Pinscher	M	83	5
41	Mixed-breed	M	36	4
42	Mixed-breed	M	110	15,85
43	Border Collie	F	18	22
44	Mixed-breed	Fs	118	18
45	Siberian Husky	F	102	24
46	Siberian Husky	Fs	138	26
47	Labrador	Fs	30	28
48	Mixed-breed	M	117	7
49	Labrador	M	6	32
50	Labrador	M	80	43
51	Labrador	M	52	35
52	Labrador	F	122	40
53	Labrador	F	24	32
54	Labrador	F	85	35
55	Labrador	F	24	39
56	Labrador	F	24	30
57	Labrador	F	35	35
58	Labrador	F	50	35
59	Labrador	M	22	43
60	Miniature poodle	Fs	29	6
61	Jack Russell Terrier	M	51	7
62	Cocker Spaniel	M	47	14
63	Rottweiler	F	5	20
64	Mixed-breed	F	21	22,3
65	Miniature poodle	M	95	5
66	Siberian Husky	Fs	51	22,5
67	Jack Russell Terrier	M	69	9,7
68	Mixed-breed	F	25	29,5

The work up of the erythrocyte membrane glycerophospholipids to obtain the corresponding fatty acid methyl esters (FAME) followed the procedure described in Figure 4, and each step is detailed in the Experimental section.

The FAMEs were separated, recognized and quantified by gas chromatography (GC) as previously described (see Materials and Methods, chapter 2.1.3.4). The cluster of 10 fatty acids contains the following molecules (see Figure 1 for their structures): palmitic (C16:0) and stearic (C18:0) acids as SFA; palmitoleic (C16:1), oleic (9c,C18:1) and vaccenic (11c,C18:1) acids as MUFA; linoleic (LA, C18:2), dihomogammalinolenic (DGLA;C20:3) and arachidonic (AA, C20:4) acids as PUFA omega-6; eicosapentaenoic (EPA, C20:5) and docosahexaenoic (DHA, C22:6) acids as PUFA omega-3.

Evaluation of the 10 peaks of the GC chromatogram was performed using calibration curves (see Materials and Methods, chapter 2.1.3.4.1), obtaining μg/mL values and then expressing each fatty acid as relative quantitative percentage (% rel. quant.) of the total quantities of the 10 fatty acids recognized and calibrated in the GC analysis.

2.2.1.1 Interval Values

The intervals and the median values obtained for our cohort of healthy dogs are reported in Table 6, together with lipid indexes, derived from the 10 fatty acid values, as follows: omega-6/omega-3 ratio, PUFA balance (omega-3/omega-3 + omega-6), SFA/MUFA ratio, unsaturation index (UI = MUFA tot × 1 + C18:2 × 2 + C20:3 × 3 + C20:4 × 4 + C20:5 × 5 + C22:6 × 6) and peroxidation index (PI = MUFA tot × 0.025 + C18:2 × 1 + C20:3 × 2 + C20:4 × 4 + C20:5 × 6 + C22:6 × 8) (Hulbert et al. 2006).

Table 6. Interval values of the 10 fatty acids cohort expressed as relative percentages of the μg/mL quantities detected by gas chromatographic analyses[1] after isolation and work-up of erythrocyte membrane glycerophospholipids of healthy dogs (n=68). The corresponding values of fatty acid families and lipid indexes are also reported.

Fatty acids[1]	**Interval values[2] (min-max)**	**Median Value**
C16:0 – palmitic acid	8.2 - 25.8	15.4
C16:1 – palmitoleic acid	0.08 - 0.55	0.25
C18:0 – stearic acid	15.6 - 27.3	20.2
9c,C18:1 – oleic acid	6.9 - 14.3	9.2
11c,C18:1 – vaccenic acid	1.2 - 2.7	1.95
LA omega-6 – C18:2 – linoleic acid	9.2 - 21	14.5
DGLA omega-6 – C20:3 dihomogammalinolenic acid	0.4 - 2.3	1.26
ARA omega-6 C20:4 – arachidonic acid	17.5 - 43.7	35
EPA omega-3 – C20:5 – eicosapentaenoic acid	0.2 - 1.5	0.66
DHA omega-3 – C22:6 – docosahexaenoic acid	0.2 - 2.5	0.9
Total SFA[3]	27.8 - 43	35.4
Total MUFA[4]	8.7 - 16.7	11.6
PUFA omega-3[5]	0.5 - 4	1.75
PUFA omega-6[6]	34.9 - 60.8	51
Total PUFA[7]	32.9 - 61.8	53
SFA/MUFA[8]	2 - 3.9	3
Omega-6/omega-3 ratio[9]	12.5 - 83.7	28.4
PUFA balance[10]	0.9 - 7.4	3.3
UI[11]	130 - 234	194.8
PI[12]	101 - 214	171.1

[1]Fatty acids are evaluated as FAME (fatty acid methyl esters) after membrane isolation, lipid extraction and derivatization as described in the Experimental section. Structures are shown in Figure 3.

[2] The values are expressed as percentage of the found quantities (calculated as μg/mL) from the gas chromatographic analysis, using calibration and quantitation protocols and standard reference compounds for each FAME, as described in Materials and Methods. The GC peak areas of the 10 fatty acids cohort corresponds to ca. 97% of the total peak areas of the chromatogram. The minimum and maximum values obtained from the population of the healthy dogs are shown for each FAME, together with the median value and the corresponding distributions as shown in Figures 11 and 12.

[3] Total SFA (Saturated Fatty Acids) = % C16:0 + % C18:0.

[4] Total MUFA (MonoUnsaturated Fatty Acids) = % C16:1 + % 9c,C18:1 + % 11c,C18:1.

[5] PUFA (PolyUnsaturated Fatty Acids) omega-3 = %EPA + %DHA.

[6] PUFA (PolyUnsaturated Fatty Acids) omega-6 = %LA + %DGLA + %ARA.

[7] Total PUFA = %LA + %DGLA + %ARA + %EPA + %DHA.

[8] SFA/MUFA = (% C16:0 + % C18:0)/(% C16:1 + % 9c,C18:1 + % 11c,C18:1).

[9]Omega-6/omega-3 ratio = (%LA + %DGLA + %ARA)/ (%EPA + %DHA).

[10] PUFA balance = [(%EPA + %DHA) / Total PUFA] × 100.

[11]UI = (%MUFA × 1) + (%LA × 2) + (%DGLA × 3) + (%ARA × 4) + (%EPA × 5) + (%DHA × 6).

[12] PI = (%MUFA × 0.025) + (%LA × 1) + (%DGLA × 2) + (%ARA × 4) + (%EPA × 6) + (%DHA × 8).

A few fatty acids of healthy dog erythrocyte membranes (as control groups) were previously reported (Mehta et al. 1991; Fuhrmann et al. 2006; Stoeckel et al. 2011) and their values are listed in following Table (7).

Table 7. Data of the fatty acids of erythrocyte membranes of our healthy dog cohort (see Table 6 in the main text) together with the data reported in previous literature.

Fatty acids	**Interval values[2] (min-max)**	**Ref. 1**	**Ref. 2**	**Ref. 3**
Number of samples	68	5	8	30
LA omega-6 – C18:2 – linoleic acid	9.2 - 21	7.80	10.13	-
DGLA omega-6 – C20:3 dihomogammalinolenic acid	0.4 - 2.3	-	1.55	-
ARA omega-6 C20:4 – arachidonic acid	17.5 - 43.7	28.29	28.05	28.6 - 30.8
EPA omega-3 – C20:5 – eicosapentaenoic acid	0.2 - 1.5	-	-	0.364 - 0.42
DHA omega-3 – C22:6 – docosahexaenoic acid	0.2 - 2.5	-	-	0.22 - 0.28
Total SFA	27.8 - 43	-	41.14	-
MUFA	8.7 - 16.7	-	-	-
Total PUFA omega-3	0.5 - 4	-	1.87	1.35 - 1.65
PUFA omega-6	34.9 - 60.8	-	42.31	44.4- 44.8

1. Mehta JR, Braund KG, Hegreberg GA, Thukral V. Lipid Fluidity and Composition of the Erythrocyte Membrane for Healthy Dogs and Labrador Retrievers with Hereditary Muscular Dystrophy. *Neurochem Res* (1991) 16:129-35.
2. Fuhrmann H, Zimmermann A, Gück T, Oechtering G. Erythrocyte and plasma fatty acid patterns in dogs with atopic dermatitis and healthy dogs in the same household. *Can J Vet Res* (2006) 70: 191–196.
3. Stoekel K, Nielsen LH, Fuhrmann H, Backmann L. Fatty acid patterns of dog erythrocyte membranes after feeding of a fish-oil based DHA-rich supplement with a base diet low in n-3 fatty acids versus a diet containing added n-3 fatty acids. *Acta Vet Scand* (2011) 53:57. doi: 10.1186/1751-0147-53-57.

In Figure 8 the distribution graphics for each fatty acid (% rel. quant.) are shown together with their families (SFA, MUFA and PUFA omega-6 and omega-3), whereas Figure 9 shows the distribution graphics of the total PUFA and lipid indexes obtained from the 10 fatty acid values.

Figures 8 and 9 also show minimum and maximum values, as well as the median value of each measured parameter, as listed in Table 6.

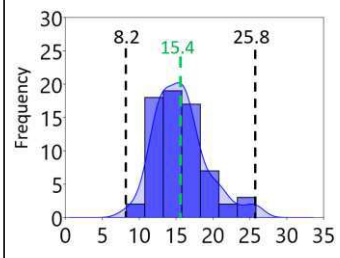
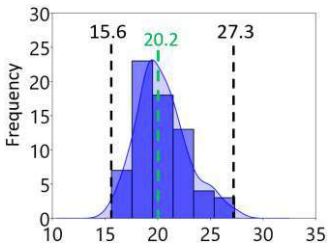
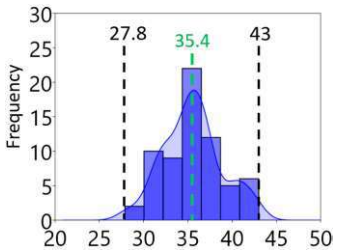
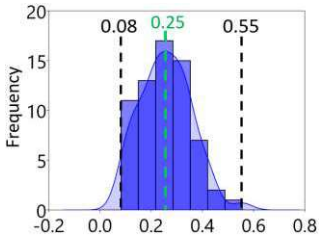
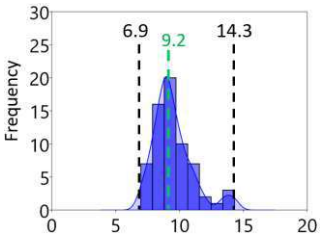
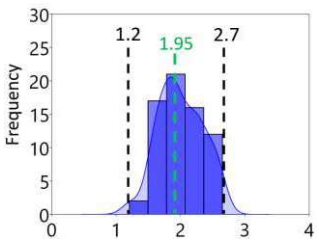
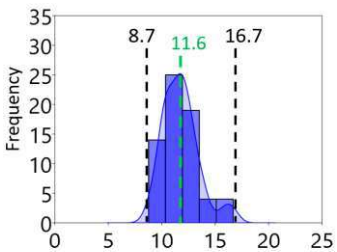
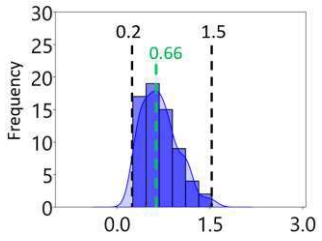
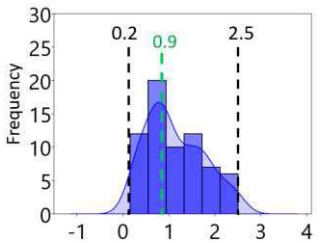
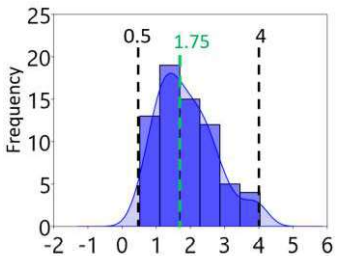
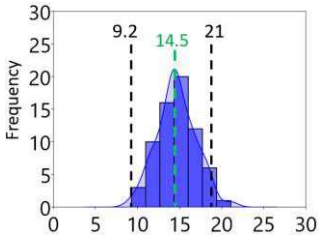
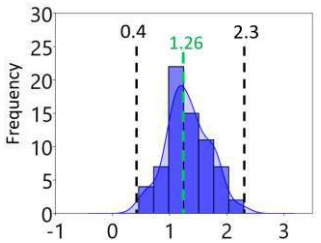
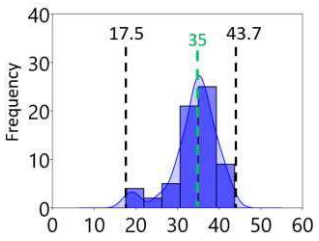
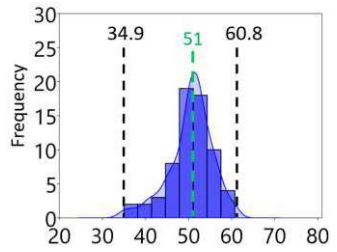
	Individual Fatty Acids			Sum of families
SFA				
MUFA				
PUFA-ω3				
PUFA-ω6				

Figure 8. Distribution of the values in the population of Healthy Dogs (n=68) for the individual fatty acids and the corresponding families using the data in Table 6 with 95% confidence interval. Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family. Black: the minimum and the maximum value obtained. Green: the median value.

