UNIVERSITÀ DEGLI STUDI DI MILANO

SCUOLA DI DOTTORATO IN SCIENZE VETERINARIE E DELL'ALLEVAMENTO
(XXXII CICLO)
DIPARTIMENTO DI MEDICINA VETERINARIA

THE INTERPLAY BETWEEN HOST DEFENSES AND SYSTEMIC PATHOGENS IN PROMOTING DISEASES OF COMPANION ANIMALS

SARA MEAZZI
R11566

TUTOR: Prof.ssa Alessia Giordano

COORDINATORE: Prof.ssa Valeria Grieco

A.A. 2018-2019

SCIENTIFIC BACKGROUND

GUT MICROBIOTA

The intestinal microbiota is the consortium of all living microorganisms that inhabit the gastrointestinal (GI) tract. The whole amount of genes of microbiota is known as the "microbiome", which is far more larger than the human genome (Adak et Khan, 2019). The insight into the complexity of the intestinal microbiota and its intimate relationship with the host has spurred researchers to better understand the importance of a balanced microbial ecosystem for the regulation of host health and immunity (Suchodolski, 2016). Indeed, microbiota plays different roles contributing to the digestion and energy harvest from feed, producing metabolites pivotal for enterocytes nutrition, acting as a physical barrier against pathogens and shaping and interact with the immune system (Neish, 2009).

METHODS FOR THE INVESTIGATION OF GUT MICROBIOTA

There are two main methods that could be used to study microbiota, that are culture-dependent and culture-independent methods.

Culture-dependent methods were firstly introduced in 1881 by Robert Koch, which was the father of the plating technique used to culture and identify microorganism. These methods are still largely used in microbiology; however, they detect only 30-50% of the totality of bacteria. There are two main issue: the first is related to the bacteria properties, indeed several bacteria requires specific environment condition to grow. On the other hand, the growth of predominant bacteria could mask the isolation of the less abundant one (Adak et Khan, 2019; Sarangi et al., 2019).

Culture-independent methods include both sequencing and non-sequencing methods. The first ones relies on Sanger sequencing of the 16S rRNA (firstly introduced in 1970 by Carl Woese and colleagues) also known as "first generation sequencing". This new insight allows to investigate

intestinal microbiota exploiting the conserved sequence of the hypervariable region V1-V9 in the small ribosomal subunit 16S rRNA (**Figure 1**). Since these techniques do not require bacterial culture, they allow to identify those microorganisms that do not grow well, besides the culturable ones.

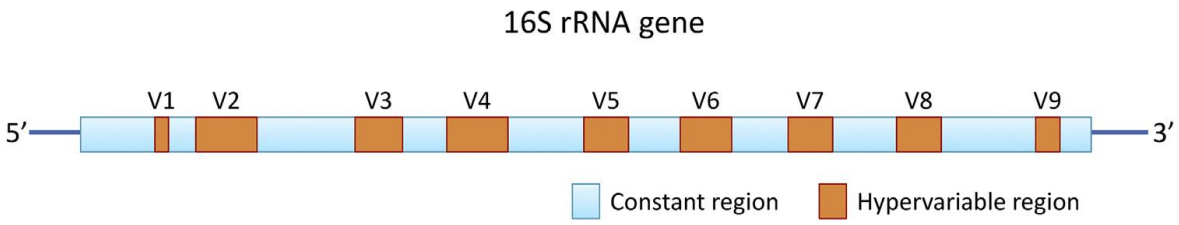

Figure 1. 16S ribosomal RNA gene showing the nine hypervariable regions (in brown colour) and constant regions (in blue) (Sarangi et al., 2019)

The non-sequencing method rely on the identification of the hypervariable region too, which is highlighted through the use of electrophoresis based on fragment length or based on the presence of a specific nucleotide sequence (FISH-flow). However, these methods lack in resolution, because the differences are too small to be correctly identified. Moreover, the less abundant bacteria are generally missed (Sarangi et al., 2019). For these reasons, they have been replaced by sequencing techniques. Sequencing methods provide a reliable picture of the relative abundance of each bacterial group (Sarangi et al., 2019). Sanger was the first sequencing technique, but it was limited to the sequencing of one DNA strand at a time, so it was very expensive and time consuming (Costa et Weese, 2019). Several techniques had been proposed during the years and in 2005 a high throughput sequencing technique was developed. This was also called next generation of sequencing (NGS) and relies on a bridge amplification that produced cluster of clonal populations which, thanks to fluorescent labelled deoxyribonucleotide, could be in turn identified (Metzker, 2005; Nie et al., 2019). NGS technology provide an enormous amount of data that needs dedicated computational software tools to be analyzed (Sarangi et al., 2019). They are in continuous evolution and become more and more accurate during the years. Nowadays, the most used are Illumina MiSeq (250-300 base length reads, lower output) and Illumina HiSeq (150 base lengh

reads, higher output) (Sarangi et al., 2019). However, even PCR has some limitations: the detected genome could come from dead bacteria, moreover, due to the extreme sensitivity, it is extremely prone to error and contamination in clinical setting (Adak et Khan, 2019).

Intestinal microbiota composition could be assessed on different specimens, for example tissue biopsies or stool. The first one gives a reliable picture of microbiota composition in association with the histologic appearance of the mucosal layer; however, it requires an endoscopic procedure, which is invasive and require, for small animals, general anaesthesia. Faecal sample, on the other hand, is more representative of the distal gut, but it is very easy to obtain. All the sample should be collected and stored in the same way and, ideally, processed simultaneously by the same laboratory, in order to decrease any batch effect (Sarangi et al., 2019).

Independently on the type of study performed, the evaluation of microbiota usually starts with the assessment of alpha and beta diversity. Alpha diversity is an evaluation of the diversity into a single sample and it is usually represented through rarefaction curves, which express richness and diversity. Microbiota with more diverse communities will have a greater slope at the beginning of the curve, because for every sequence read, a new "microorganism" (Operational Taxonomic Unit) is found. Conversely, beta diversity is referred to the comparison between community or groups (Costa et Weese, 2019).

HUMAN GUT MICROBIOTA

The gastrointestinal tract is divided both functionally and anatomically in several compartment (stomach, small and large intestine) with different microenvironmental conditions that reflects in differences in the microbiota composition (Adak et Khan, 2019). Nevertheless, healthy human gut microbiota is composed by seven main phyla: *Firmicutes, Bacteroidetes, Actinobacteria, Fusobacteria, Proteobacteria, Verrucomicrobia* and *Cyanobacteria* (Bäckhed et al., 2005).

Bacteroidetes and *Firmicutes* together constitute over 90% of the total population (The Human Microbiome Project Consortium, 2012; Marchesi et al., 2016). Gut microbiota explains different roles in the human body. Firstly, a metabolic role, indeed large intestine microbiota, especially bacteria belonging to *Firmicutes* and *Bacteroidetes* phyla, own a large variety of genes encoding for the use of different polysaccharides as a source of energy (e.g. lignin or other oligosaccharides). The fermentation of dietary fibers results in the release of gas, small chain fatty acid (SCFAs), some organic acids (lactate and succinate) and alchools (methanol and ethanol) (Adak et Khan, 2019). Specifically, SCFAs are absorbed in the colon and meet about 10% of caloric demand in people (McNeil, 1984). Bacteria influence also the protein catabolism into peptides and amino-acids. Moreover, it seems that the sources of dietary protein could shape the microbiota composition (e.g. beef meat increase the population of *Bacteroides* and *Clostridia*, while pea and whey proteins enhance the growth of *Bifidobacterium* and *Lactobacillus*, suppressing the pathogenic *Bacteroides fragilis* and *Clostridium perfringens*) (Hentges et al., 1977; Swiatecka et al., 2011). For what concerns the lipid metabolism, a high-fat diet seems to induce microbial dysbiosis (with a decrease in *Bacteroidetes* and an increase in *Firmicutes* and *Proteobacteria*), to enhance adiposity and to sustain a low-grade inflammation in the adipose tissue (Murphy et al., 2015). Although it is known that there is a correlation between gut microbiota and host health status, it is still unclear if dysbiosis could be a cause or an effect (Nie et al., 2019).

Moreover, there is a relationship between gut microbiota and immunity. Indeed, the immune system is not fully developed at birth and appropriate microbial stimulation in early life has a strong effect on the immune system maturation. This has been demonstrated on sterile mice which showed a compromised intestinal and systemic immune system (Round et Mazmanian, 2009; Lee et Mazmanian, 2010; Kamada et al., 2013). In humans, cesarean born seems to have a greater chance to develop immune disorders in early life compared with those born naturally. The same is

reported also for infants treated with antibiotics (Bokulich et al., 2016; Kristensen et Henriksen, 2016). In both these situations, a simpler microbiota composition has been found.

It seems that gut microbiota could influence at different levels the defenses in the gastrointestinal tract. For example, it has been demonstrated that the intestinal mucous layer of two mice colonies with different bacterial composition are quite different too in terms of penetrability to bacteria, indicating that the intestinal microbiota plays a role in the formation of the mucous layer (Jakobsson et al., 2015). Moreover, bacteria gut colonization enhances the mucosa immunity tolerance by inducing the generation of regulatory T cells (Treg) through specific metabolites or structural components. This mechanism is the basis of the symbiotic relationship between bacteria and host (Nie et al., 2019).

MICROBIOTA AND DYSBIOSIS

Due to the relationship with innate immunity and the ability to trigger Treg and T helper (Th) lymphocytes (Gosalbes et a., 2011), it seems that gut microbiota could also play a role in the development of autoimmune diseases.

For example, during Inflammatory Bowel Disease (IBD), especially Crohn's disease, the presence of a complicated genetic background causes an abnormal immune response and a damaged intestinal epithelial barrier. It seems that, both in mice and people, there is an expansion of IgA-coated bacteria with a positive correlation with the colitis-causing capability (Palme et al., 2014; De Souza et al., 2017).