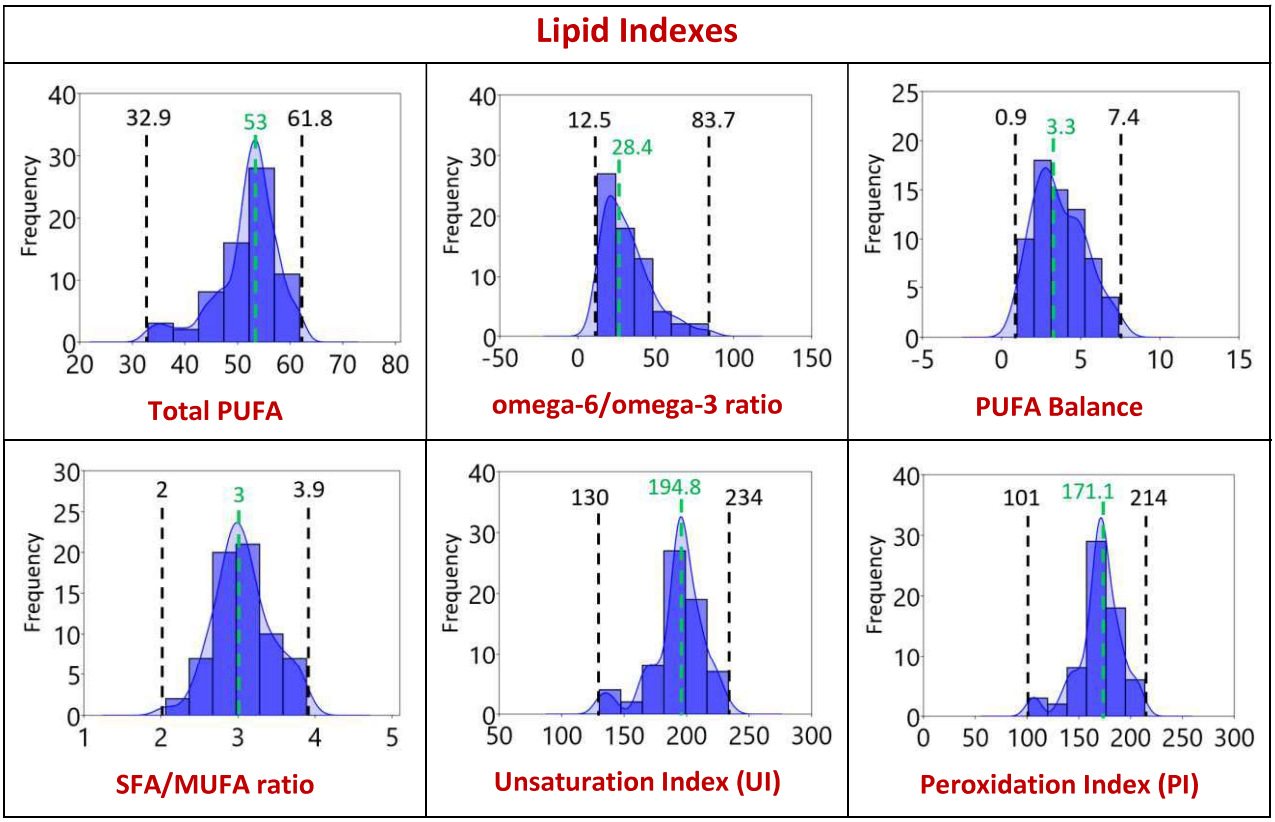


Figure 9. Distribution of the lipid indexes of the population of Healthy Dogs (n=68) with 95% confidence interval as reported in Table 6. 1st row: total PUFA, omega-6/omega-3 and SFA/PUFA ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes. Black: the minimum and the maximum value obtained. Green: the median value.

2.2.1.2 The correlations of fatty acids with healthy dog characteristics

We used the above described interval values to evaluate any possible correlations between FAME types, families and lipid indexes with the dog characteristics and the results are demonstrated in the following figures. (breed, sex, age, and bodyweight).

2.2.1.2.1 The correlations of fatty acids with age

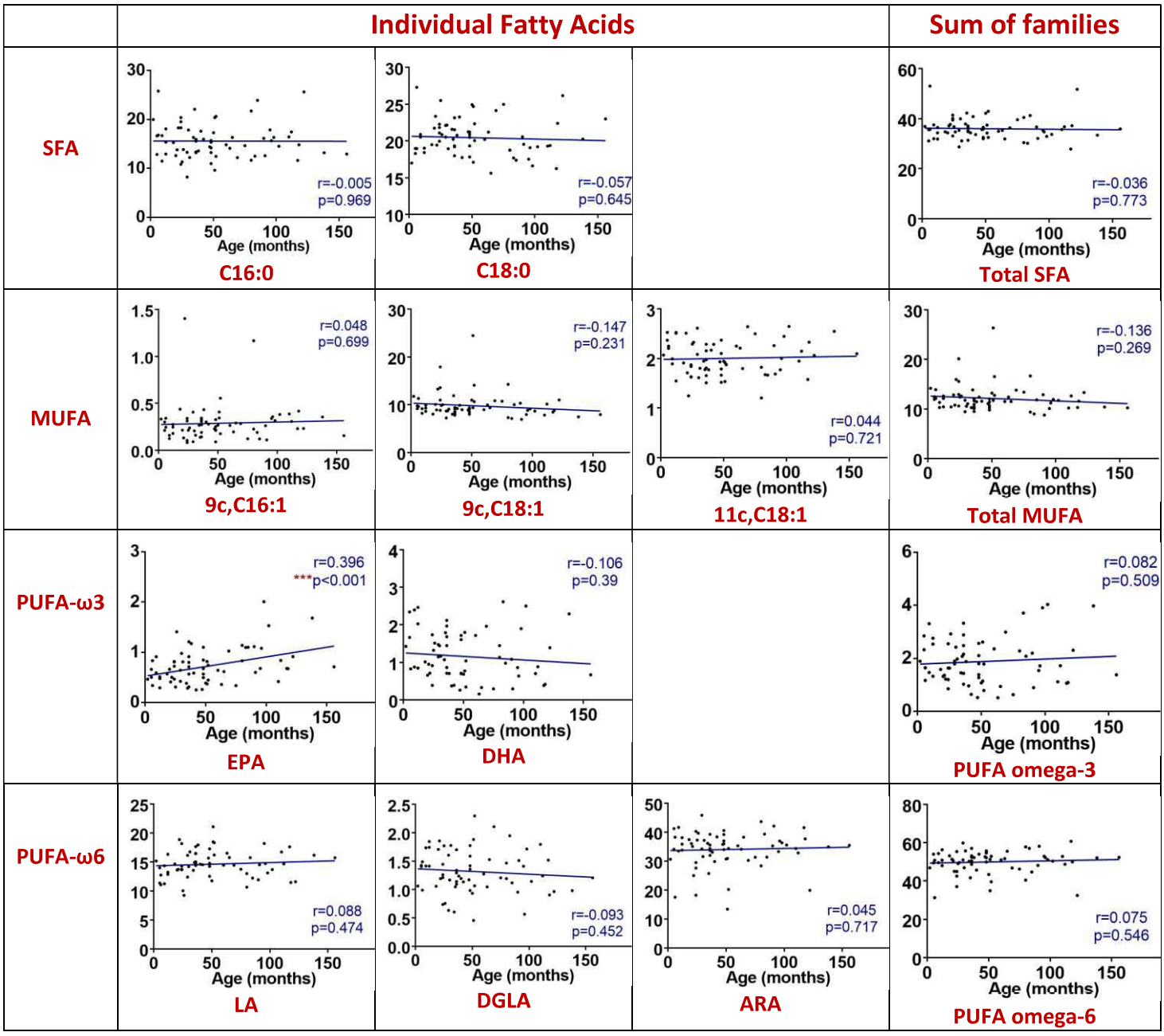


Figure 10. Pearson correlation with linear regression and parameters for healthy dogs (n=68) using age and each fatty acid type and family obtained from erythrocyte membranes (data are reported in Tables 2 and 3 in the main text). Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family.

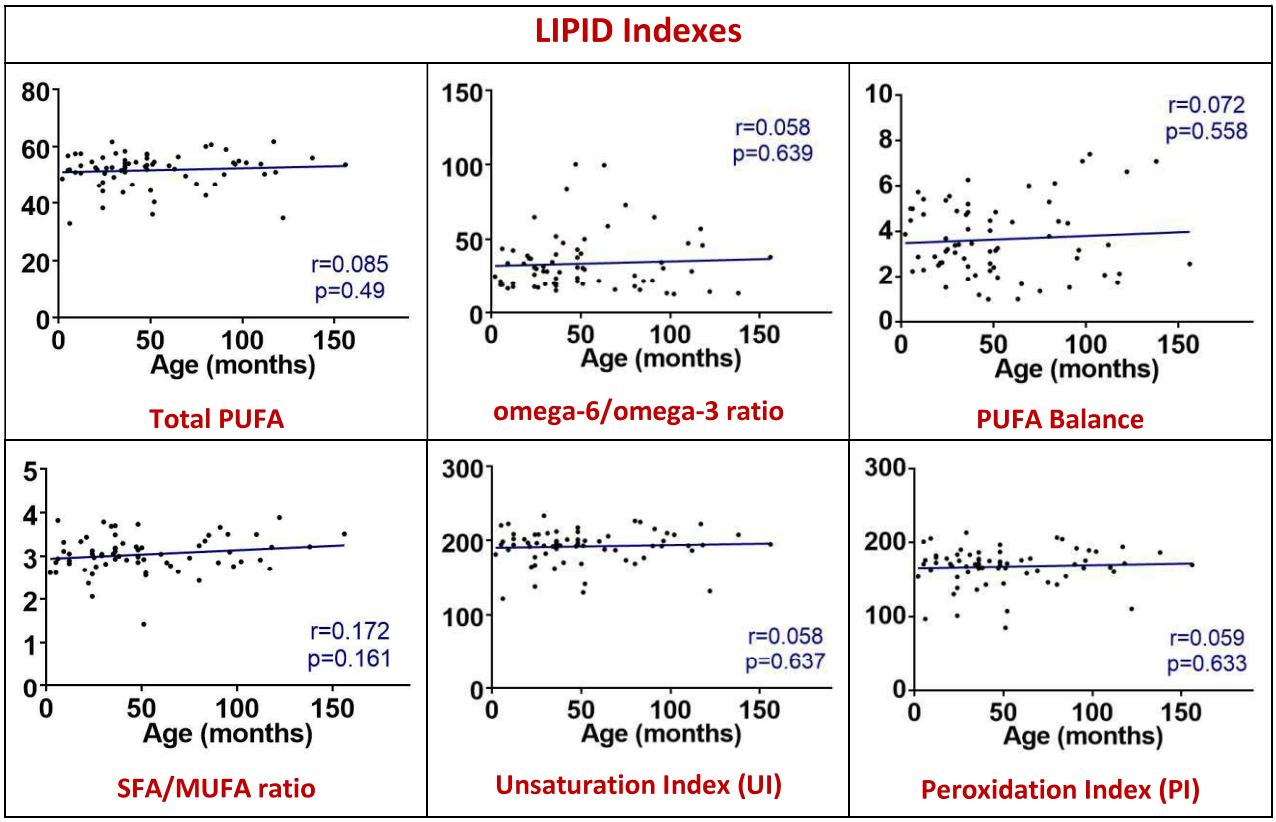


Figure 11. Pearson correlation with linear regression and parameters for healthy dogs (n=68) using age and lipid indexes obtained from erythrocyte membranes (data are reported in Tables 2 and 3 in the main text). 1st row: total PUFA, omega-6/omega-3 and PUFA balance ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes.

A correlation with age, was found with eicosapentaenoic acid (EPA, omega-3), that show that along with age the levels of EPA increase ($p<0.001$, $r=0.396$).