The relationship between the host immune response and the microbiota occurs not only in the intestine, but it seems to extend also to other organs (Nie et al., 2019) (**Figure 2**)

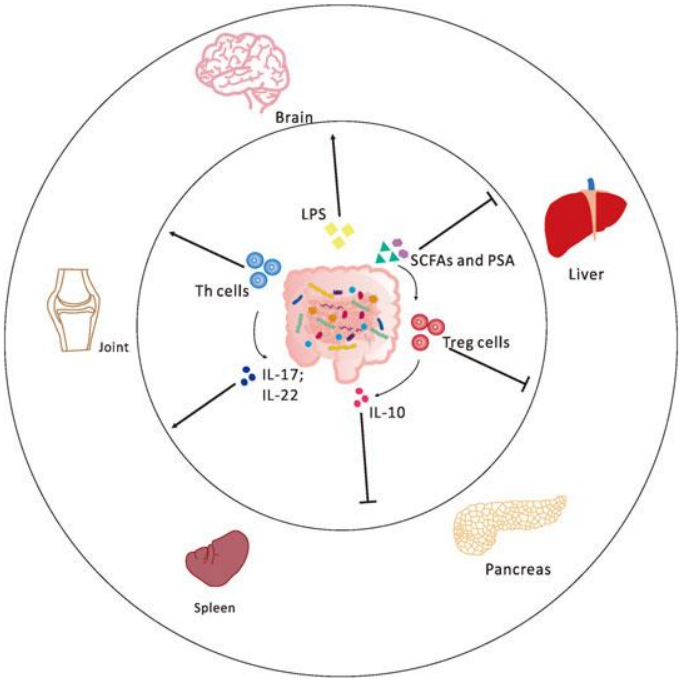

Figure 2. Schematic representation of the systematic immune effect of intestinal microbiota (Nie et al., 2019)

The role of gut microbiota has been studied also in other autoimmune disorder such as type 1 diabetes (Wen et al., 2008), multiple sclerosis, arthritis and in other metabolic disorders in which obesity could be a predisposing factor (cardiovascular disease, type 2 diabetes). In all these studies significant differences in the gut microbiota composition have been found with respect of the healthy control (Nie et al., 2019).

For example, children affected by type 1 diabetes, showed a lower *Firmicutes/Bacteroidetes* ratio, concurrently with an increase in *Prevotella* genera, a higher representation of *Clostridum* and a lower abundance of *Lactobacillus* and *Bifidobacterium* (Murri et al., 2013). It is also possible to observe in microbiota composition some early signs promoting the development of type 1 diabetes, before the actual production of autoantibodies, particularly with the expansion of pro-inflammatory microorganisms (Alkanani et al., 2015).

The relationship between obesity and gut microbiota has been of great interest. Several studies confirmed the presence of an altered microbiota composition of obese subject compared to the lean one (both in mice and in people), with a higher *Firmicutes/Bacteroidetes* ratio and a simpler

microbiota composition (Ley et al., 2006; Turnbaugh et al., 2009). Moreover, with fecal transplantation, it has been demonstrated that the obese phenotype associated with a concurrent peculiar metabolism, could be transferred from an obese mouse to a germ-free mouse, through fecal microbiota transplantation (Nie et al., 2019).

Finally, gut microbiota seems to be also involved in carcinogenesis, particularly in colorectal cancer and liver cancer in people. Indeed, colorectal cancer patients show a higher abundance of *B. fragilis* and other opportunistic pathogens, while butyrate-producer bacteria are reduced (Wang et al., 2012). These changes seem to be present before the precancerous lesions progress to invasive cancer (Dejea et al., 2018).

The use of microbiota analyses in the diagnosis of a disease is still controversial, because it requires a sampling in a specific site (according to the location of the lesion) being stool sampling, even though not invasive, not always providing the best results (Nie et al., 2019). Some changes in the intestinal microbiota could occur before the onset of tissue damage or clinical signs of a disease, but to obtain this information there is a need for longitudinal monitoring of genetically predisposed population (for example as for type 1 diabetes) (Kostic et al., 2015). Moreover, alterations in gut microbiota are not taxonomically uniform and could be influenced by diet or other environmental factors (Nie et al., 2019). So, to date, changes in gut microbiota composition are mostly indicators of an unhealthy state, more than a specific disease (Duvallet et al., 2017).

Several studies investigate the exact relationship between immune response to viral systemic diseases and microbiota. For example, type I interferon (IFN) response is demonstrated to be decreased in antibiotic-treated mice, both locally and at distal non-gastrointestinal site. Specifically, the expression of IFN and other pro-inflammatory cytokines such as TNF-α and IL-6 is decreased, as well as the ability of macrophages and dendritic cells to respond to IFN-γ, lipopolysaccharides (LPS) or different virus infections (Ito et al., 1976; Abt et al., 2012).

Moreover, the decrease in type I IFN response is associated with an increase in virus titers and/or enhanced susceptibility of mice to lethal infection (Winkler et Thackray, 2019). Finally, the commensal microbiota plays a pivotal role in the development of secondary lymphoid tissues and the differentiation, maturation and function of CD4+ and CD8+ T lymphocytes, Treg, Th17 cells and B lymphocytes (Winkler et Thackray, 2019), even though the exact mechanism is still not clear.

The importance of microbiota for the host is highlighted by altered immune responses that have been recorded in several studies carried out on germ-free mice: in the absence of commensal bacteria both systemic and gut-associated lymphoid tissues (GALT) were less developed. Moreover, these subjects had a reduced number of T and B lymphocytes and a dysregulation of Th1/Th2 balance. These studies clearly indicate an important reciprocal regulation between intestinal microbiota and T cell development (Williams et al., 2006; Round et Mazmanian, 2009; Lopes et al., 2016).

GUT MICROBIOTA IN VETERINARY MEDICINE

Due to the increasing interest in gut microbiota in human medicine, the number of veterinary studies focused on the microbiota analyses in pets has dramatically increase.

Firmicutes, Bacteroidetes, Actinobacteria and *Proteobacteria* are the most represented phyla (99% of all the gut microbiota) both in dogs and cats (Handl et al., 2011).

Consistent changing in diet has been demonstrated to play a pivotal role in the microbiota composition of dogs. Specifically, there is a significant difference between commercial diets, enriched in fibers and carbohydrates, and BARF (Biologically Appropriate Raw Food), typically containing more animal proteins and fat: dogs fed with commercial diets usually show a lower

abundance of *Bacteroidetes* and *Fusobacteria* and a higher proportion of *Firmicutes* (Schmidt et al., 2018; Alessandri et al., 2019).

Most of the veterinary literature investigates variations of gut microbiota in association with dysbiosis (with different underlying causes). The word dysbiosis is referred to an alteration in the composition and/or in richness of the intestinal microbiota (Suchodolski, 2016) and it has been associated with various gastrointestinal disorders (e.g. acute diarrhea, IBD) (Suchodolski et al., 2012a; Zheng et al., 2018) but also with an inflammatory status in general. More specifically, it seems that during IBD, both dogs and people showed a decrease in relative abundance of *Fusobacteria*; while during episodes of acute diarrhea there is an increase in *Proteobacteria* and a decrease in *Bacteroidetes* (specifically those members producing SCFA, important for intestinal health) and *Firmicutes* both in small intestine and fecal sample. This demonstrate that despite the differences in the microbial composition in the different GI tract, dysbiosis in the small intestine could still be identified in fecal samples that actually were considered as an appropriate reflection of the distal intestinal tract (Suchodolski et al., 2012b).

FELINE INFECTIOUS PERITONITIS

EPIDEMIOLOGY AND ETIOLOGY

Feline infectious peritonitis is a severe and usually fatal disease that affects mostly young cats, especially those that live in a multi-cat environment. It has been observed firstly in the late 1950s and the causative agent is a Coronavirus (Pedersen, 2009). It is an enveloped, positive, single strand RNA virus and its genome consist of 11 open reading frames (ORFs) encoding for both structural and non-structural proteins, together with some accessory proteins. The first group include four protein: spike (S), nucleocapsid (N), membrane (M) and envelope (E), while the latter is related with viral replicase (Kipar et Meli, 2014). Among all these proteins, S protein, arranged in peplomers, plays a pivotal role in entering target host cells (Bosch et al., 2003). Due to its RNA genome, as other Coronaviruses, it is prone to high error rates during its replication, with the consequent formation of different quasispecies. Feline Coronavirus (FCoV) exists in two antigenically different serotype: type I, ubiquitous and difficult to grow in cell culture, and type II, which is a recombination of type I with canine Coronavirus (CCoV), mostly diffused in Asia and that grow easily in cell cultures (Pedersen et al., 1984; Herrewegh et al., 1998). Moreover, both serotypes could occur in two different pathotypes or biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV), that can not be distinguished each other serologically, nor morphologically (Kipar and Meli, 2014). Even though both of them could spread systemically through monocyte-associated viremia, only FIPV is capable to cause FIP (Kipar et al., 2006a).

Despite the extremely high prevalence of FCoV infection (over 90% in multi-cat environments such as shelters), FIP morbidity is low (about 5%) (Pedersen, 2009; Drechsler et al., 2011). FIP affect most frequently young cats (younger than 2 years). Some purebreds (Abyssinian, Bengals,

Birmans, Himalayans, Ragdoll and Rex cats) and male intact cats seems to have a higher predisposition (Pesteanu-Somogyi et al., 2006) to the infection. After a fecal-oral transmission, FCoV firstly replicates inside the enterocytes, so cats could become shedders for the virus for a long time (persistent carriers represented by 13% of cats), or be re-infected with the same or a different strain of the virus itself, even in absence of any clinical signs (Addie et Jarrett, 2001; Kipar et Meli, 2014).