2.2.1.2.2 The correlations of fatty acids with Bodyweight

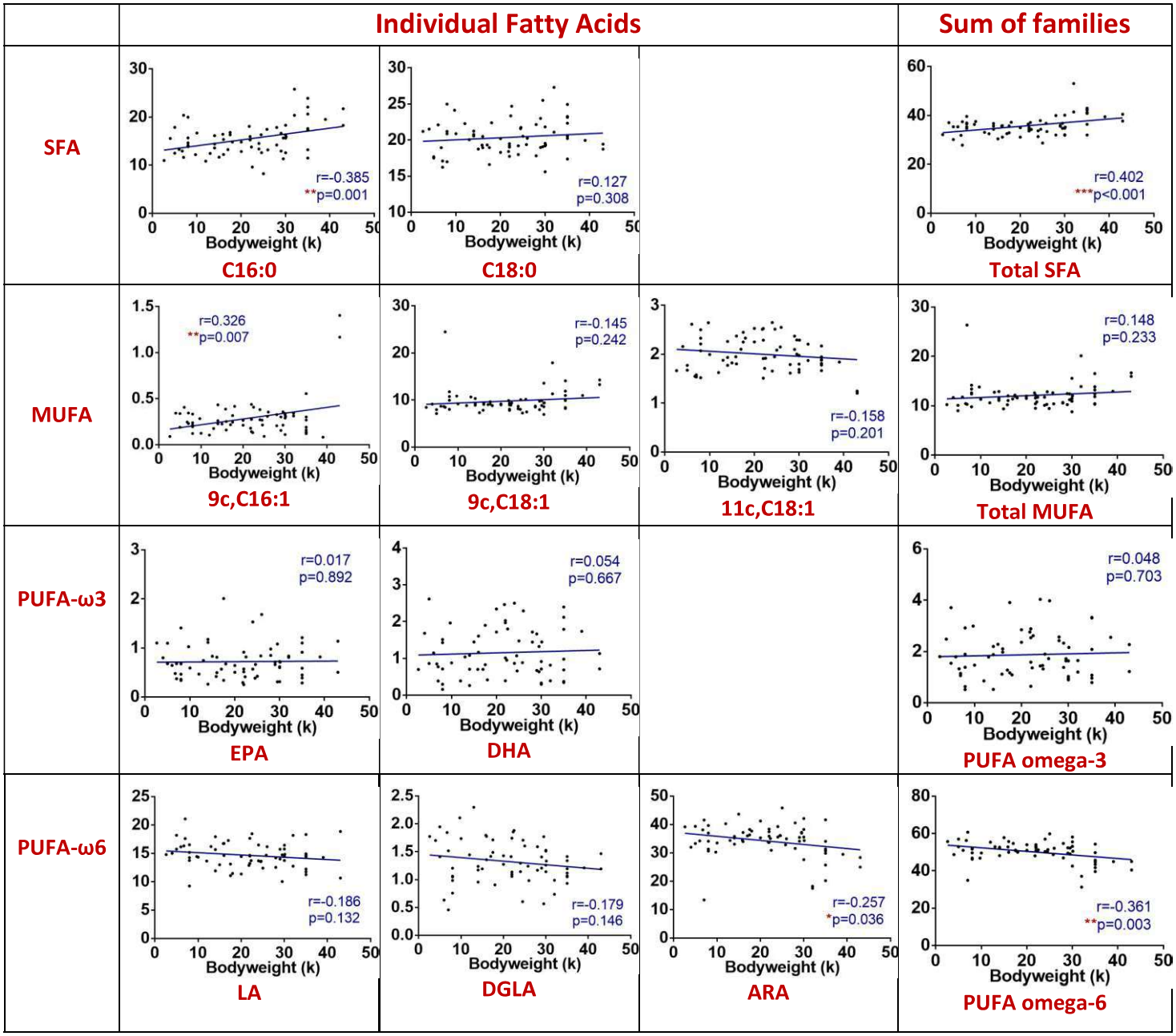


Figure 12. Pearson correlation with linear regression and parameters for healthy dogs (n=68) using bodyweight and each fatty acid type and family obtained from erythrocyte membranes (data are reported in Tables 2 and 3 in the main text). Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family.

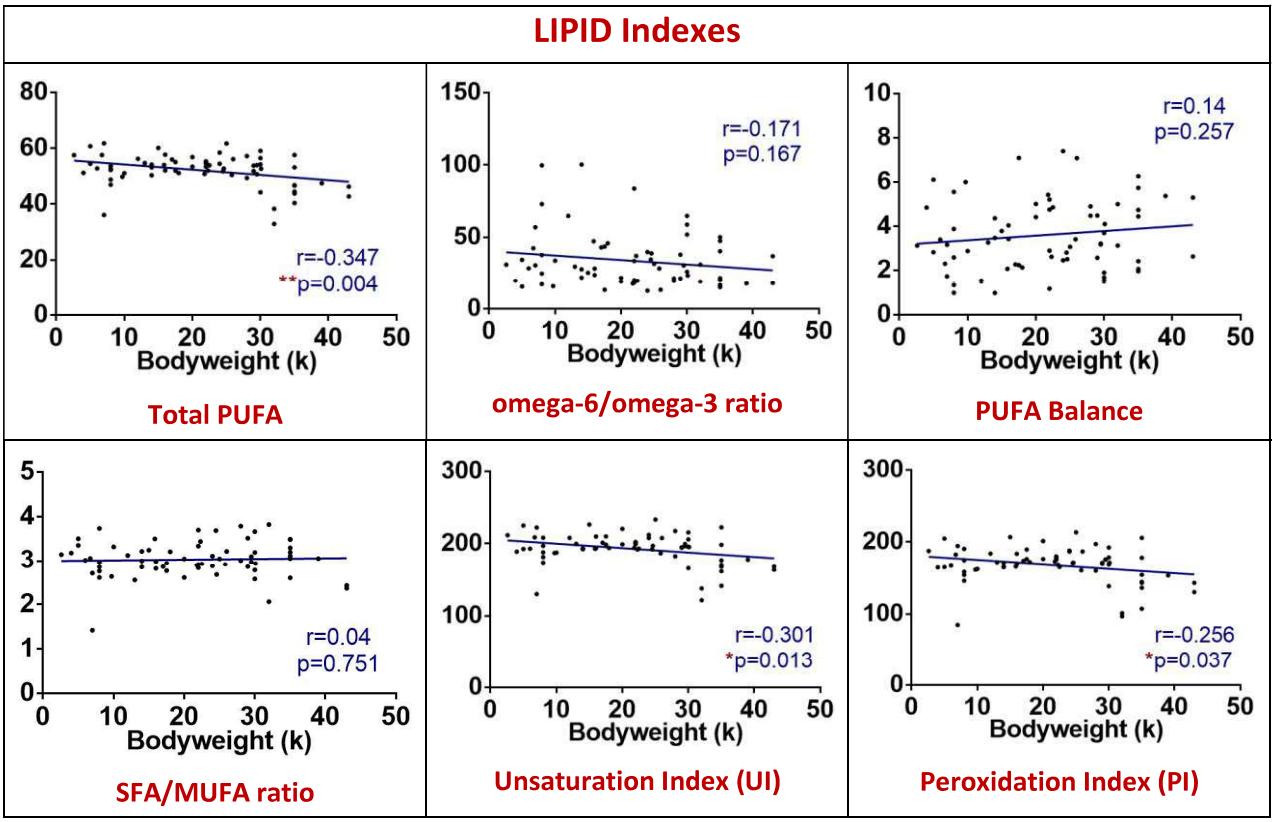


Figure 13. Pearson correlation with linear regression and parameters for healthy dogs (n=68) using bodyweight and lipid indexes obtained from erythrocyte membranes (data are reported in Tables 2 and 3 in the main text). 1st row: total PUFA, omega-6/omega-3 and PUFA balance ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes.

From the analysis according to the bodyweight, several fatty acid parameters correlated significantly, as follows: positive correlation with palmitic acid (C16:0; p = 0.001, r = 0.385) and the total amount of SFA (p = 0.001, r = 0.402). The MUFA palmitoleic acid (C16:1) significantly correlated in a positive manner to bodyweight (p = 0.007; r = 0.326). The bodyweight negatively correlated with arachidonic acid (p = 0.036, r = 0.257), the total content of the omega-6 PUFA (p = 0.003, r = 0.361) and the total amount of PUFA (p = 0.004, r = 0.347). Moreover, both UI and PI showed negative correlation with bodyweight (p =0.031, r = 0.301 and p = 0.037, r = 0.256, respectively). A statistical analysis based on the different breeds of the healthy dogs was not possible because of the limited number of samples for each breed and in addition 24 out of the total 68 (35%) of the healthy dogs recruited for this study were mixed-breed.

2.2.1.2.3 Examination of FA between Male and Female dogs

Subsequently, two groups of male and female healthy dogs were examined to envisage differences of the membrane RBC fatty acid composition between the two genders. Because of the small number of sterilized females (n=12) and neutered males (n=6) we did not consider this additional characteristic to further discriminate groups. The values for the individual FAME and the lipid indexes for male and female healthy dogs are reported as mean ± SD values in Table 8, always expressed as quantitative relative percentages (% rel. quant.) of each value over the total 10 fatty acids values, as explained above. Significant differences were found for the lower levels of stearic and linoleic acids ($p = 0.0126$ and $p = 0.0266$, respectively) and higher levels of palmitoleic acid ($p = 0.0344$) in female than in male dogs.

Table 8. Cohort of 10 fatty acids expressed as percentages of the found µg/mL quantities**,** detected by gas chromatographic analyses of the fatty acid methyl esters (FAME)[1] after isolation and work-up of erythrocyte membrane glycerophospholipids, of male dogs (M=30) and female dogs (F=38). The corresponding values of fatty acid families and lipid indexes are also reported. The significance as p values is also indicated; italic denotes significativity.

Fatty acids[1]	**M (µg/mL %)[2] n=30**	**F (µg/mL %)[2] n=38**	**M vs F p value[c]**
C16:0 – palmitic acid	15.88 ± 3.52	15.38 ± 3.53	0.5639
C16:1 – palmitoleic acid	0.35 ± 0.27	0.24 ± 0.10	*0.0266*
C18:0 – stearic acid	19.62 ± 2.45	21.09 ± 2.23	*0.0126*
9c,C18:1 – oleic acid	10.07 ± 3.31	9.62 ± 1.85	0.4793
11c,C18:1 – vaccenic acid	1.93 ± 0.35	2.06 ± 0.33	0.1209
LA omega-6 – C18:2 – linoleic acid	15.29 ± 2.65	14.11 ± 1.84	*0.0344*
DGLA omega-6 – C20:3 dihomogammalinolenic acid	1.36 ± 0.35	1.28 ± 0.39	0.4075
ARA omega-6 C20:4 – arachidonic acid	33.65 ± 7.06	34.33 ± 5.25	0.6506
EPA omega-3 – C20:5 – eicosapentaenoic acid	0.76 ± 0.39	0.70 ± 0.32	0.4933
DHA omega-3 – C22:6 – docosahexaenoic acid	1.10 ± 0.61	1.20 ± 0.67	0.5296
Total SFA[3]	35.50 ± 4.60	36.46 ± 4.12	0.3697
Total MUFA[4]	12.35 ± 3.36	11.92 ± 1.84	0.5062
PUFA omega-3[5]	1.86 ± 0.89	1.90 ± 0.83	0.8450
PUFA omega-6[6]	50.29 ± 6.80	49.72 ± 5.16	0.6944
Total PUFA[7]	52.15 ± 6.84	51.62 ± 5.30	0.7194

Fatty acids[1]	M (µg/mL %)[2] n=30	F (µg/mL %)[2] n=38	M vs F p value[c]
SFA/MUFA ratio[8]	2.98 ± 0.48	3.09 ± 0.36	0.2544
Omega-6/omega-3 ratio[9]	34.33 ± 19.32	32.78 ± 18.96	0.7423
PUFA balance[10]	3.60 ± 1.67	3.69 ± 1.55	0.8229
Unsaturation Index (UI)[11]	191.98 ± 25.47	192.00 ± 20.24	0.9977
Peroxidation Index (PI)[12]	166.26 ± 29.09	168.09 ± 22.31	0.7705

[1] FAME (fatty acid methyl esters) are expressed as percentage of the found quantities from the gas chromatographic analysis using calibration and quantitation protocols and standard reference compounds for each FAME, as described in Experimental section and Supplementary Information.

[2] The values are expressed as percentage of the found quantities (calculated as µg/mL) ± standard deviation (sd) obtained from the gas chromatographic analyses, using calibration and quantitation protocols and standard reference compounds for each FAME, as described in Supplementary Information. The GC peak areas of the 10 fatty acids cohort corresponds to ca. 97% of the total peak areas of the chromatogram.

[3] Total SFA (Saturated Fatty Acids) = % C16:0 + % C18:0.

[4] Total MUFA (MonoUnsaturated Fatty Acids) = % C16:1 + % 9c,C18:1 + % 11c,C18:1.

[5] PUFA (PolyUnsaturated Fatty Acids) omega-3 = %EPA + %DHA.

[6] PUFA (PolyUnsaturated Fatty Acids) omega-6 = %LA + %DGLA + %ARA.

[7] Total PUFA = %LA + %DGLA + %ARA + %EPA + %DHA.

[8] SFA/MUFA = (% C16:0 + % C18:0)/(% C16:1 + % 9c,C18:1 + % 11c,C18:1).

[9] Omega-6/omega-3 ratio = (%LA + %DGLA + %ARA)/ (%EPA + %DHA).

[10] PUFA balance = [(%EPA + %DHA) / Total PUFA] × 100.

[11] UI = (%MUFA × 1) + (%LA × 2) + (%DGLA × 3) + (%ARA × 4) + (%EPA × 5) + (%DHA × 6).

[12] PI = (%MUFA × 0.025) + (%LA × 1) + (%DGLA × 2) + (%ARA × 4) + (%EPA × 6) + (%DHA × 8).

The corresponding graphical representations are shown below in Figures 14 and 15.

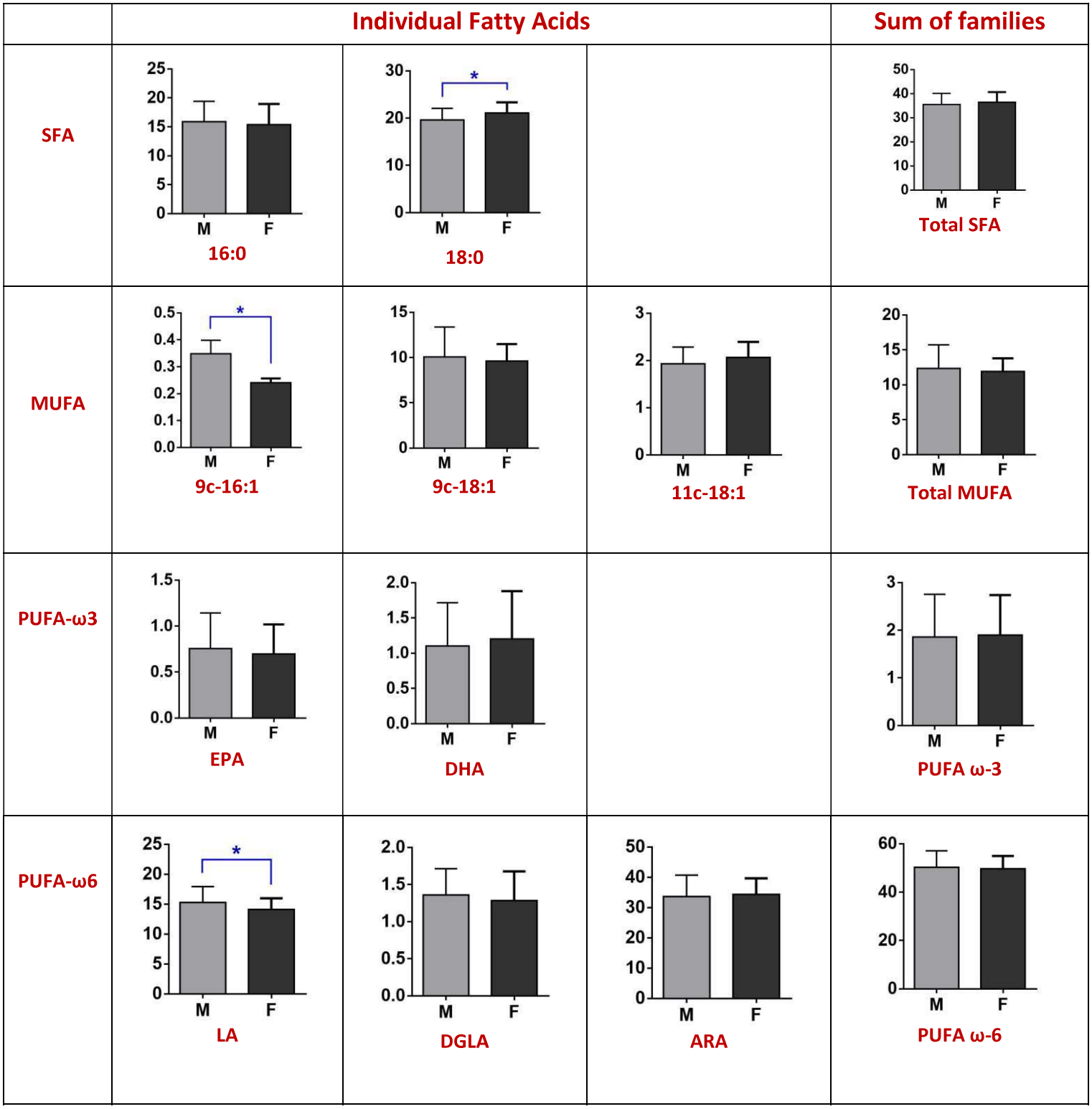


Figure 14. Relative quantitative percentage differences between Male (M, grey, n=30) and Female (F, black, n=38) healthy dogs for each type of fatty acid in the erythrocyte membranes. The values are given as mean ± SD. Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family. Values significantly different when compared to with each other: (*) $p < 0.05$. For healthy dogs' characteristics see Table 2. For specific values see Table 8 in the main text.

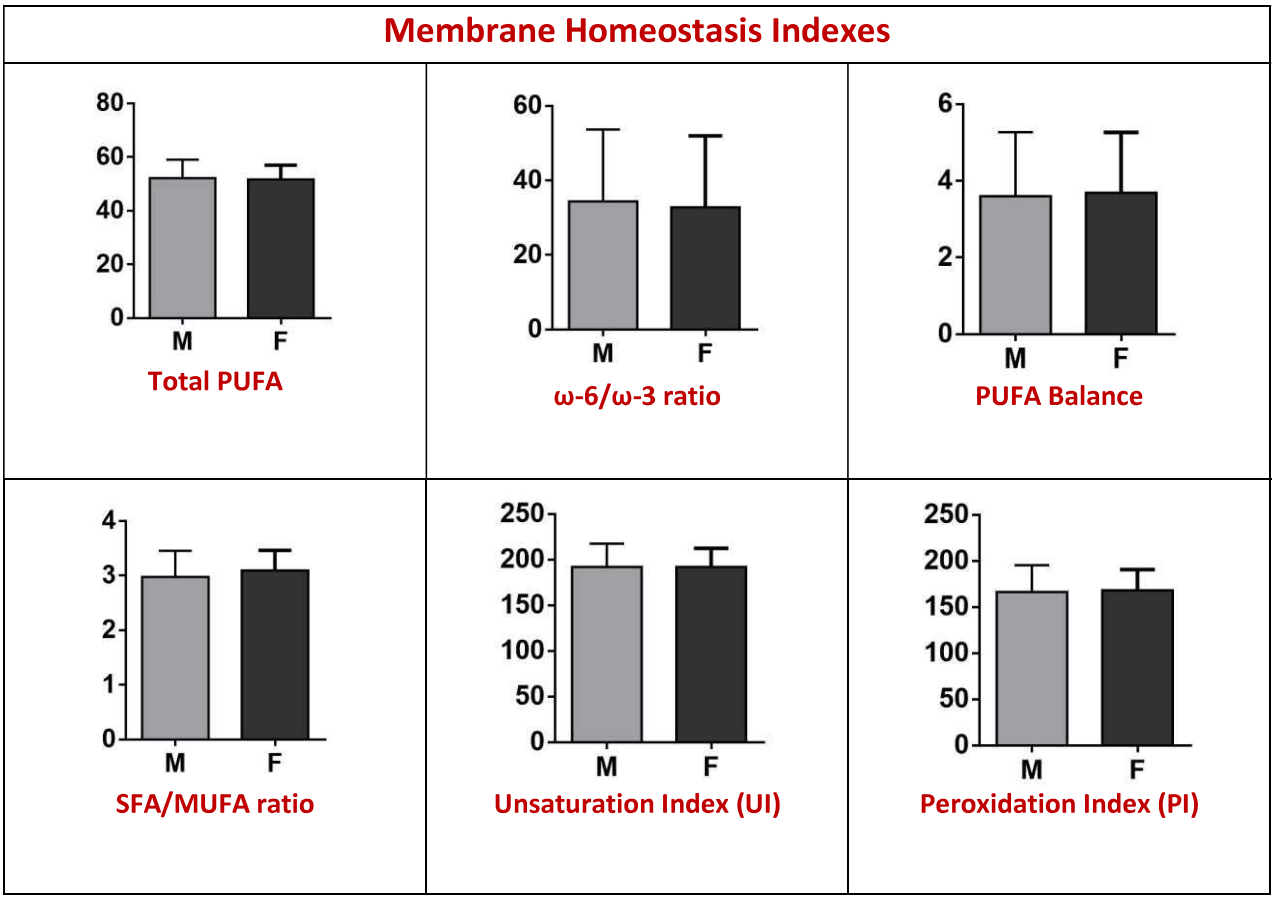


Figure 15. Relative quantitative percentage differences between Male (M, grey, n=30) and Female (F, black, n=38) healthy dogs for the membrane homeostasis indexes. 1st row: total PUFA, omega-6/omega-3 and SFA/PUFA ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes. The values are given as mean ± SD. For healthy dog characteristics see Table 2. For specific values see Table 8.

2.2.2 The erythrocyte membrane lipidome in dogs with CE

A total of 117 dogs were included in the present study, of these 49 were diagnosed with CE, while 68 were healthy dogs enrolled as control group in the previous study (see Results 2.1). The CE group comprised 17 females (7 spayed) and 32 males (1 neutered), with a median age of 47 months (range 4–144). Table 9 shows age, sex, bodyweight and breed of the cohort of CE dogs.

Table 9. Characteristics of the CE dogs (n=49) involved in the study.

n	Breed	Sex	Age (months)	Bodyweight (kg)
1	Miniature Poodle	M	84	5,3
2	Labrador	M	12	35,0
3	French Bulldog (Bouledogue)	M	15	10,3
4	Staffordshire Terrier	M	108	23,0
5	Border collie	M	72	25,2
6	English Setter	M	48	16,0
7	American Staffordshire Terrier	M	47	23,1
8	French Bulldog (Bouledogue)	Fs	22	10,6
9	Mixed-breed	F	42	18,5
10	Jack Russell Terrier	F	10	4,4
11	English Bull terrier	M	35	26,0
12	Pitbull	M	66	22,0
13	Dogo Argentino	M	96	35,5
14	Boxer	M	87	17,0
15	French Bulldog	M	13	7,1
16	German Shepherd	Fs	41	25,0
17	Miniature Poodle	M	83	6,0
18	Malinois	M	24	22,0
19	Maltese	Fs	114	4,0
20	German Shepherd	M	14	28,0
21	Mixed-breed	M	37	36,3
22	Mixed-breed	F	32	12,5
23	Labrador	Fs	76	28,2
24	Dobermann	M	11	30,5
25	Boxer	M	12	32,3
26	Dachshund	F	96	4,0
27	Mixed-breed	F	96	25,0
28	Maltese	F	36	4,0
29	Golden Retriever	M	24	31,0
30	Bolognese	M	104	4,6
31	Italian Segugio	F	48	15,0
32	German Shepherd	M	14	34,5

n	Breed	Sex	Age (months)	Bodyweight (kg)
33	Bull terrier	F	33	15,5
34	Labrador	M	10	31,0
35	Portuguese Sheepdog (Cao da Serra de Aires)	M	131	12,6
36	Jack Russell Terrier	Fs	48	5,5
37	Labrador	F	24	29,7
38	Belgian Shepherd Dog (Groenendael)	Mc	45	28,0
39	Basset Hound	M	55	23,0
40	Golden Retriever	Fs	48	28,3
41	Mixed-breed	F	84	7,3
42	Mixed-breed	M	96	17,0
43	Miniature Poodle	M	144	6,7
44	Setter Inglese	M	4	21,5
45	Mixed-breed	Fs	96	22,5
46	Kurzhaar	M	72	20,2
47	Pitbull	M	12	30,0
48	Golden Retriever	M	144	36,0
49	Boxer	M	18	30,0

Values of single fatty acid, total fatty acid contents (total SFA, total MUFA, total PUFA), ratio between the families (SFA/MUFA, omega-6/omega-3), and indexes (unsaturation index, peroxidation index, PUFA balance, EI, D6DI) of study dogs are reported in the following table (10).

Table 10. Cohort of 10 fatty acids expressed as percentages of the found μg/mL quantities**,** detected by gas chromatographic analyses of the fatty acid methyl esters (FAME)[1] after isolation and work-up of erythrocyte membrane glycerophospholipids, of CE dogs (n=49) The corresponding values of fatty acid families and lipid indexes are also reported.