PATHOGENESIS

Even if not completely clear, it seems that FIP relies on the presence of a systemic infection with the virulent pathotype of FCoV (FIPV) associated with both viral replication inside blood monocyte/macrophages and an activation of these cells. Cats show individual differences in monocyte susceptibility to infection, highlighting that host factors play a role in the infection too (Kipar et Meli, 2014). Even though both FECV and FIPV could replicate inside blood monocytes, the viral load is usually higher during FIPV infection (Kipar et al., 2006a). It has been suggested that blood monocytes could be the site in which FECV to FIPV mutation occurs, allowing a viral adaptation to replication in monocyte/macrophages. The presence of FIPV infection induce a generalized monocytes/macrophages activation, that, in turn, produce several pro inflammatory cytokines (TNFα and IL-1β**),** adhesion molecules (CD18) and enzymes (metalloproteinase-9) that allows the interaction with endothelial cells, causing a granulomatous phlebitis, which is the typical lesion of FIP (Kipar et Meli, 2014). Several attempts had been made to investigate the role of host immunity in the development of FIP. FCoV infection, regardless of the pathotype, starts with a humoral response and the consequent increases in the antibody titer against the virus (Vogel et al., 2010). For what concerns specifically FIPV infection, it is thought that the cell mediated immunity is protective, while antibodies, even in presence of high titers, are not enough to

eliminate the virus (Kipar et Meli, 2014). Indeed, through a still not clear mechanism, FIPV inhibits the complement-mediated lysis of infected monocyte, even in presence of viral antigen on their surface (Cornelissen et al., 2009). For what concerns lymphocyte populations, while healthy FCoV-infected cats show a lymphoid follicular hyperplasia, FIP affected cats are usually lymphopenic both in blood and in lymphoid organs, with a decrease in circulating CD4+ and CD8+ T-cells and a depletion in natural killer cells, that causes a reduced ability to contain the inflammatory process (de Groot-Mijnes et al., 2005; Vermeulen et al., 2013). An experimental study evaluated the role of cytokines in FIP infection and results show that IL-10, which decreases macrophage activation and increases cytotoxic immune response, limits the viral infection. On the other hand, a lack of IL-12 decreases the efficacy of a protective immunity, allowing monocyte activation and the development of the clinical disease (Kipar et al., 2006b). The immunopathogenic mechanism underlying the development of FIP it is thought to be an immune-complexes mediated type III hypersensitivity, even if FIP does not show all the typical features of an immune mediated vasculitis (such as the involvement of arteries and the main presence of neutrophils) (Pedersen et Boyle, 1980; Kipar et al., 2005).

The immune response has a pivotal role not only in the protection against FIP, but also in the development of different clinical presentations. Indeed, a strong humoral response lead to the effusive form of the disease, due to the huge amount of circulating immune complexes, while a weak non-protective cell mediated immunity is usually associated with a non-effusive presentation (Pedersen, 2009).

Finally, environmental factors and particularly stressors, such as overcrowding or surgery, seems to play a role as a trigger for the disease (Tasker, 2018).

CLINICAL SIGNS AND PATHOLOGICAL FINDINGS

Clinical features of FIP could be very variable due to both the form (effusive or not) and to the target organs, but all of them are caused by a vasculopathy (Tasker, 2018). In most of the cases, there is an overlap between effusive (wet) and non-effusive (dry) forms (Pedersen, 2009; Kipar et Meli, 2014). Fever unresponsive to treatments, anorexia, weight loss and jaundice are some of the most common, even if very unspecific, clinical signs (Addie et al., 2009). In the wet form, clinical signs are strictly dependent on the effusion site: abdominal enlargement, dyspnoea, lethargy or, rarely, scrotal enlargement (with effusions respectively in abdomen, thorax, pericardium or scrotum). Generally, the effusive form has a rapid and fatal progression. Pyogranulomatous lesions, typical of the dry form, could affect different organs leading possibly to: nephromegaly, diarrhoea or vomiting, lymph node enlargement, uveitis or neurological signs (Addie et al, 2009). The clinical course of the non-effusive form could last for several weeks or months (Pedersen, 2009).

During the necropsy, the main feature of FIP is a fibrinous-granulomatous serositis. Sometimes granulomas can be so small that only histological examination could identify them.

DIAGNOSIS

Obtaining a definitive diagnosis of FIP is quite a challenge and could be achieved only through the evidence of the coronavirus highlighted with immunohistochemistry (IHC), inside the typical histopathological lesions on biopsies (Pedersen 2014; Kipar et Meli, 2014; Tasker 2018).

However, in the absence of a definitive diagnosis, it is still possible to obtain a high index of suspicion of FIP. As already state above, signalment and clinical observation is the starting point of a FIP suspicion.

Haematology is usually unspecific and the most common findings are lymphopenia (49.5 – 77%) with neutrophilia (39 – 57%) and a mild normocytic to microcytic normochromic anaemia (37 – 54%) (Riemer et al., 2016). However, it should be kept in mind that some atypical presentation may occur (for example in association with immune-mediated haemolytic anaemia) (Norris et al., 2005).

Clinical biochemistry may show some abnormalities that could support FIP suspicion even if none of them is sufficiently specific for the disease. For example, hyperglobulinemia (89% of cases) and hypoalbuminemia (near to 65%), together with an overall hyperproteinaemia (which however not always occurs) (Riemer et al., 2016). To evaluate the globulin classes, a serum protein electrophoresis is needed: usually there is an increase in α2 and γ-globulins, even though in the last few years the typical pattern became less common (Stranieri et al., 2017a). In literature is reported that an albumin:globulin ratio lower than 0.4 is very suggestive of FIP (Tasker et al., 2018). Other common biochemical findings are represented by hyperbilirubinemia (21 - 36%), without a significant increase of hepatic enzymes.

Alpha-1-acid glycoprotein (AGP) is a positive acute phase protein which significantly increases in cats affect by FIP. If values higher than 0.5 mg/mL are not specific for FIP, an AGP higher than 1.5 mg/mL is quite accurate for the disease (Duthie et al., 1997; Giori et al., 2011; Hazuchova et al., 2017). Depending on pre-test probability, higher value could be needed to "confirm" the disease, especially in cats with a low pre-test probability (Paltrinieri et al., 2007). Two years ago, the only validated kit for determining the serum concentration of AGP was retired from commerce. The role of other feline acute phase protein as diagnostic marker for FIP, such as serum amyloid A, has been investigated, but resulted non sufficiently specific. For this reason, new biomarkers such as paraoxonase-1 have been investigated for diagnostic purposes (see below).

For what concerns serology, even though it seems that FIP affected cats usually had higher antibody anti-FCoV titers, there is still some overlapping with non-affected cats. So, serology is of limited use in clinical practice for the diagnosis of FIP. Moreover, healthy cats living in multi-cat environment may have high antibody titers and, on the other hand, 10% of FIP affected cats are seronegative (Addie et al., 2009) due to the presence of immune-complexes that lower the concentration of circulating antibodies. These results further decrease its usefulness.

In the wet form of the disease, the analyses of effusion is very helpful. FIP effusion is usually yellow, clear, viscous and protein rich ([total protein] > 33 g/L). Albumin to globulin ratio, electrophoretic profile and AGP concentration are similar to those of the serum. The cellular component is generally, but not always, scarce and represented mainly by macrophages, non-degenerated neutrophils and a low number of lymphocytes. Cytological examination highlights the presence of a typical eosinophilic granular proteinaceous background (Tasker, 2018). Some test could identify the coronavirus inside the sample effusion, such as PCR or immunocytochemistry, even though it is not possible to differentiate between enteric and FIP virus (Felten et al., 2017a, 2017b). Finally, on effusion samples, the Rivalta's test could be performed. This is a rapid and inexpensive test which is not specific for FIP but resulted generally positive during this disease. A drop of effusion is added to a solution of distilled water and acetic acid: a positive result is obtained when the drop does not dissolve inside the solution, but, conversely, could be clearly identify as a firm clot, and it is helpful to differentiate a transudate from an exudate (Fischer et al., 2013). Particularly in FIP effusions, this is due to the high concentration of high molecular weight proteins which clot in contact with the acetic acid. Rivalta's test provides a qualitative finding, but a quantitative result, based on the same principle, could be obtained evaluating the Δ total nucleated cells (ΔTNC) using the automated haematology analyser Sysmex XT-2000iV. Specifically, a value of ΔTNC higher than 1.7 strongly enhances the probability to

diagnose FIP and the specificity maximizes with values higher than 2.5 (Giordano et al., 2015; Stranieri et al., 2017b).

In cats presenting neurological signs, an evaluation of cerebrospinal fluid (CSF) could reveal an increase in the protein content (>0.3 g/L from cisternal puncture samples and >0.46 from lumbar sample) and/or in cell count (8x106 cells/L), even though sometimes it could be unremarkable. When present, cells are mainly represented by neutrophils and macrophages. Nevertheless, CSF may be useful for PCR evaluation, with some limitation here below reported (Crawford et al., 2017).

RT-PCR is used to identify FCoV but it is not specific for FIPV (neither are the PCR targeting the specific mutation of the Spike gene, since it is just a marker of systemic diffusion of the coronavirus and not specific for the identification of FIPV). PCR could be performed on any kind of specimens, including blood, tissues, effusions, CSF or aqueous humour. Tissue samples should not be stored in formalin, since it degrades RNA, decreasing PCR sensitivity (Tasker, 2018).

On tissue biopsies it is possible to perform histopathology in order to find the typical lesions. Moreover, on the same sample, it is advisable to perform an immunostaining (immunohistochemistry – IHC) using anti-FCoV antibodies, in order to confirm the diagnosis (Tasker, 2018). Immunostaining could be performed, as immunocytochemistry, also on effusions (sensitivity 57 -100%) or CSF (Ives et al., 2013; Felten et al., 2017a).

Paraoxonase-1

Paraoxonase-1 (PON-1) belongs to a group of enzymes (paraoxonases) capable to metabolize the organophosphate paraoxon. PON-1 is a glycoprotein, synthetized by liver, that circulates in the blood stream associated with HDL particles, specifically the apolipoprotein-1 (Furlong et al., 2016; Shunmoogam et al., 2018). During inflammation, especially when associated with a marked

oxidative stress, the HDL particles loses the apolipoprotein-1 and many associated enzymes, including PON-1, that are replaced by serum amyloid A and ceruloplasmin (Khovidhunkit et al., 2004). Moreover, liver production of PON-1 during inflammation is inhibited. For this reasons, PON-1 acts as a negative acute phase reactant, with PON-1 activity decreasing during strong inflammation in people and in several animal species (Giordano et al., 2013; Rossi et al., 2013). Cats seem to be more prone than other species to the oxidative stress damage. A recent work has demonstrated the intimate relationship between inflammation and oxidative stress during FIP (Tecles et al., 2015). Further studies are required to investigate the role of PON-1 as a biomarker for FIP diagnosis.

CANINE LEISHMANIASIS

EPIDEMIOLOGY AND ETIOLOGY

Canine leishmaniasis is a systemic infectious zoonotic disease of dogs. It is caused by a protozoan named *Leishmania* and the most common species is *Leishmania infantum* (or, in the New World, *Leishmania chagasi)* (Paltrinieri et al., 2016). The canine species represents the definitive host for the parasite. It is transmitted by a phlebotomine sand fly, which acts as a vector, even though the transmission could be also transplacentar, transfusional and sexual (with a marginal epidemiological role) (Saridomichelakis, 2009). The disease distribution worldwide is influenced by the presence of this particular vector (Maroli et al., 2013). The parasite could be present in two different forms: amastigote (without a proper flagellum) and promastigote (with the flagellum). Specifically, females of sand fly ingest the amastigote form during the blood meal on infected dogs. The parasite repeatedly divides and the promastigotes mobile forms are subsequently inoculated in the host skin during the following blood meal. Here, the promastigotes are phagocytized by macrophages and then turn again into the amastigote form. The shift from amastigote to promastigote takes from 4 to 20 days, depending on *Leishmania* and vector species (Saridomichelakis, 2009). The vector is ubiquitous in most of the tropical, subtropical and Mediterranean regions and several species are involved in *Leishmania* transmission (in Italy the most common one is *Phlebotomus perniciosus*) (Ready, 2013). During the last year, due to an increase in the climate change the area of vector diffusion is changing (Ready, 2008). Moreover, the spread of the disease is enhanced by the animal movements and this also explain the reported cases of canine leishmaniasis in United Kingdom or United States, which are still not considered as endemic (Di Muccio et al., 2015).

PATHOGENESIS

The development of the disease depends both on the protozoan features and the host immunity response (Paltrinieri et al., 2016). During the blood meal, the sandfly inoculates the promastigotes into the skin, where, due to the small vessel rupture, there is a draw of macrophages, neutrophils, dendritic cells and fibroblasts (de Menezes et al., 2016). Neutrophils act with their proteolytic enzymes and oxygen reactive species (ROS) and even though most of the promastigotes survive, the presence of neutrophils and their cytokine secretions, helps in recruiting more specialized immune cells such as macrophages, lymphocytes or dendritic cells. Parasites are then phagocytized into a parasitophorous vacuole by macrophages, where they can resist to the phagolysosomal digestion due to a defective production of nitric oxide (Brandonisio et al., 1996; Vouldoukis et al., 1996) and survive inside these cells, spreading the infection (Engwerda et al., 2004). Depending on several factors, as reminded above, parasites may be eliminated directly in the skin (self-limited infection), may remain located in the skin and lymph nodes (asymptomatic infection) or they could spread systemically (both symptomatic or asymptomatic infection) depending on the resistance of the host (Saridomichelakis, 2014).

The macrophages have also a pivotal role in presenting the parasite antigens to T cells through the major histocompatibility complex molecules. The activation of a cell-mediated immunity is mandatory for an efficient defence against Leishmania infection (Baneth, 2005). For this reason, T cell subsets have a crucial role in the host susceptibility (Saridomichelakis, 2009). In most of the studies CD4+ T helper lymphocytes in peripheral blood are decreased in susceptible dogs, as well as CD8+ T cells (even if there are some exception) (Pinelli et al., 1994; Pinelli et al., 1995; Reis et al., 2006). T helper cells could be classified into two classes depending upon the specific cytokines they secrete in response to antigenic stimulation. Th1 cells primarily produce interferon (IFN)-γ and interleukin (IL)-2, whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. For a long time, it has been thought that Th1 response would be protective, while Th2 activation is

correlated with susceptibility (Locksley et al, 1987) to canine leishmaniasis. However, this is not completely true, since it seems that both Th1 and Th2 responses are present in an infected dog, even though the former seems to be predominant in resistant dogs (Saridomichelakis, 2014). For what concerns the cytokine profiles, IL-2 and IFN-γ have been linked with resistance and Th-1 profile (even though some results are controversial (Manna et al., 2006; Correa et al., 2007). On the other hand, IL-4 and IL-6 seem to be associated with a Th-2 profile (Santos-Gomes et al., 2002; de Lima et al., 2007). The total number of B lymphocyte in peripheral blood of susceptible dogs may be increased or normal due to the migration into target organs (Saridomichelakis, 2014). The activation of B lymphocytes is not protective against the parasite and results in an overproduction of immunoglobulins, generally IgG (Marinez-Moreno et al., 1993; Almeida et al., 2015; Cardoso et al., 2007). The antibody titer is usually positively associated with the presence and severity of clinical signs (da Costa et al., 2007). Indeed, the presence of antibodies causes the formation of immune-complexes that deposit in different sites and, in turn, enhance the inflammation with the complement activation, which is the real responsible for the clinical signs (Lopez et al., 1996).

CLINICAL SIGNS AND PATHOLOGICAL FINDINGS

Clinical signs of canine leishmaniasis could be quite heterogeneous based on the targeted organs. It is a chronic disease and the most common clinical features are cutaneous lesion, lymph nodes enlargement, anorexia, muscular atrophy, polyuria, polydipsia, ocular lesion, epistaxis, onycogryphosis, lameness, diarrhoea and vomiting (Solano-Gallego 2009; Koutinas et Koutinas, 2014). However, as previously reminded, dogs infected may also present as asymptomatic.

Skin lesions are very common and could be observed alone or together with other clinical signs. Moreover, they could present in a different range of patterns: most frequently there is an exfoliative dermatitis with the presence of dry white scales and dry coat, associated with periocular, ear or

nasal planum alopecia and sometimes erythema and hypopigmentation (Koutinas et Koutinas, 2014). Histologically, it is possible to observe hyperkeratosis and follicular keratosis. On the bony prominences and foot pads it is not rare to observe an ulcerative dermatitis secondary to the local inflammatory process associated with vasculitis. Uncommon is the nodular, pustular or papular dermatitis. All these findings could be associated with secondary bacterial infection (piodermitis) mostly caused by *Staphylococcus* (Ciaramella et al., 1997; Saridomichelakis et Koutinas, 2014). Ocular lesions, usually signs of the systemic diffusion of the parasites, include several different presentations such as conjunctivitis, kerato- conjunctivitis sicca, blepharitis and uveitis (Di Pietro et al., 2016). Enlarged lymph nodes are not warm or painful and histologically it is possible to see a reactive lymphoid hyperplasia with macrophages containing amastigotes (Ciaramella et al., 1997; Mylonakis et al., 2005). Polyuria and polydipsia are generally correlated with a severe renal damage, generally a glomerulonephritis due to immune-complexes deposition that could evolve in a tubular damage (Zatelli et al., 2003). The renal damage could be associated with proteinuria and since this is the first cause of death in dogs affected by leishmaniasis, it is mandatory to perform a correct staging according to IRIS guidelines (International Renal Interest Society Canine Guidelines, 2013). The musculo-skeletal system could be affected both for the deposition of immune-complexes and for the catabolic nature of this disease. Temporal muscle could be atrophic and there could also appear lethargy, asthenia and lameness. Common are also the polyarthritis that could be erosive or not and should be investigated with imaging (Ciaramella et al., 1997).

The typical histopathological pattern is the presence of a granulomatous-pyogranulomatous inflammation associated with vasculitis. (Paltrinieri et al., 2016)

DIAGNOSIS

There are several clinico-pathological abnormalities that could support the suspicion for *Leishmania* infection (Paltrinieri et al., 2016). Alterations in the complete blood cell count are usually unspecific, but the most common findings are anaemia and neutrophilia (that could be marked in case of secondary bacterial infection) (Nicolato et al., 2013), sometimes associated with lymphopenia or lymphocytosis. Anaemia is typically mild to moderate normocytic normochromic, related to the chronicity of the disease, even though there could be other underlying mechanisms such as, a reduced erythropoietin production due to renal failure, or a haemolytic component (Ciaramella et al., 1997; Nicolato et al., 2013). Finally, mild to moderate thrombocytopenia is quite frequent in dogs with leishmaniasis, even if in case of marked thrombocytopenia is advisable to exclude the presence of other concurrent vector-borne diseases (Paltrinieri et al., 2016). The finding od amastigotes in the peripheral blood smear is very uncommon (<0.5%) (Giudice et Passantino, 2011). For what concern the serum biochemical evaluation, since the kidney is one of the most frequently affected organs, it is very common to find increased creatinine and urea (or BUN), as well as phosphorous, while serum albumin, in case of a severe protein-losing nephropathy, could be lower than the range of normality. Moreover, albumin could decrease also due to its role as a negative acute phase protein, which tend to decrease during inflammation. Biomarkers of hepatobiliary and pancreatic dysfunction could be increased, as well as CK (creatine kinase) and LDH (lactate dehydrogenase) enzymes related with the musculoskeletal system (Paltrinieri et al., 2016). Urinalysis is necessary to complete the renal function evaluation, as suggested by the IRIS guidelines (IRIS Canine Guidelines, 2013). In case of loss of renal function, urine specific gravity (USG) tends to decrease; the presence of glucose in urine without concurrent hyperglycaemia in serum, it is considered as a marker of tubular damage. Finally, it is necessary to assess the presence of proteinuria, since it is considered as a risk factor for the progression of nephropathy, possibly using a urine creatinine-to-protein ratio (UPC), more than just the dipstick

estimate, because UPC allows a quantification of the damage and it is also useful for the therapy monitoring (Jacob et al., 2005). Using SDS-page or SDS-age (Sodium dodecyl sulphate - polyacrylamide and agarose gel electrophoresis, respectively) it is also possible to evaluate the weight of proteins in the ultrafiltrate in order to state if the damage is mainly glomerular, tubular, or if it is a mixed form (Schultze et Jensen, 1998). Serum total proteins could be normal or increased, but generally there is a decrease in albumin, as already mentioned previously, associated with an increase in the concentration of globulins, resulting in a decreased albumin/globulin ratio. A serum protein electrophoresis is needed to investigate the different globulin fractions: generally, an increase in α2 and γ-globulins is observed, with a polyclonal arrangement. In these fractions migrate both acute phase proteins (α2-globulins) and immunoglobulin (γ-globulins). Occasionally, a peak in the β2 region could be observed, due to the presence of IgM and some acute phase proteins. The *Leishmania* induced gammopathy is usually polyclonal, although, rarely, monoclonal peaks have been described (Font et al., 1994). Finally, positive acute phase proteins (APPs) such as C reactive protein (CRP), haptoglobin (Hp), ceruloplasmin (Cp), serum amyloid A (SAA) and ferritin have been shown to increase in diseased dogs (Martinez-Subiela et al., 2002). On the other hand, albumin, transferrin and paraoxonase-1 (PON-1) decrease, since they are negative acute phase proteins (Ibba et al., 2015). Modifications in APPs concentration is not diagnostic but could be very useful as a monitoring tool for treatments and as prognostic factor (Rossi et al., 2014a).