CE dogs (n=49)		
Fatty acids[1]	**Mean**	**SD**
C16:0 – palmitic acid	10,87	1,77
C16:1 – palmitoleic acid	0,47	0,42
C18:0 – stearic acid	23,60	2,00
9c,C18:1 – oleic acid	9,76	1,40
11c,C18:1 – vaccenic acid	2,12	0,60
LA omega-6 – C18:2 – linoleic acid	13,23	2,31
DGLA omega-6 – C20:3 dihomogammalinolenic acid	1,67	0,54
ARA omega-6 C20:4 – arachidonic acid	35,99	2,59
EPA omega-3 – C20:5 – eicosapentaenoic acid	0,90	0,45
DHA omega-3 – C22:6 – docosahexaenoic acid	1,39	0,62
Total SFA[3]	34,47	2,39
Total MUFA[4]	12,34	1,68
PUFA omega-3[5]	2,29	0,87
PUFA omega-6[6]	50,89	3,17
Total PUFA[7]	53,18	3,31
SFA/MUFA[8]	2,84	0,38
Omega-6/omega-3 ratio[9]	26,03	11,91
PUFA balance[10]	4,29	1,59
UI[11]	200,63	11,11
PI[12]	177,38	12,53

[1]Fatty acids are evaluated as FAME (fatty acid methyl esters) after membrane isolation, lipid extraction and derivatization as described in the Experimental section. Structures are shown in Figure 1.

[2] The values are expressed as percentage of the found quantities (calculated as μg/mL) from the gas chromatographic analysis, using calibration and quantitation protocols and standard reference compounds for each FAME, as described in Materials and Methods. The GC peak areas of the 10 fatty acids cohort corresponds to ca. 97% of the total peak areas of the chromatogram.

[3] Total SFA (Saturated Fatty Acids) = % C16:0 + % C18:0.

[4] Total MUFA (MonoUnsaturated Fatty Acids) = % C16:1 + % 9c,C18:1 + % 11c,C18:1.

[5] PUFA (PolyUnsaturated Fatty Acids) omega-3 = %EPA + %DHA.

[6] PUFA (PolyUnsaturated Fatty Acids) omega-6 = %LA + %DGLA + %ARA.

[7] Total PUFA = %LA + %DGLA + %ARA + %EPA + %DHA.

[8] SFA/MUFA = (% C16:0 + % C18:0)/(% C16:1 + % 9c,C18:1 + % 11c,C18:1).

[9]Omega-6/omega-3 ratio = (%LA + %DGLA + %ARA)/ (%EPA + %DHA).

[10] PUFA balance = [(%EPA + %DHA) / Total PUFA] × 100.

[11]UI = (%MUFA × 1) + (%LA × 2) + (%DGLA × 3) + (%ARA × 4) + (%EPA × 5) + (%DHA × 6).

[12] PI = (%MUFA × 0.025) + (%LA × 1) + (%DGLA × 2) + (%ARA × 4) + (%EPA × 6) + (%DHA × 8).

Dogs affected by chronic enteropathies, regardless to the diagnosis, had higher values of palmitic acid ($p < 0.0001$) and lower levels of stearic acid ($p < 0.0001$) compared to healthy dogs, with an overall reduction of total SFA observed in CE dogs ($p = 0.02$). No differences were observed between healthy and CE dogs in regards of single (palmitoleic, oleic and vaccenic acids) and total MUFA values. CE dogs showed higher EPA ($p = 0.02$) and DHA ($p = 0.02$) content, as well as an increased content of total omega-3 PUFA ($p = 0.01$). In regards of omega-6 PUFA, CE dogs showed reduced content of linoleic acid ($p = 0.0006$) and increased dihomo-gamma-linoleic acid ($p = 0.0001$) compared to healthy dogs, while no differences were observed in arachidonic acid levels and total content of omega-6 PUFA between CE and healthy patients.

CE dogs showed membranes homeostasis indexes patterns different compared to healthy dogs. In particular, CE dogs had reduced values of omega-6/omega-3 ($p = 0.034$) and SFA/MUFA ($p = 0.001$) ratios, and increased PUFA balance ($p = 0.035$), UI ($p = 0.013$) and PI ($p = 0.014$).

No significant differences were observed in fatty acid contents, membrane homeostasis indexes among dogs affected by different forms of CE, or between CE dogs with and without PLE.

A comparative analysis between HD and CE dogs is demonstrated on the following Figures (16 and 17):

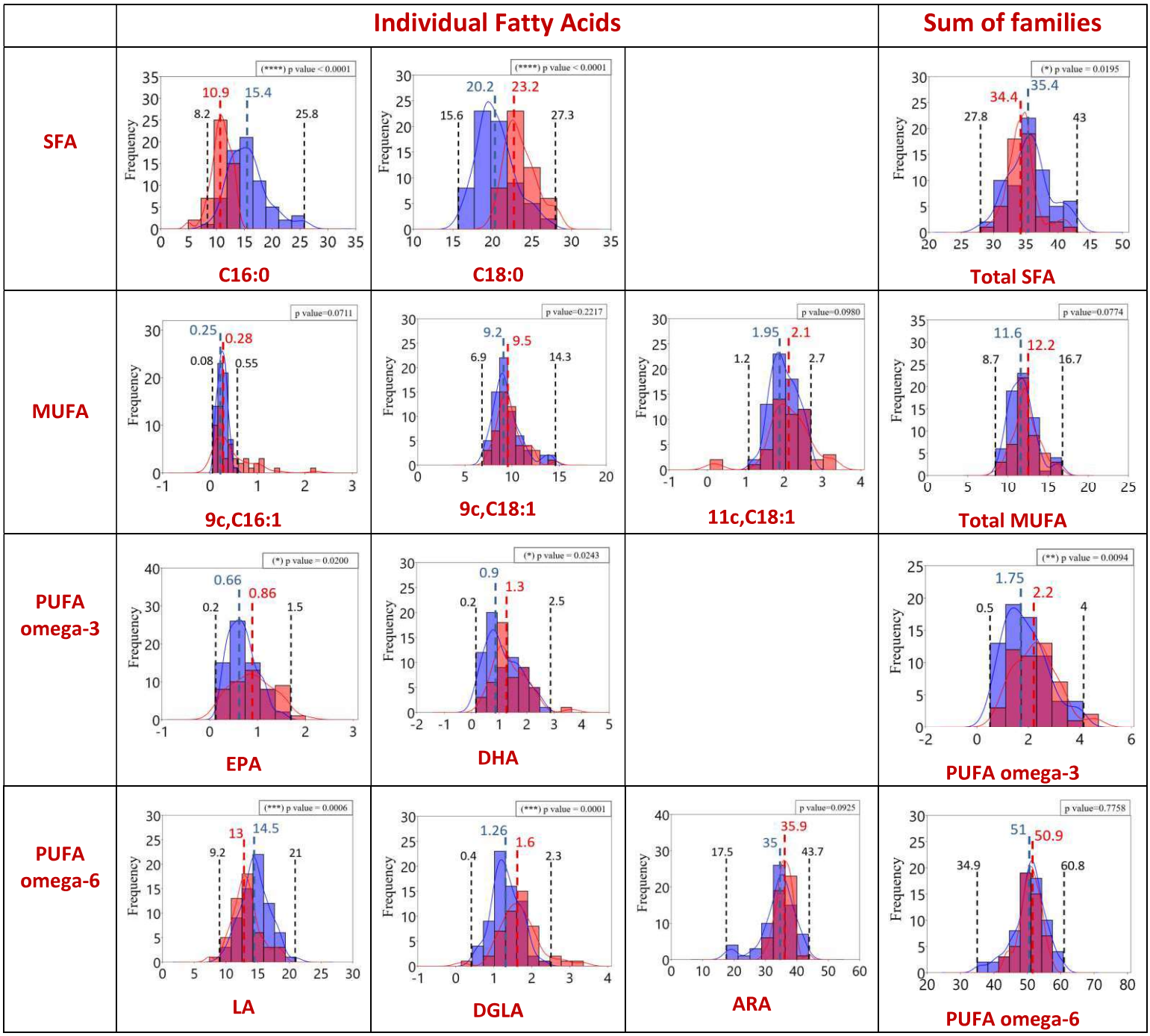


Figure 16. Distribution of the lipid indexes of the population of CE dogs (n=49) with the reference interval values obtained from HD (see fig 9). for the individual fatty acids and the corresponding families using the data in Table 3 with 95% confidence interval. Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family. Black: reference interval values (see fig 9). Blue: the median value of HD. Red: the median value of CE dogs. A Mann-Whitney test has been performed for the comparison between HD and CE dogs. The p-value is indicated on the top right. Values significantly different when compared to with each other: (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (****) $p < 0.0001$.

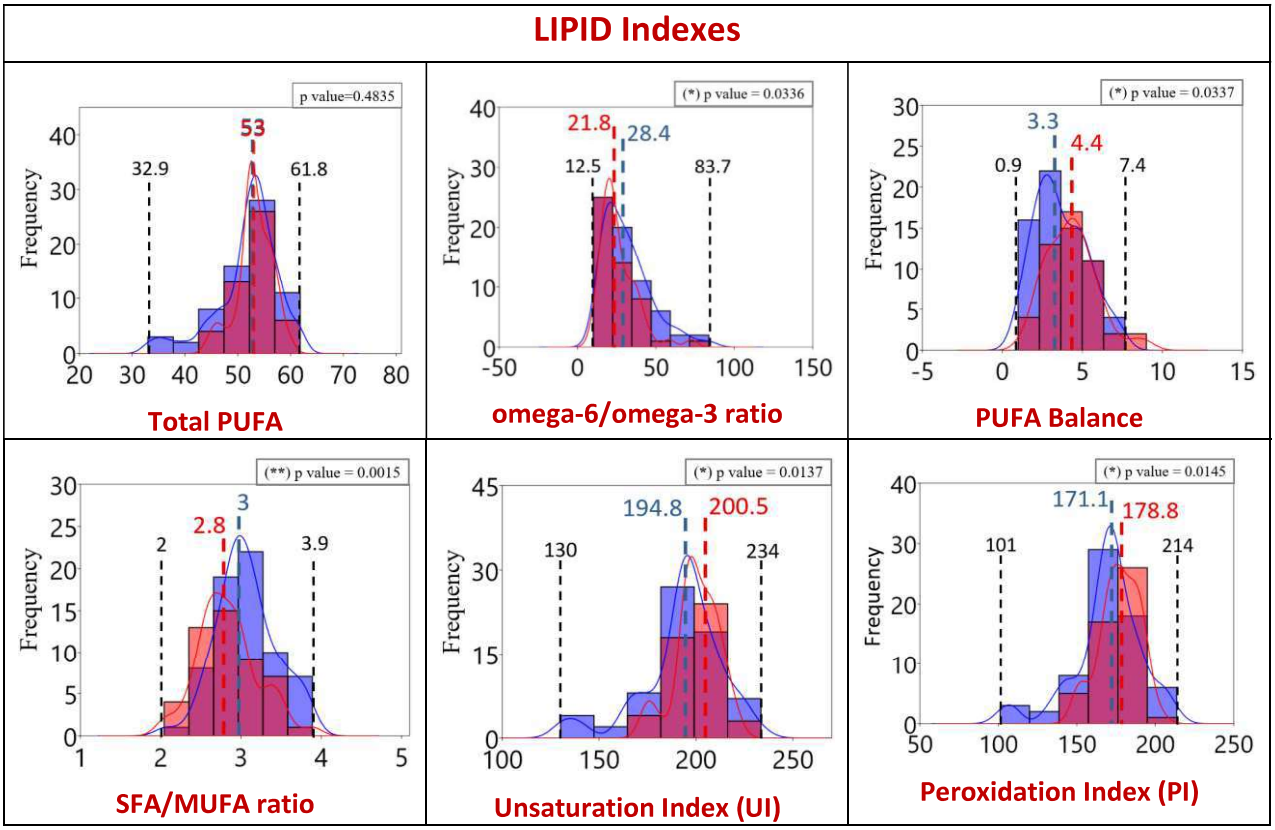


Figure 17. Distribution of the lipid indexes of the population of CE dogs (n=49) with the reference interval values obtained from HD (see fig 10). Healthy Dogs (n=68) with 95% confidence interval. 1st row: total PUFA, ω-6/ω-3 and SFA/PUFA ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes reference interval values (see fig 10 Blue: the median value of HD. Red: the median value of CE dogs. A Mann-Whitney test has been performed for the comparison between HD and CE dogs. The p-value is indicated on the top right. Values significantly different when compared to with each other: (*) $p < 0.05$, (**) $p < 0.01$.