To confirm the diagnosis of leishmaniasis, both direct and indirect tests could be used (Paltrinieri et al., 2016). Direct tests are those that clearly identify the parasite, such as PCR and cytology. The most used PCR methods are conventional, nested and real-time (Reis et al., 2013). The last one is the only one that could give some quantitative information and, since it run in a closed system, it is less prone to contamination. Sensitivity and specificity may change according to the method and the target DNA sequence. PCR could be performed on any tissue or fluid; it could be run also on

peripheral blood and even though the sensitivity is lower, it is a less invasive sampling than tissue biopsies (Almeida et al., 2013). It should not be forgot that the presence of the parasite is related only with an infection and not with the clinical signs of the disease. Hence, the diagnosis should be supported by the presence of clinical signs or clinico-pathological abnormalities. Cytology is a simple and less expensive analyses that should always be performed at least in case of skin lesions, lymph nodes enlargement or the atypical presence of nodular lesions. The same evaluation could be performed also on fluid specimens such as joint fluids, effusions, CSF or on bone marrow smears. The detection of the amastigotes, both inside the macrophages cytoplasm or free on the background, associated with the clinical signs, is an evidence of the disease. The typical cytological pattern associated is characterized by macrophages and neutrophils, with a lower degree of lymphocytes and plasma cells (Paltrinieri et al., 2016).

On the other hand, some indirect tests are available, highlighting the contact between the host and the parasite thanks to the presence of specific anti-*Leishmania* antibodies (Paltrinieri et al., 2016). The most commonly used techniques are based on immunofluorescence (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunochromatography (ICT) (Solano-Gallego et al., 2014). The last one is the base for any rapid assay which are diffused for the in-clinic use, but it and provide only a qualitative result. Nevertheless, the specificity of rapid test is usually quite good, whereas the sensitivity is usually low (30-70%). The other assays give, on the contrary, a quantitative information. IFAT is considered as the reference method for anti-*Leishmania* serology in dogs and it has both sensitivity and specificity near to 100%, except for analyses done in some area of the new world where it may cross-react with *Trypanosoma cruzii*, giving some false positive results. However, IFAT is operator-dependent, so it is advisable to always monitor the patient in the same laboratory. ELISA, on the other hand, is easier to standardize since the results are read by a spectrophotometer and it is characterized by a good specificity and sensitivity too. Antibody titers are considered as high when they are four-fold higher than the laboratory threshold.

A lower value should be interpreted carefully in light of the clinical signs or of the presence of a possible vaccination that could sometimes interfere with the test. It should be remembered that the median time for seroconversion is around 5 months in natural conditions (Solano-Gallego et al., 2014; Paltrinieri et al., 2016).

Based on clinical signs and clinic-pathological data, dogs should be classified as (Paltrinieri et al., 2016):

- Exposed: clinically unremarkable, low-titer positive serology, but negative by PCR and/or cytology
- Infected: clinically unremarkable, with normal haemato-chemistry, but a positive PCR and/or cytology
- Sick: infected with clinical signs or clinic-pathological abnormalities
- Severely sick: with severe chronic condition such as proteinuric nephropathy, chronic renal failure or other concurrent diseases.

AIMS OF THE PhD PROJECT

AIMS OF THE PhD PROJECT

The general aim of this thesis was to investigate the presence of possible correlation between gut microbiota and the presence of systemic infectious disease in dog and cats. Since it is known that microbiota could influence the development and shape the immunity system, in some cases the immunity status was evaluated too. The development of the diseases investigated along this project (feline infectious peritonitis and canine leishmaniasis in cats and dogs respectively) is known to be strictly correlated with the type of immune response mounted by the host, as described in the sections above. Moreover, for what concerns FIP, standing the difficulties in diagnosing the disease *in vivo* the performances of a new biomarker have been evaluated, as well as the correlation between different diagnostic techniques on tissues, in order to ease the enrolment and the appropriate grouping of cats in future studies. Specific aims and steps of the PhD project are listed here below.

SPECIFIC AIMS

I. To compare the fecal microbiota composition, evaluated through next generation sequencing (NGS), of FCoV negative clinically healthy cats with that of FCoV positive clinically healthy cats and of cats with FIP. It is known that gut microbiota could shape the immune response and the development of feline infectious peritonitis has for sure a huge component in the host immune response. The availability of information on this topic would give new insight on the microbiota-immunity relationship and possibly on the mechanisms of susceptibility or resistance to the disease.

II. To determine the reference intervals of the paraoxonase-1 (PON-1) activity on a healthy feline population using a paraoxon-based method. This method was already validated in cats and

the presence of an established reference interval would allow any further hypothesis on its role as a biomarker for specific diseases.

III. To evaluate the potential of PON-1 as a diagnostic biomarker of FIP in a group of cats showing clinical signs consistent with the disease and enrolled regardless of the final diagnosis. Since oxidative stress has a pivotal role for the development of FIPV infection, so lower values of PON-1 activity in serum was expected in FIP affected cats. Since *in vivo* diagnosis of FIP is still quite challenging, the availability of a marker supporting the clinical suspicion could be useful for future researches on this disease as well as for clinical purposes.

IV. To evaluate the agreement among histology, immunohistochemistry and RT-PCR on different organs for the diagnosis of feline infectious peritonitis. The use of RT-PCR instead of histology and immunohistochemistry on biopsies would ease the organs investigation for the presence of the virus and possibly the *in vivo* diagnosis of this disease. As for PON-1, the availability of reliable *in vivo* assays will be useful for both clinical and research settings.

V. To compare the fecal microbiota composition evaluated through next generation sequencing (NGS) of healthy dogs with that of clinically healthy dogs exposed to *Leishmania* and of dogs with clinical leishmaniasis. The presence of clinical signs of leishmaniasis depends mostly on the host immune response. Microbiota seems to have a role in shaping the immune system. Results of this study would provide new insights on the microbiota-immunity relationship and eventually on the possible susceptibility to clinical leishmaniasis in dogs.

VI. To evaluate the leukocyte population, using flow cytometry, in peripheral blood of healthy dogs, clinically healthy dogs exposed to *Leishmania* and dogs with clinical leishmaniasis. Moreover, to correlate the results with the fecal microbiota composition evaluated through next generation sequencing (NGS) in order to evaluate the presence of possible significant association between microbiota composition and the immunity asset. The concurrent evaluation of immunity

and microbiota could highlight relationship that would help in better define possible aspects of susceptibility for clinical leishmaniasis.

DESCRIPTION OF THE STUDIES (I – VI)

I. FELINE GUT MICROBIOTA COMPOSITION IN ASSOCIATION WITH FELINE CORONAVIRUS INFECTION

MATERIALS AND METHODS

Animals/Caseload

Twenty-one cats, submitted for clinical examinations to both the Veterinary Teaching Hospital of our University and to private practitioners, were recruited.

The inclusion criteria applied after the screening analyses, in order to reduce possible confounding factors, were: (1) Private owned cats living indoor. (2) No administration of antibiotics in the previous sixty days. (3) Negative serology for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV). (4) Age ≤2.5 years. (5) Availability of fresh feces (6) Absence of clinical signs (for inclusion in the groups of clinically healthy FCoV positive or negative cats) or presence of clinical signs such as effusions or neurological signs and clinicopathological abnormalities consistent with FIP (Stranieri et al., 2018), followed by a post-mortem confirmation of diagnosis (for the inclusion in FIP group). (7) Fed with a similar diet based on dry and canned commercial food.

Sample collection

At admission, 2 mL of whole blood was collected from each cat by venipuncture of the jugular vein: 1 mL was transferred into an EDTA tube and 1 mL into a plain tube (Venoject, Terumo Italia Srl, Rome, Italy). When effusion was present, it was sampled by ultrasound-guided drainage and stored in EDTA tubes. According to the standard operating procedures of our laboratory whole

blood and effusion were analyzed within 12-18 hours from the sampling. From each animal, a fresh fecal sample (at least 15 g) was collected and immediately stored, frozen at -20°.

The FIP cats, due to the severe course of the disease, were humanly euthanized, after owner's consent. A complete necropsy followed by routine histology and immunohistochemistry and RT-PCR for Feline Coronavirus was performed. Immunohistochemistry and RT-PCR were performed on tissue biopsies from liver, spleen, mesenteric lymph node, intestine, kidney and lung and, in cats with neurological signs also on brain and cerebellum, in order to confirm the clinical suspicion of FIP. In one case, since the owner declined the necropsy, the diagnosis of FIP was confirmed using immunocytochemistry on the effusion sample. The samples had been collected for diagnostic purposes by referring veterinarians according to standard veterinary procedures. Therefore, according to the decision of the Ethical Committee of the University of Milan, residual aliquots of samples or tissues collected under informed consent of the owners can be used for research purposes without any additional formal request of authorization to the Ethical Committee (EC decision 29 Oct 2012, renewed with the protocol n° 02-2016).