2.2.3 The erythrocyte membrane lipidome in dogs with DM

A total of 80 dogs were included in the present study, of these 12 were diagnosed with DM, while 68 were healthy dogs enrolled as control group in the previous study (see Results 2.1). The 12 dogs affected with diabetes mellitus (DM), were 7 females (5 spayed) and 5 males (1 neutered), with a median age of 131 months (range 94–181). Table 11 shows age, sex, bodyweight and breed of the cohort of the DM dogs.

Table 11. Characteristics of the DM dogs (n=12) involved in the study.

n	Breed	Sex	Age (months)	Bodyweight (kg)
1	Mixed-breed	M	96,00	
2	Mixed-breed	F	158,00	5
3	Mixed-breed	F	156,00	5,40
4	Mixed-breed	Fs	110,00	
5	Mixed-breed	M	144,00	
6	Yorkshire Terrier	M		
7	Mixed-breed	Fs	181,00	8,60
8	Mixed-breed	Fs	94,00	4,90
9	Yorkshire Terrier	Fs	135,00	4,80
10	Cavalier king charles spaniel	M	113,00	12,60
11	Mixed-breed	Fs	112,00	21,90
12	Mixed-breed	Mc	151,00	22,50

Table 12. Cohort of 10 fatty acids expressed as percentages of the found µg/mL quantities, detected by gas chromatographic analyses of the fatty acid methyl esters (FAME)[1] after isolation and work-up of erythrocyte membrane glycerophospholipids, of DM dogs (n=12) The corresponding values of fatty acid families and lipid indexes are also reported.

DM dogs (n=12)		
Fatty acids[1]	**Mean**	**SD**
C16:0 – palmitic acid	14,97	1,61
C16:1 – palmitoleic acid	0,53	0,32
C18:0 – stearic acid	21,43	4,19
9c,C18:1 – oleic acid	10,24	1,12
11c,C18:1 – vaccenic acid	2,05	0,40
LA omega-6 – C18:2 – linoleic acid	13,64	1,70
DGLA omega-6 – C20:3 dihomogammalinolenic acid	1,21	0,24
ARA omega-6 C20:4 – arachidonic acid	33,69	3,63
EPA omega-3 – C20:5 – eicosapentaenoic acid	0,91	0,82
DHA omega-3 – C22:6 – docosahexaenoic acid	1,32	0,64

Fatty acids[1]	Mean	SD
Total SFA[3]	36,40	4,25
Total MUFA[4]	12,82	1,33
PUFA omega-3[5]	2,23	1,29
PUFA omega-6[6]	48,54	4,26
Total PUFA[7]	50,78	3,75
SFA/MUFA[8]	2,88	0,56
Omega-6/omega-3 ratio[9]	29,21	17,02
PUFA balance[10]	4,47	2,79
UI[11]	190,98	13,28
PI[12]	167,18	12,19

[1]Fatty acids are evaluated as FAME (fatty acid methyl esters) after membrane isolation, lipid extraction and derivatization as described in the Experimental section. Structures are shown in Figure 1.

[2] The values are expressed as percentage of the found quantities (calculated as µg/mL) from the gas chromatographic analysis, using calibration and quantitation protocols and standard reference compounds for each FAME, as described in Materials and Methods. The GC peak areas of the 10 fatty acids cohort corresponds to ca. 97% of the total peak areas of the chromatogram.

[3] Total SFA (Saturated Fatty Acids) = % C16:0 + % C18:0.

[4] Total MUFA (MonoUnsaturated Fatty Acids) = % C16:1 + % 9c,C18:1 + % 11c,C18:1.

[5] PUFA (PolyUnsaturated Fatty Acids) omega-3 = %EPA + %DHA.

[6] PUFA (PolyUnsaturated Fatty Acids) omega-6 = %LA + %DGLA + %ARA.

[7] Total PUFA = %LA + %DGLA + %ARA + %EPA + %DHA.

[8] SFA/MUFA = (% C16:0 + % C18:0)/(% C16:1 + % 9c,C18:1 + % 11c,C18:1).

[9]Omega-6/omega-3 ratio = (%LA + %DGLA + %ARA)/ (%EPA + %DHA).

[10] PUFA balance = [(%EPA + %DHA) / Total PUFA] × 100.

[11]UI = (%MUFA × 1) + (%LA × 2) + (%DGLA × 3) + (%ARA × 4) + (%EPA × 5) + (%DHA × 6).

[12] PI = (%MUFA × 0.025) + (%LA × 1) + (%DGLA × 2) + (%ARA × 4) + (%EPA × 6) + (%DHA × 8).

A comparative analysis between HD and DM dogs is demonstrated in the following distribution Figures (18 and 19):

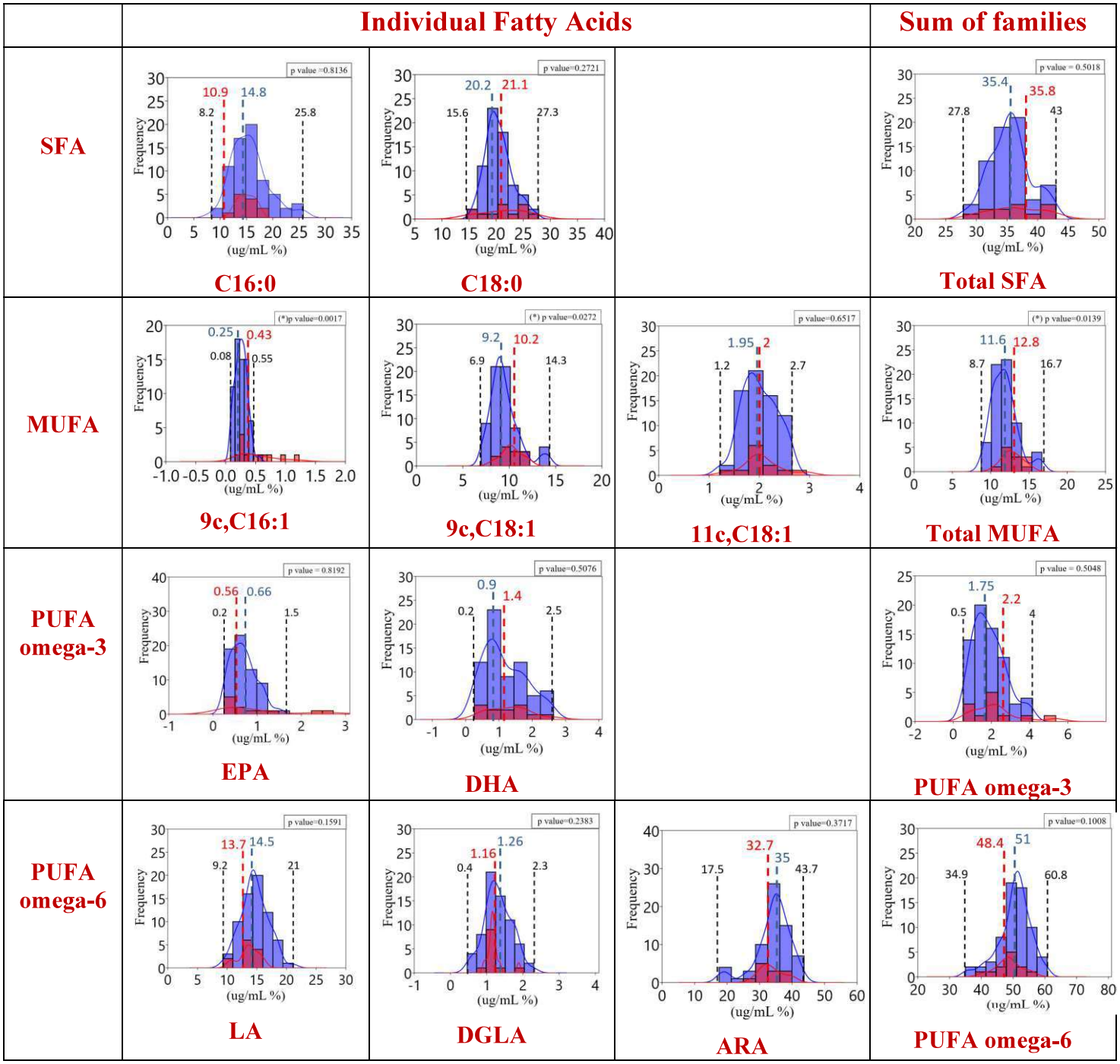


Figure 18. Distribution of the lipid indexes of the population of DM (n=12) dogs with the reference interval values obtained from HD (see fig 9). for the individual fatty acids and the corresponding families using the data in Table 3 with 95% confidence interval. Each member of the fatty acid family is given in a row, the last column being the sum of the corresponding fatty acid family. Black: reference interval values (see fig 9). Blue: the median value of HD. Red: the median value of DM dogs. A Mann-Whitney test has been performed for the comparison between HD and DM dogs. The p-value is indicated on the top right. Values significantly different when compared to with each other: (*) $p < 0.05$.

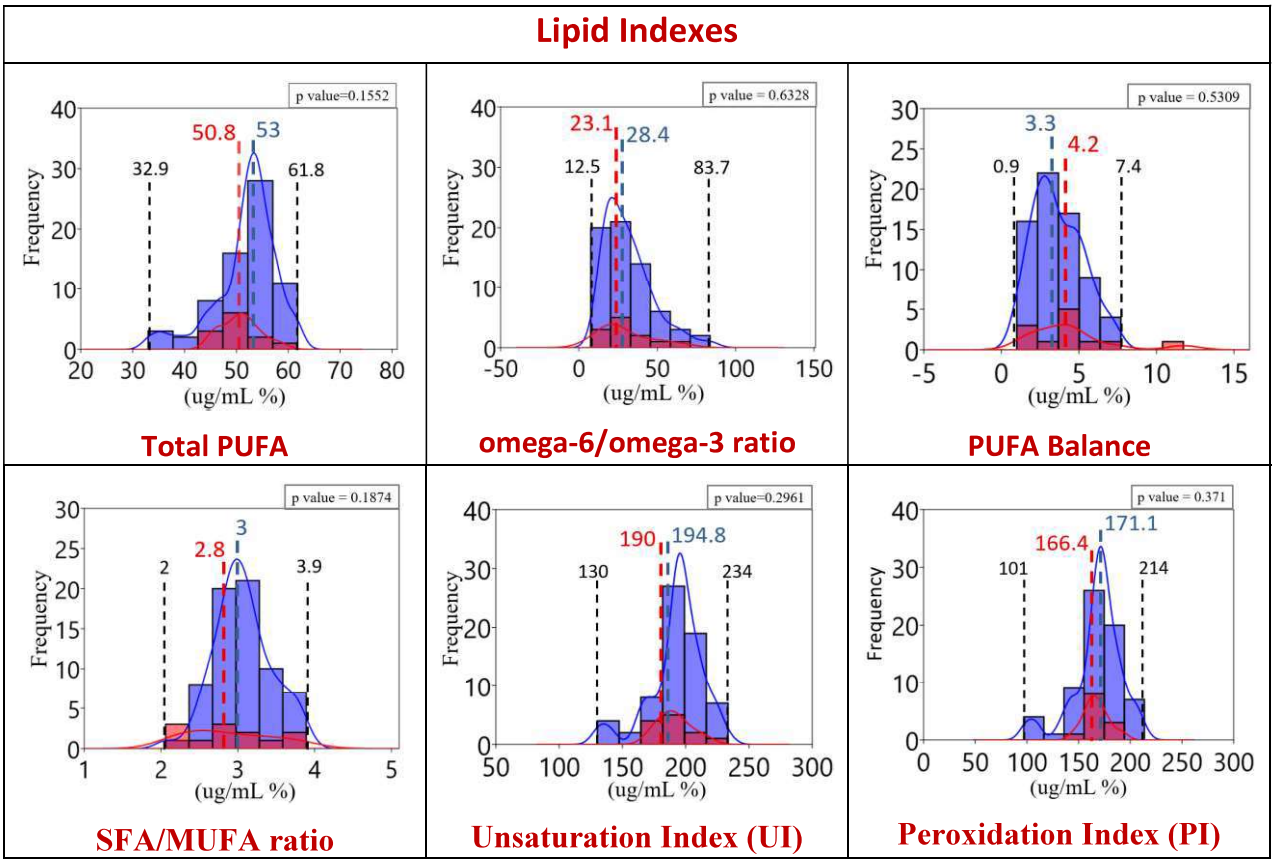


Figure 19. Distribution of the lipid indexes of the population of DM dogs (n=12) with the reference interval values obtained from HD (see fig 10). Healthy Dogs (n=68) with 95% confidence interval. 1st row: total PUFA, ω-6/ω-3 and SFA/PUFA ratios; 2nd row: SFA/MUFA ratio, unsaturation and peroxidation indexes reference interval values (see fig 10). Blue: the median value of HD. Red: the median value of DM dogs. A Mann-Whitney test has been performed for the comparison between HD and DM dogs. The p-value is indicated on the top right.