Screening Analyses

The purpose of clinicopathological tests was to confirm the absence of subclinical changes in healthy cats and to evaluate the clinical status of those affected by FIP. A complete blood cell count was performed on whole blood in EDTA using the Sysmex XT-2000iV hematology laser analyzer (Sysmex Corporation, Kobe, Japan), along with a blood smear evaluation. Serum samples were obtained by centrifugation (3750 rpm x 5min) of blood collected in plain tubes and used to run a panel of routine biochemical tests (including urea, creatinine, total bilirubin, total protein, albumin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase) with an automated spectrophotometer (RX Daytona, Randox Laboratories, Crumlin, United Kingdom),

using reagents provided by the manufacturer. A rapid serological test for FIV and FeLV was performed on serum with a lateral flow ELISA kit (SNAP FIV/FeLV Combo Test, IDEXX Veterinary Diagnostic, Hoofddorp, Netherlands).

Additional laboratory tests were performed for cats suspected of FIP, to further support the clinical diagnosis of the disease (Pedersen, 2014a; Tasker, 2018). Specifically, serum protein electrophoresis was performed on agarose gel using the automated analyzer Hydrasis (Sebia Italia, Bagno a Ripoli, Florence, Italy) and the specific manufacturer's reagents (Hydragel 7/15 β1–β2, Sebia Italia), as previously reported (Stranieri et al., 2017). Based on the total protein concentration, measured with the biuret method, and on the percentages of the electrophoretic fractions, the concentration (g/L) of each electrophoretic fraction was calculated. Moreover, feline α-1-acid-glycoprotein (AGP) was measured on serum using a radial immunodiffusion (SRID) kit (Tridelta Development Ltd, Maynooth, Kildare, Ireland), following the manufacturer's instructions (Duthie et al., 1997; Paltrinieri et al., 2007; Hazuchova et al., 2017). When present, effusion was analyzed by measuring total protein concentration with a refractometer, and cell counts using the Sysmex XT-2000iV hematology laser analyzer mentioned above. A cytologic evaluation was also performed. Particular attention was given to ΔTNCC due to its high diagnostic accuracy for FIP-related effusion (Giordano et al., 2015; Stranieri et al., 2017b). In two cases, due to the severe health conditions, it was not possible to perform an adequate blood sample for the screening analyses. However, a complete necropsy followed by tissue biopsies was performed in order to confirm the suspicion for FIP.

For molecular testing, each frozen fecal specimen was diluted in 400 μL of PBS. The mixture was vortexed, incubated at 40 °C for 5 minutes and then centrifuged (5500 rpm × 4 min). The supernatant was transferred into a new sterile Eppendorf tube, incubated at 95 °C for 5 minutes and then centrifuged (11000 rpm x 1 min). RNA was extracted from the supernatant using a kit for viral RNA extraction (NucleoSpin® RNA Virus, Macherey-Nagel, Düren, Germany),

following manifacturer's instructions. Amplification of a 177bp fragment of the conserved 3' untranslated region (3' UTR) using a nested RT-PCR was performed as previously described (Herrewegh et al., 1995). FCoV RNA was used as positive control and RNase-free water as negative control. RT-PCR amplicons were visualized under an ultraviolet transilluminator following electrophoresis through a 2% agarose, ethidium bromide stained gel. Based on the presence or absence of amplicons, samples were considered as positive or negative for FCoV, respectively.

For immunohistochemistry, the biopsy samples were fixed in 10% isosmotic formalin. The samples were processed as already described in other studies (Zini et al., 2018). The primary antibody, manually added by an operator, was a mice monoclonal antibody against FCoV (clone FIPV3-70 Serotec, Oxfork UK). The immunocytochemistry was performed only on one sample using a similar procedure, except for the antigen unmasking step.

Microbiota Analyses

Microbiota analyses were outsourced to an external laboratory. Investigation of microbial communities (Metabarcoding analyses) in each frozen fecal sample was performed by amplicon sequencing of a hypervariable genomic region (V3-V4 region 16SrRNA gene amplification) using an NGS approach on Illumina Platform. Total DNA was extracted from each fecal sample using a commercial kit (QIAamp DNA Stool Mini Kit, QIAGEN S.r.l. Milan, Italy).The quality of the genomic DNA was verified using a 2200 TapeStation DNA Screen Tape device (Agilent, Santa Clara, CA, USA) and an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) and its concentration ascertained using a Qubit assay (Life Technologies). The DNA was normalized to 5 ng/uL and then 2.5 uL was used for PCR amplification. Indexed NGS libraries were evaluated with the D1000 screen Tape (Agilent Tape Station 2200) and then quantified with ABI9700 qPCR

instrument using the KAPA Library Quantification Kit in triplicates, according to the manufacture's protocol (Kapa Biosystems, Woburn, MA, USA). Five uL of the pooled library at a final concentration of 4 nM were used for sequencing using Illumina Miseq with a 250 Paired end-read sequencing module.

Statistical Analysis

Pre-processing steps were performed and the raw reads quality of the extracted sequences were checked (using FastQC v0.11.2). Reads were trimmed with Phred scale quality threshold of 19 (which represent the probability of an incorrect base call) allowing the reads to be truncated after base quality drops below 18 (Phred scale). Reads IDs were edited (multiple_split_libraries_fastq.py, Qiime script) in order to be compatible for the following QIIME pipeline scripts. The 97% clustered Qiime formatted Greengenes v.13.8 reference database was used. Before the statistical analyses, alpha rarefaction and beta diversity were evaluated. Alpha rarefaction was an evaluation of within-sample diversity by species richness. A graphical alpha-rarefaction plot has been created using Qiime pipeline. Beta diversity is an estimation of between-sample diversity by microbial profile. A graphical representation has been calculated using Bray Curtis Qiime beta-diversity pipeline.

Finally, statistical analyses were performed with "R" statistical software (R v3.2; packages edgeR v3.10.5, Robinson M.D., 2010 and Phyloseq v1.14.0. McMurdie et Holmes, 2013). Samples were assigned to groups relying on samples' metadata and the Taxonomy table (genus level) was normalized. For each group of samples, a statistical analysis was performed using the edgeR ExactTest function, to generate a list of statistically relevant taxonomies that are differentially present among the conditions. Exact test specifications for differential expression between two groups of taxonomy tables was performed. It implements the exact test proposed by Robinson and

Smyth (Robinson et Smyth, 2008) for a difference in mean between two groups of random variables following a negative binomial distribution.

Finally, comparison of different phyla, classes and orders relative abundance among groups, was performed using Analyse-it for Microsoft Excel. Specifically, a Kruskall-Wallis test was performed, followed, when statistically relevant results were found, by a Wilcoxon Mann-Whitney test, for the comparison between two groups. Statistical significance was set at $P < 0.05$

RESULTS

Screening analyses

Based on the results of the screening analyses, after the application of inclusion criteria, 15 cats were selected and classified as follows: five healthy negative for FCoV PCR on feces (H group), five healthy positive for FCoV PCR on feces (COR group) and five affected by FIP, confirmed by post-mortem analyses (FIP group). Of the 15 animals enrolled, eight were female and seven were male. Breeds were quite variable, even though the domestic shorthair was the most represented (six cats), followed by two ragdolls, an exotic, a holy birman, a nowegian forest, a bobtail, a maine coon, a Scottish fold and a sphynx. Hematology and biochemistry, together with the clinical examination, were unremarkable for all the clinically healthy cats, regardless the FCoV positivity, while cats of the FIP group showed changes consistent with the disease (**Table 1.1**).

ID	Hematology	Biochemistry	SPE	AGP	ΔTNCC	IHC
1F	na	na	na	Na	Na	+
2F	Na	Na	na	na	Na	+
3F	Microcytic hypochromic anemia	↑total protein, ↑bilirubin	↓Albumin, ↑α2, β1, β2, γ globlulin	4.86	21.0	+
4F	Mild leukopenia	Mild azotemia, ↑ALT	↑ γ globulin	0.54	Na	+
5F	Nr	Mild azotemia, ↑total protein, ↑ALT ↑bilirubin	↓Albumin, ↑β2, γ globlulin	2.0	7.70	+(ICC)

Table 1.1. Clinicopathological abnormalities of cats belonging to the FIP group. Cats 1F and 2F were humanly euthanized in an external clinic due to the severe clinical conditions and subsequently referred to the University for necropsy. For this reason, no information regarding routine hematology and biochemistry were available for these animals. ID = identification number; SPE = serum protein electrophoresis; AGP = alpha-1-acid glycoprotein; ΔTNCC = delta total nucleated cell count; IHC = FIPV immunohistochemistry; nr = not relevant; na = not available; ALT = alanine aminotranferase; ICC = FIPV immunocytochemistry

Microbiota analyses

A total of 3,231,916 sequences, with an average of 215,461 sequences/sample (median 219,276, range 195,516.5 – 235,660.5) were of adequate quality and were subsequently analyzed. The alpha-diversity rarefaction curves did not reach a proper plateau for almost all the samples (**Figure 1.1**). This means that the sequencing depth was enough to identify only the most abundant bacteria for each sample, excluding the rarest ones.

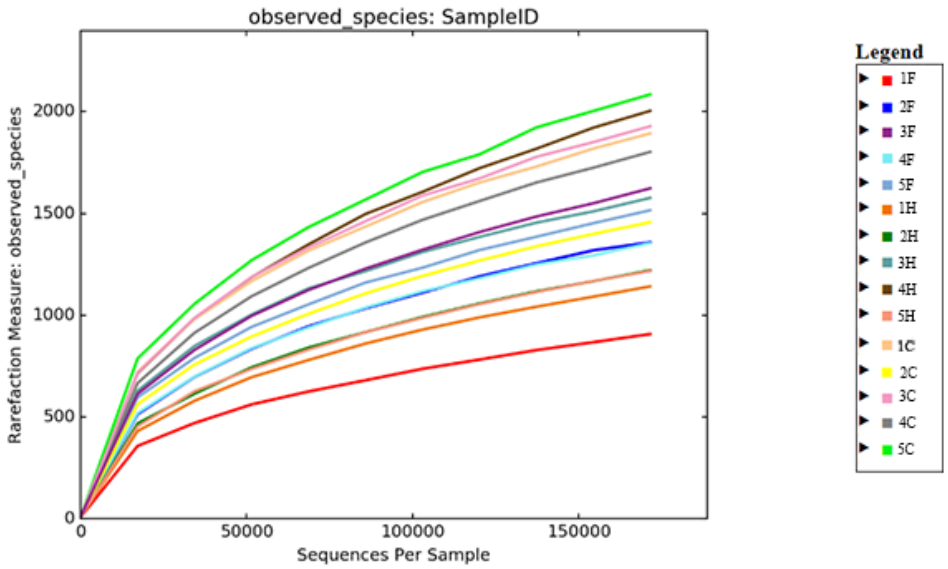

Figure 1.1. Alpha rarefaction for the entire caseload. Each sample is represented in a different colour.

The evaluation of beta-diversity showed that the samples were not grouped perfectly by category. However, the COR group seemed to have a hybrid microbial composition, between the microbial composition of the H and the FIP groups (**Figure 1.2**).

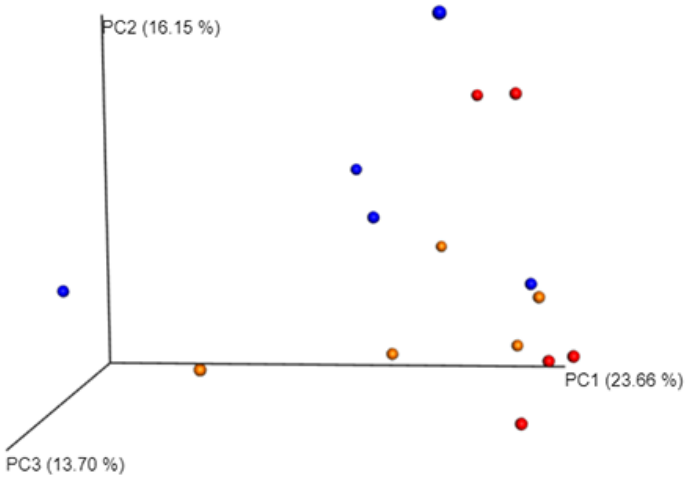

Figure 1.2. Beta diversity. Principal coordinates analyses of weighted distances. Proportions of variance explained by each principal coordinate axis is denoted in the corresponding axis label. In red cats belonging to healthy Coronavirus positive group (COR), in blue cats affected by FIP (FIP) and in orange healthy cats, negative for Coronavirus (H).

According to the statistical analyses there were no taxa significantly linked to the different conditions (zero differential taxa found for FIP vs H, FIP vs COR, COR vs H.). However, it was possible in some cases, to identify specific pattern between groups or for single animals. Eleven

different phyla were identified, even if the majority was represented by six of them (**Figure 1.3**). *Firmicutes* was the main represented phylum, followed by *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*. Nevertheless, COR group showed a major abundance of *Firmicutes* and minor of *Bacteroidetes*, compared to the other groups. This was also confirmed by the *Bacteroidetes:Firmicutes* ratio (0.9 in H group, 0.5 in FIP group and 0.1 in COR group; P = 0.13. If we compare COR group to H and FIP group taken together, the P value gets closer to the significance level p = 0.055). Even though the phylum relative abundance were not significantly different among the three groups, *Bacteroides* and *Firmicutes* were close to the significance level (P =0.13 and 0.08 respectively).

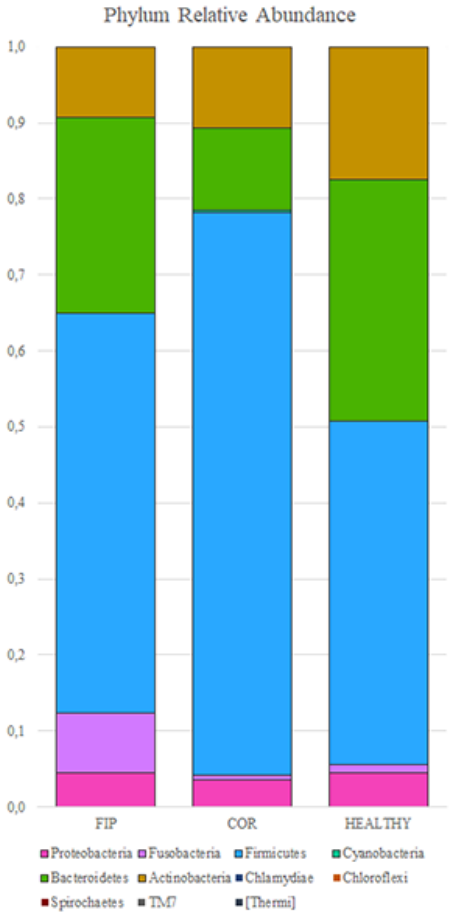

Figure 1.3. Relative abundance of the predominant phyla in the fecal microbiota in the three groups (y-axis). Group of FIP affected cats (n =5), healthy coronavirus negative (n =5) and positive (n =5) on x-axis. Each colour corresponds to different phylum.

In the FIP group three cats shared a similar microbiota composition, while cat 1F showed a completely different composition and cat 2F showed a lower number of diverse phyla (**Figure 1.4**).

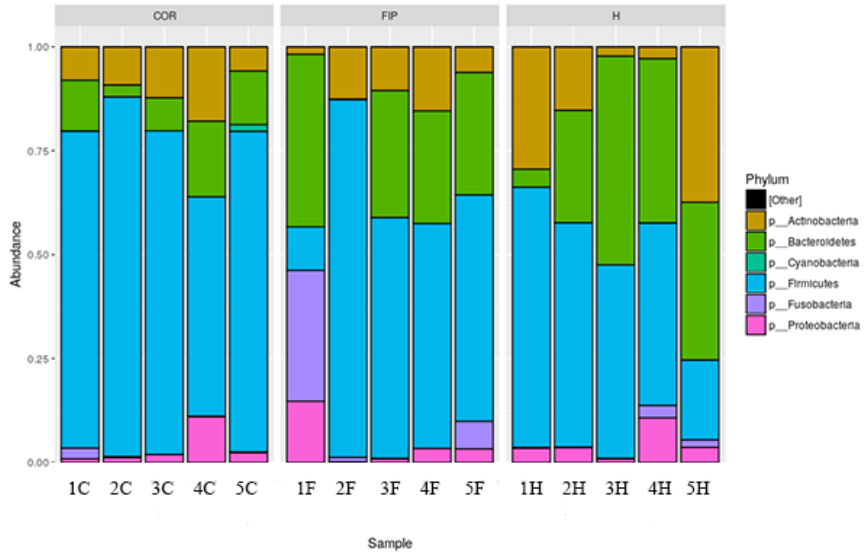

Figure 1.4. Relative abundance of the predominant phyla for each subject in the fecal microbiota. FIP affected cats (n =5), healthy coronavirus negative (n =5) and positive (n =5). Each colour corresponds to different phylum.

The same pattern could also be observed in relative class abundance (**Table 1.2**). COR group had a major proportion of *Erysipelothrichi* and *Clostridia* and lower *Bacteroidia* with respect of the other two groups (P =0.05; 0.10 and 0.13 respectively). Again, cat 1F showed a completely different pattern of microbiota composition and cat 2F had a lower number of different classes (**Figure 1.5**). The same trend was observed also in relative order abundance. For family and genus, the great amount of data made impossible to observe any evident pattern. *Lactobacillus* presence was quite variable in healthy cats. However, in FIP group, they were present only in one cat (4F) which had a dry form of FIP.

Class (%)	FIP (*n* =5)	COR (*n* =5)	H (*n* =5)
Actinobacteria	0.0024	0.0007	0.0900
Coriobacteriia	0.1024	0.0908	0.0624
Bacteroidia	0.2948	0.1221	0.3795
Bacilli	0.0002	0.0303	0.0105
Clostridia	0.4988	0.5244	0.3677
Erysipelotrichi	0.0055	0.1630	0.0367
Fusobacteriia	0.0108	0.0018	0.0021
Betaproteobacteria	0.0020	0.0017	0.0055
Deltaproteobacteria	0.0014	0.0011	0.0006
Epsilonproteobacteria	0.0011	0.0023	0.0061
Gammaproteobacteria	0.0218	0.0135	0.0082

Table 1.2. Median relative abundance of the most common classes in the fecal microbiota.

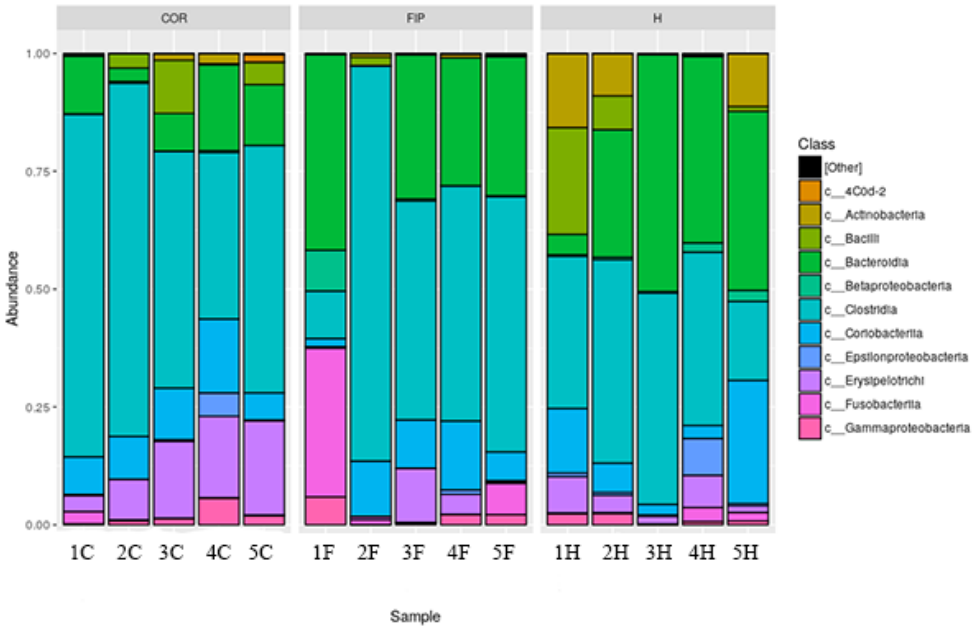

Figure 1.5. Relative abundance of the predominant classes for each subject in the fecal microbiota. FIP affected cats (n =5), healthy coronavirus negative (n =5) and positive (n =5). Each colour corresponds to different class.