The results showed an increased level of the MUFA palmitoleic acid (9cis-16:1) and oleic acid (9c,C18:1) as well as the total MUFA content on DM dogs in erythrocyte membrane when compared to the HD.

2.3 DISCUSSION

2.3.1 Healthy Dogs

For the first time the erythrocyte membrane lipidome characterization of the fatty acid content of glycerophospholipids is provided in a cohort of clinically healthy dogs (n=68). The rationale for the choice of 10 fatty acids takes into account the most representative components in terms of structural and functional roles played by such hydrophobic moieties in membrane phospholipids, as well as the nutritionally important polyunsaturated fatty acids (see Figure 1). We arbitrarily chose this cohort for the application to veterinary analyses, taking into consideration previous work done in humans with physiological and pathological conditions (Heude et al. 2003; Hodson et al., 2008; Harris et al. 2012; Sansone et al. 2016; Giacometti et al. 2017; Amézaga et al. 2018; Gai et al. 2019; Pallot et al. 2019;). It is worth underlining that studies reported in literature on fatty acids of erythrocyte membranes in both humans and animals do not report the same cohort of fatty acids. For humans, the meta-analysis reported by Hodson et al, (2018) highlighted 13 SFA, MUFA and PUFA moieties in erythrocyte membrane phospholipids obtained from 9 studies of 321 men and 70 women. In another study on infants, only the total SFA and MUFA values are given without specifying each fatty acid type, whereas for the omega-6 pathway together with the C18 and C20 PUFA long chain fatty acids such as 22:4 and 22:5 are evaluated, which are not commonly reported in other studies (Makrides et al. 1994). With the emerging field of lipidomics, new fatty acid structures are evidenced, such as for example the presence of the n-10 MUFA family, with 6cis-C16:1 (sapienic acid) as positional isomer of palmitoleic acid, and the corresponding geometrical trans isomer (6trans-C16:1). These new markers indicate the metabolic partition of delta-6 desaturase enzymes between palmitic and linoleic acids and the free radical-based transformations (Ferreri et al. 2016) respectively, but it is not possible to define yet their participation and effects for the membrane composition. In model animals as well as in humans, increased or decreased fatty acid levels were found in kidney (Zhao et al. 2012) or liver diseases (Svegliati-Baroni et al. 2019), respectively, and such alterations were correlated with other biochemical markers, however further research are needed to establish normal and altered levels for newly identified fatty acid markers. Considering such complexity, it was evaluated of strategic importance to concentrate on a specific number of fatty acids, i.e., the 10 fatty acid

cohort, and on a specific cell compartment, i.e., the erythrocyte membrane, in order to get a simplified, yet representative and significative, approach for the examination of known metabolic and nutritional influences on the biophysically regulated homeostatic control of membrane formation and remodeling. The cohort includes fatty acids that cannot be missed in biological membranes, that are characteristic for each tissue type (Pradas et al. 2018; Ferreri and Chatgilialoglu 2012; Abbott et al. 2012; Harayama and Riezman 2018) and their interval values (minimum and maximum values) in the specific membrane type, such as in erythrocytes, derive from population studies, as previously mentioned.

It is worth mentioning that, when fatty acid analysis is performed, it is very important to ascertain that the subjects did not follow any supplementation during the last 4 months. In the canine diets, it is becoming more and more popular the use of "omega" supplements or "omega-enriched" commercial diets, that lead to the uptake of these essential fatty acids that cannot be prepared by de novo biosynthesis. It is known that such supplementations can change the results of lipid analyses (Walker et al. 2015), therefore we asked this specific information to the owners during the recruitment.

The analytical conditions are also important to ensure the efficient separation of the 10 fatty acid cohort especially of unsaturated fatty acids with the same fatty acid chain length but different geometry and position of the double bonds. In this respect the library of geometrical and positional isomers in our hands plays an important role to check peak superimposition (Sansone et al. 2013; Ferreri et al. 2016; Sansone et al. 2016; Amézaga et al. 2018). The overall fatty acid distribution reported in Table 1 and Figures 2-3 highlights that in the dog erythrocyte membrane lipidome the omega-6 PUFA content is prevalent followed by SFA and MUFA. It is worth underlining that omega-3 fatty acids are present in minimal concentrations, marking the difference with human lipidome (Abbott et al. 2012; Harris et al. 2012; Hall et al. 2012; Ferreri et al. 2016; Sieber-Ruckstuhl et al. 2019; Boretti et al. 2020). Moreover, in healthy dogs the SFAs, stearic and palmitic acids, and the omega-6 PUFAs, arachidonic and linoleic acids, sum up to nearly the 94% of the total RBC fatty acids, as already observed in other mammalian species (Abbott et al. 2012). In the distribution graphs of Figure 2, large interval ranges for the palmitic acid percentage (8.2-25.8) and arachidonic acid (17.5 - 43.7) can be appreciated. It is also interesting to note that 80% of these values describes a narrower range (palmitic acid: 8.5-20%, arachidonic acid: 30-40%). On the other hand, the distribution of linoleic acid values follows a gaussian-like behavior, with a probability distribution symmetric about the mean, showing that data near the mean are more frequent in occurrence than data far from the mean.

The different distributions of SFA, C18 and C20 PUFA omega-6 indicate that it is necessary to gather large sets of these data for mathematical treatment to model their roles in the membrane compositions (Mar 2019). It is also worth pointing out again that the omega-6 are essential fatty acids (EFA), not prepared directly by biosynthesis, and also dogs need the intake of linoleic acid as precursor of other biosynthetically prepared omega-6 fatty acids. The importance of ascertaining the omega-6 dietary supply to animals represents an historical highlight in veterinary medicine, having been noted in canine dermatological problems by Burr and Burr in 1929 (Burr and Burr 1973). Therefore, it is of extreme importance to follow-up the intakes of linoleic acid and its metabolic transformations to DGLA and arachidonic acid, together with their incorporation at the level of cell membranes, since they regulate fundamental structural and functional properties for health in the whole. This information can be integrated in a panel with other biochemical, biomolecular, clinical parameters for a thorough evaluation of health in dogs.

The strength of our cohort of fatty acids is to provide a valuable set of metabolic information to examine: i) data on the PUFA pathways that start from precursors that must be necessarily taken from the diet thus reporting on the omega-6 and omega-3 fatty acid balance, that in dogs such balance is shifted towards the omega-6 family; and ii) data on the SFA-MUFA pathway that starts from the de novo biosynthesis of the first fatty acid in the body, that is the SFA palmitic acid, thus including the effects of insulin response and liver conditions (Han 2016; Abbott et al 2012). From these crucial data sets the influence of enzymatic activities, hormones and nutrients, can be studied and it is possible to use a specific SFA-MUFA-PUFA cohort to examine metabolic profiles, decide nutritional-based strategies and follow-up the changes during physiological and pathological conditions, including oxidative stress (Zhao et al. 2014; Zhao et al. 2015; Ferreri et al. 2016; Han 2016; Ni et al. 2019;). The limitations are due to the small size of our data, compared with the large data set provided by other powerful lipidomic techniques, although the balance cost-effectiveness are important parameters to evaluate in wide applications to population.

The omega-6 EFA linoleic acid and its transformation to DGLA and ARA, which is predominant in dogs, affect not only from the point of view of the membrane structural properties but also affects the membrane response to stimuli. In fact, upon different kinds of stimuli, phospholipase A2 is activated and liberates the fatty acid moieties from the cell membrane phospholipids (Murakami et al. 2010), therefore the membrane fatty acid composition can be considered a precious information of the pro- and anti- inflammatory

predisposition of the organism (Adler et al. 2018; Loef et al. 2019). Indeed, liberation of SFA, MUFA and PUFA fatty acids generates potent mediators, such as prostaglandins, leukotrienes, endocannabinoids and many others, that influence dog metabolism in health and diseases (Bauer et al. 2008; Abramo et al. 2014; Kalenyak et al. 2019). There is a growing attention to the lipidomic analysis in animal studies, as reported recently for variations of phospholipids of whole blood and plasma in canine inflammatory bowel disease (Kalenyak et al. 2019). In this context we believe that the present work on erythrocyte membrane fatty acid cohort in healthy animals could represent the benchmark for assessment of healthy dog molecular profile.

A focus of our approach in dogs was to observe the omega-3 value and its distribution in healthy dogs, since omega-3 are essential fatty acids and EPA and DHA levels derive from the dietary intake of the precursor (alpha-linolenic acid). It is well known that this pathway in animals is less prevalent than omega-6 (Abramo et al. 2014). Our data confirmed low levels and a narrow range (0.5-4) (Table 1) together with a significant age-related increase of EPA (Supplementary Figure 3). It is worth noting that in humans EPA and DHA levels are strongly influenced by dietary intakes, but some studies have shown that age remains a significant determinant of EPA and DHA status regardless of dietary intake (Johnson et al. 2015). Omega-3 are known to have specific anti-inflammatory activities and, being the omega-6/omega-3 ratio naturally high in dogs (12-7-83.7), it is evident that their increase in the cellular environment, influencing this ratio, can exert significant metabolic effects. In this context the establishment of omega-3 levels under various dog health conditions can be interesting to precisely individuate levels of omega-3 to introduce in the regular diets of animals. Limitations of our study are in the non-homogeneous distribution of male and female dogs and in the diversity of breeds, but the prosecution of data collection will certainly compensate such weaknesses.

Considering the correlations found in our work, it is worth underlining that de novo lipogenesis with formation of palmitic acid and palmitoleic acid are known biomarkers of bodyweight increase in animals and humans (Sansone et al. 2016; Guo et al. 2012) and their increase over the normal levels are indicators of metabolic derangements in humans at older ages (Zong et al. 2012). We found positive correlation between bodyweight and the values of palmitoleic, stearic acids and total SFA of our healthy dog cohort. At the same time the negative correlation between bodyweight with all unsaturated indicators (arachidonic acid, PUFA omega-6, UI, PI) indicates a shift of membrane components from unsaturated to saturated moieties, that will be worth of further deepening especially in the dog obesity conditions. An increase of SFA and

MUFA components has been correlated with suppression of inflammatory response in animal models (Guo et al. 2012).

Correlations of the fatty acid data with different breeds need more data, whereas interesting observations could be done related to dog sex. The lower levels of stearic acid and the higher levels of palmitoleic acid detected in male dogs indicate that SFA to MUFA pathway functions more than in females. Palmitoleic acid is a lipokine that travels to muscles and liver, where it improves cell sensitivity to insulin, blocks fat accumulation in the liver (Cao et al. 2008) and seems to be involved in regulating muscle mass (Cao et al. 2008; Lipina et al. 2017). Although preliminarily, this different distribution of FA indirectly indicates differences in metabolism of dogs according to the sex, to be deepened also considering larger numbers of sterilized females and neutered males.

2.3.2 Dogs diagnosed with Chronic enteropathy

The current study suggests that there is a peculiar pattern of red blood cells membrane lipidome in dogs affected by chronic enteropathy, however red blood cells lipidomic analysis does not seem useful to distinguish the different forms of CE at the time of the first visit. This shift in fatty acid patterns are not completely explained by alterations in dietary intake and may be a response to inflammation rather than an etiological factor. Indeed, circulating fatty acids seems correlated with intestinal pro-inflammatory cytokines levels (Wiese et al 2016). Despite increased SFA levels have been observed in rats with experimentally induced ulcerative colitis (Fernández-Bañares et al. 1997; Nieto et al 1998), in the present study CE dogs showed lower total SFA levels compared to healthy dogs. This finding is consistent with the results of a previous study on human ulcerative colitis, in which patients have significantly lower serum percentages of SFA (Wiese et al 2016). In regards of single SFA, the relative quantitative percentage stearic acid (C18:0) was significantly higher and that of palmitic acid (C16:0) was significantly lower than those observed in the healthy subjects, as already observed in erythrocytes membranes of Japanese people affected by Chron disease (Uchiyama et al 2013). This finding, together with higher elongase-6 activity observed, suggest that in the CE dogs, the pathway to form stearic acid from palmitic acid seems hyperactivated.

On the other hand, despite palmitoleic acid levels were higher in the CE group compared to healthy dogs, trending toward significance ($p = 0.07$), it can be hypothesized that the parallel

Delta-9 desaturase transformation of palmitic to the MUFA palmitoleic acid is reduced in dogs affected by CE. As matter of fact, it is worthy to note that the liver expression of stearoyl-coenzyme A desaturase 1 (SCD1, Delta-9 desaturase), the key enzyme controlling the desaturation of saturated fatty acids, is dramatically inhibited in colitis mice (Chen et al 2008; Wang et al 2016).

It has been hypothesized that IBD patients would have decreased blood and tissue n-3 PUFA due to the inflammatory state. However, looking at the membrane asset in CE dogs higher EPA, DHA and total n-3 PUFA levels were found. Our results indicate to a n-3 accumulation. Indeed, the data observed in this canine cohort are consistent with pattern previously observed in active IBD patients showing a significantly higher fraction of the n-3 PUFA, and DHA (Esteve-Comas et al 1992; Esteve-Comas et al 1993). A further prior study found plasma n-3 PUFA concentrations increased in patient with Chron disease compared to control patients; moreover, in these patients, total n-3 PUFA and EPA levels directly correlates with pro-inflammatory cytokines levels (Scoville et al 2019).

On the other hand, on the present canine cohort is presented an accumulation of n-3 fatty acids accompanied by altered levels of n-6.

The total n-6 PUFA and AA levels did not differ between CE and healthy dogs. On the other hand, the percentage weight of LA was significantly lower and that of DGLA was significantly higher, than those of the healthy subjects. These results point to the similarity of dog with human IBD (Uchiyama et al 2013; Sitkin and Pokrotniesks 2018), in which the metabolism of linoleic acid supported by the Delta-6 desaturase seems to be accelerated than that of healthy individuals (Uchiyama et al 2013; Ito et al 2015). The profile of CE dogs showed an hyperactivation of this enzymatic pathway with faster metabolization of linoleic acid to DGLA in canine CE. Interestingly, in humans, genetic polymorphisms associated with alterations in the metabolism of long chain PUFA from dietary linoleic acid and alpha-linolenic acid have been associated with the risk of developing Chron disease (Costea et al 2010; Costea et al. 2014; Kettunen et al 2012; Ananthakrishnan et al 2017).

In the fatty acid profile of healthy dogs we found that SFAs, stearic and palmitic acids, and the omega-6 PUFAs, arachidonic and linoleic acids, constitute ca. 94% of the total RBC fatty acids, with the palmitic acid (8.2-25.8) and arachidonic acid (17.5 - 43.7) percentages that are the largest in the membrane lipidome with the widest interval ranges. Therefore, the significant changes of palmitic, stearic and linoleic acids levels mirror a relevant metabolic derangement.

On the other hand, in the omega-6 pathway the elevated levels of DGLA assumes a meaning if one considers that in dog metabolism the linoleic acid transformation to gamma linolenic and DGLA acids is the main anti-inflammatory control of the animal, leading to potent antinflammatory eicosanoids, trespassing the role of omega-3 that have much lower levels. Indeed, since the first historical observation of dermatological canine problems (Burr et al. 1973), it should be always recalled that in dogs the omega-3 have not the same physiological role that in human metabolism in controlling inflammation.

We are aware that further and larger studies are needed to asses membrane profiles for dogs health and also to evaluate the role of gene encoding desaturase enzymes in dogs. In addition, should be taken into account that desaturases are usually controlled by feedback regulation and it cannot be excluded that the feedback regulation of PUFA could be altered in canine CE. To further understand the complex interaction occurring in PUFA metabolism is worth to note that DGLA is metabolized by cyclooxygenase (1 and 2) and arachidonate 15-lipoxygenase, into prostaglandins and anti-inflammatory eicosanoids with the ability to antagonize synthesis of AA-derived pro-inflammatory eicosanoids (Sergeant et al 2016).

It is worth noting that also membrane homeostasis indexes are altered in CE dogs, and in particular the n-3 PUFA/n-6 PUFA ratio (n-3/n-6 ratio) that is known to be influenced through diet therapy as important factor for reducing human IBD activity. PUFA are essential elements in the diet since they are not prepared by enzymatic systems as precursors omega-6 linoleic acid and omega-3 alfa-linolenic acid. A high vital membrane n-3/n-6 ratio was effective in maintaining remission in IBD patients (Uchiyama et al 2010). In consequence of this, it could be argued that it is pivotal to establish optimal n-6/n-3 ratio and PUFA balance values also in dogs and diet therapy and nutraceutical interventions should be investigated in detail in canine CE.

The unsaturation and peroxidation indexes are useful parameters for estimating the homeostasis of membranes in terms of protection of the tissues against oxidative damage while maintaining an appropriate environment for membrane function. A low degree of fatty acid unsaturation is present in longevous species and membrane peroxidizability index is inversely related to maximum life span in mammals (Pamplona et al. 1998; Puca et al. 2008). PUFAs may integrate into phospholipids and modify the physical properties of cell membranes, thus increasing membrane fluidity and permeability. Indeed, PUFAs affect in vitro presence of occludin and Zonula occludens-1 (ZO-1) intensity, and DHA limited the effect of the

inflammatory stimulus on occludin, ZO-1 and barrier function, suggesting a role of PUFAs in trans-cellular permeability (Beguin et al. 2013).

Oxidative damage in dogs with IBD has been recently demonstrated (Minnamoto et al 2015; Rubio et al. 2017) and the increased peroxidation index observed in CE fogs of the present study suggests a higher susceptibility of the membranes to peroxidability and oxidative damage. The usefulness of antioxidant treatment alongside the traditional therapeutic approaches, should be investigated in detail in canine CE.

Finally, the positive correlation between folate and arachidonic acid levels can be evaluated also in view of the known correlation in humans between folate and PUFA (Das 2008; Umhau et al. 2006) which brings also important correlation with neurological functions.

2.3.3 Dogs diagnosed with Diabetes Mellitus

Interestingly, this group of diseased dogs showed the values of MUFA palmitoleic acid (9cis-16:1) and oleic (C18:1) different from normality, with an increased content of both of these fatty acids in erythrocyte membrane. These fatty acids cannot come from the diet and their biological meaning is directly derived from the lipid metabolism. In fact, it derives from the enzymatic transformation of palmitic acid to palmitoleic acid and to oleic acid by the delta-9 desaturase (fig 20).

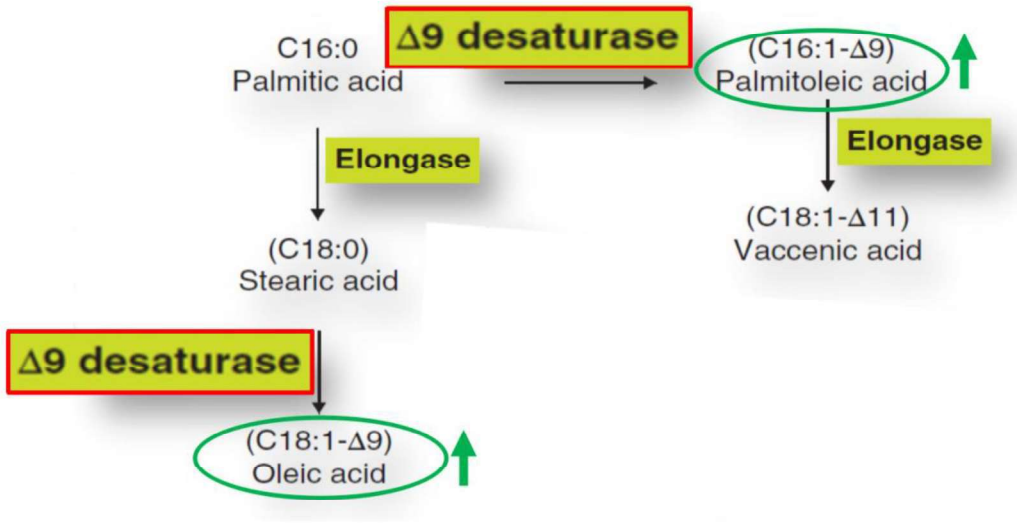


Figure 20. The main transformations of the SFA-MUFA pathway. Along the palmitic acid transformation, there is an increase in the formation of palmitoleic and oleic acid due to an accelerated Δ9 desaturase enzymatic activity on the DM dogs.

The activation of this enzymatic transformation is known to be related to the insulin response and the carbohydrate management, which is connected to lipid biosynthesis through the pyruvate and acetyl-CoA pathways.

The erythrocyte membrane lipidome of DM dogs may be successfully applied to expand the molecular evaluations in veterinary medicine providing important information of the cell membrane status in physiological and pathological conditions, involving structural and signaling properties.

CHAPTER 4: Conclusion

In the current study, we provided the first panel of erythrocyte membrane fatty acids in healthy dogs choosing a cohort representative for the main structural and functional roles of these hydrophobic molecules in the cell membrane compartment. The panel of 10 fatty acids represents a set of information, chosen for their biochemical, nutritional and metabolic significance, and their reference ranges for healthy dogs can represent a benchmark to examine unhealthy conditions. only by constructing a benchmark of the membrane fatty acid profile of a specific compartment of a living organism, it will be possible to individuate in a holistic and sustainable way the differences between healthy and unhealthy conditions. It is worth to remark that the effects of fatty acids in cellular metabolism departing from the membrane release and remodeling processes are more and more studied also for immune functions (Alarcòn et al. 2020) therefore the knowledge of the membrane asset can be a useful indication for the examination of animal response at molecular level. Indeed, the protocol herein described, including calibration and quantitation procedures, can be applied to large animal populations, with molecular information that are ready to be combined with other medical data sets for mining techniques, which are nowadays under study for the exploitation of disease risks and prevention plans (Frigolet et al. 2017).

Our findings indicate that the erythrocyte membrane lipidome may be successfully applied in dogs with CE, as well as dogs with DM. The lipidomic analysis is demonstrated to provide important information on pathogenesis of the CE and potentially leading to personalized therapeutic intervention targeted to decrease inflammation and to increase protective components. A protocol has been defined for the erythrocyte membrane fatty acid analysis to be used as the methodology for evaluating a high number of animals. In this way we started to create the paradigm of fatty acid-based lipidomic analysis to evaluate health conditions in dogs and demonstrated a second utility in the assessment of the insulin response on the lipid metabolism of diabetic dogs, to be treated with appropriate diet and therapy.

Clinically speaking, since imbalances in lipid metabolism contributes to diverse animal phenotypes and disease states, ranging from inflammation and cancer to metabolic diseases, the study of membrane lipids in normal and pathological states can successfully contribute to monitor health conditions as well as the result of treatments. Membrane lipidomic research in veterinary will provide, as done for humans, deep insights in mechanisms of several diseases, helping to identify molecular targets, patient selection for better treatment response, prediction

and monitoring of treatment efficacy and response to therapeutic measures. In the light of this, membrane lipidomics may become part of the arsenal adopted for clinical diagnostics in veterinary medicine, useful for the evaluation of a disease onset and its progression, representing a potential avenue to individualized treatment and monitoring in different pathological states.

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ANNEXES

Annex 1: The erythrocyte membrane lipidome of healthy dogs: creating a benchmark of fatty acid distribution and interval values, Research publication in Frontiers in Veterinary Science, section Animal Nutrition and Metabolism

Annex 2: Proceedings of 28th Congress of European College of Veterinary Internal Medicine – Companion Animals (ECVIM-CA), abstract '*The erythrocyte membrane lipidome profile in healthy dogs and changes in dogs with diabetes mellitus',* published on Journal of Veterinary Internal Medicine – JVIM

Annex 3: Proceedings of 29th Congress of European College of Veterinary Internal Medicine – Companion Animals (ECVIM-CA), abstract '*The paradigm of erythrocyte mem-brane lipidome in healthy dogs: first evaluation of the op-timal interval values of a fatty acid cluster',* published on Journal of Veterinary Internal Medicine – JVIM

Annex 4: *Cyclin-CDK Complexes are Key Controllers of Capacitation-Dependent Actin Dynamics in Mammalian Spermatozoa*, Research Publication

Annex 5*: Graphene Oxide Improves In Vitro Fertilization in Mice with no Impact on Embryo Development and Preserves the Membrane Microdomains Architecture*. Accepted Research Publication

Annex 6: Poster presented in Annual Meeting of Doctoral Programs in Molecular and Cellular Biotechnology

Annex 7: Presentation used in the oral communication delivered in the 28th Congress of European College of Veterinary Internal Medicine – Companion Animals (ECVIM-CA)

Annex 8: Presentation used in the oral communication delivered in the 29th Congress of European College of Veterinary Internal Medicine – Companion Animals (ECVIM-CA)

Annex 9: Presentation used in the oral communication delivered in the 1st Repeat Annual Meeting

Annex 10: Presentation used in the oral communication delivered in the 2nd Repeat Annual Meeting

Annex 11: Poster presented in in the 29th Congress of European College of Veterinary Internal Medicine – Companion Animals (ECVIM-CA)

Annex 12: Abstract accepted for oral communication delivered by a colleague in the 72º Conference SISVet 2018

Annex 13: Abstract (a) accepted for oral communication delivered by a colleague in the 72º Conference SISVet 2019

Annex 14: Abstract (b) accepted for oral communication delivered by a colleague in the 72º Conference SISVet 2019