DISCUSSION

Until recently, traditional bacterial culture was commonly used to identify gut microbial population, but it is now recognized that the vast majority of intestinal microorganisms cannot be cultured using standard plating techniques (Suchodolski, 2016). In the last few years molecular methods, mostly targeting the small subunit ribosomal RNA gene (16S rRNA), have been used for identification of bacterial microbiota. This gene is ubiquitously present in all bacteria and archaea

and contains both conserved and variable sequence regions, enabling researchers to distinguish organisms at different phylogenetic levels. However, microbiota evaluation is tricky both in the execution and in the interpretation of results, and the use of next generation sequencing technique is quite expensive. For this reason, several studies involved a low number of animals (Desai et al., 2009; Płoneczka-Janeczko et al., 2017; Schmid et al., 2018).

This is the first study about the gut microbiota composition in cats with feline coronaviruses. Alpha rarefaction and beta diversity analyses are the most common and historically relevant statistics for metagenome studies aimed to determine easily and visually the presence of groups between samples. The alpha rarefaction aims to demonstrate that enough reads were sequenced for each sample for the main taxa identification. The beta diversity graphically represents the distance between microbiological communities from each sample. No differences in the fecal microbiota were observed among three groups (H, COR and FIP). However, despite the low number of animals, in several cases, P values were close to statistical significance. The analyses of a higher number of animals is needed to confirm the impact of systemic infection on the intestinal microbiota, as previously reported in people (Goedert, 2016). Moreover, the peculiar enteric tropism of FCoV could explain these results. Whether is the microbiota influenced by FCoV presence, or if certain animals with peculiar microbiota composition are predisposed to FCoV infection is unclear. In literature, only two studies investigated the correlation between coronaviral diseases and gut microbiota, specifically in swine transmissible gastro-enteritis caused by the Transmissible Gastro Enteritis virus (TGEV) (Koh et al., 2015; Liu et al., 2015). However, the results were not comparable with our findings because of the different pathogenic role of FCoV compared to TGEV, as FECV infection is usually asymptomatic, and because of the different diet and bacterial phylotypes observed in swine compared to cats.

In contrast to what previously reported in cats (Weese et al., 2015), our caseload showed a low abundance of *Proteobacteria*. In humans, increase in *Proteobacteria* relative abundance is usually

associated with “dysbiosis” and gastrointestinal symptoms, such as, for example, diarrhea (Kaakoush et al., 2012; Suchodolski et al., 2012b; Shin et al., 2015). In our study, none of the cats had gastrointestinal disorders and this could explain the *Proteobacteria* lower abundance, together with the individual variability. Interestingly, in human a low *Bacteroidetes* to *Firmicutes* ratio in considered another marker od dysbiosis (Shin et al., 2015). In our study, the COR group showed a lower *Bacteroidetes* to *Firmicutes* ratio compared to the others. This could be related to the peculiar FCoV enteric tropism (stronger than in FIP cats), even if none of the animals showed signs of gastrointestinal disorders. This findings in COR group, could be a hint for an alteration in microbiota stability, even in absence of overt clinical signs.

In the FIP group, two of the five cats enrolled showed a completely different microbiota composition compared to the others. Specifically, cat 2F showed a lower number of diverse phyla. Despite the possible individual variability, such a lack in microbiota diversity in the gut microbiota diversity has previously been reported in people treated with antibiotic (Modi et al., 2014). In our caseload, as this was an exclusion criterion, this low abundance may be associated with other variables (*e.g.* diet).

Lactobacillus seem to have a probiotic activity, however, elevated concentration had been reported in systemic disorders, such as type II diabetes in people, thus making unclear their protective role (Sato et al., 2014). In the FIP group, *Lactobacillus* was only found in cat 4F, affected by a dry form of FIP. The meaning of this finding it is unclear. Besides, few studies have investigated this genus in cats, so it could be interesting to evaluate its presence in a larger number of animals, both healthy and diseased.

II. REFERENCE INTERVALS FOR PARAOXONASE 1 (PON-1) ACTIVITY IN A HEALTHY FELINE POPULATION

MATERIAL AND METHODS

Animals/Caseload

Serum samples were collected from client-owned cats admitted at our Institution for routine visits or for diagnostic purposes. Cats were considered clinically healthy based on the lack of clinical or laboratory abnormalities as determined by physical examination and a routine panel of laboratory tests (complete blood cell counts, spectrophotometric evaluation of creatinine, urea, glucose, alanine aminotransferase, alkaline phosphatase and total protein). Cats that had recent history of diseases or of pathophysiological conditions potentially affecting blood results (pregnancy, lactation, infections, etc.) or that received any medication or surgical treatments were excluded. Blood was collected from the cephalic or jugular vein and immediately transferred to plain tubes to obtain serum by centrifugation (2500g for 15 minutes). Serum samples were processed immediately, as described in following sections. Since blood was collected for diagnostic purposes and the owners signed an informed consent that authorizes the use of leftover sera for research purposes (after the diagnostic procedures), according to the regulations of our Institution (EC decision 02-2016) a formal approval from the Ethical Committee was not required.

Measurement of PON-1 activity

Serum PON-1 activity was measured using an automated spectrophotometer (Cobas Mira, Roche Diagnostic, Basel, Switzerland) using paraoxon (an organophosphate) as substrate to ensure that only PON-1 and no other enzymes were measured. The assay has been previously validated in

dogs (Rossi et al., 2014a) adapting the enzymatic method proposed by Feingold and colleagues (Feingold et al., 1998). The reaction buffer was prepared using glycine buffer (0.05 mM, pH 10.5) containing 1 mM of paraoxon-ethyl as a substrate (purity > 90%, Sigma-Aldrich, Missouri, USA) and 1 mM of CaCl2. The reaction was initiated with 6 µL of sample, 89 µL of distilled water, and 100 µL of reaction buffer, at 37 °C. The rate of hydrolysis of paraoxon to p-nitrophenol was measured by monitoring the increase in absorbance at 504 nm using a molar extinction coefficient of 18,050 L/mol/cm-1. PON-1 activity was expressed as U/mL i.e. 1 nmol of p-nitrophenol formed per minute under the assay conditions. The same method was already validated also in cats (Rossi et al., 2014b). A single measurement of PON-1 activity was done. Results were used to determine the reference intervals according to the ASVCP guidelines (Friedrichs et al., 2012).

Statistical Analysis

Reference intervals were determined using an Excel (Excel; Microsoft Corp., USA) spreadsheet with the Reference Value Advisor (version 2.0) (Geffrè et al., 2011) that performs computations according to the IFCC-CLSI recommendations as suggested by the ASVCP guidelines (Friedrichs et al., 2012). Descriptive statistics, tests of normality according to the Anderson-Darling method with histograms and Q-Q plots, and Box-Cox transformation were calculated. Both the Dixon-Reed and Tukey tests were used to identify outliers. Following the ASVCP guidelines, outliers that were considered "suspicious" by the software were retained. Conversely, far outliers were removed from the analyses. RIs were calculated using a nonparametric bootstrap method that also calculated the 90% CI of RIs. Results recorded in male vs female cats were compared to each other with a Mann-Whitney U test (using Analyse-it software Ltd, Leeds, UK), to assess whether it would be advisable to establish separate RIs for the partitioned groups. The possible age-related differences were investigated by linear regression using the Reference Value Advisor (version 2.0) macroinstructions mentioned above.

RESULTS

A total of 71 cats were enrolled to establish the reference interval. Information about sex was available for 69 out of 71: 43 were male and 24 were female. For what concern the age: 25 cats were younger than 1 year, for 13 cats the age ranged between 1 and 2 years, while 33 cats were older than 2 years (up to 13 years old) (**Table 2.1**).

Cats n°	Breed	Gender	Age	Cats n°	Breed	Gender	Age
1	SPHYNX	F	4 MM	36	RAGDOLL	F	2,5 YY
2	DSH	M	3 YY	37	RAGDOLL	M	4 YY
3	MAINE COON	MN	1 YY	38	RAGDOLL	F	3 YY
4	RAGDOLL	M	8 MM	39	RAGDOLL	M	8 YY
5	RAGDOLL	F	1 YY	40	RAGDOLL	F	5 YY
6	Nd	nd	6 MM	41	RAGDOLL	FS	1 YY
7	DSH	F	9 MM	42	RAGDOLL	MN	2 YY
8	DSH	MN	7 YY	43	RAGDOLL	F	10 YY
9	MAINE COON	M	4 YY	44	DSH	M	1 YY
10	DSH	FS	2 YY	45	DSH	FS	1 YY
11	DSH	MN	7 YY	46	DSH	MN	1 YY
12	DSH	FS	8 YY	47	DSH	FS	2 YY
13	DSH	MN	8 YY	48	DSH	MN	3 YY
14	DSH	MN	4 YY	49	DSH	MN	6 YY
15	DSH	MN	2 YY	50	DSH	MN	1 YY
16	DSH	MN	8.8 YY	51	DSH	MN	9 YY
17	PERSIAN	M	1,5 YY	52	DSH	FS	2 YY
18	RAGDOLL	M	8 YY	53	DSH	MN	1 YY
19	DSH	MN	5 YY	54	MAINE COON	M	7 YY
20	RAGDOLL	F	1 YY	55	DSH	FS	1 YY
21	RAGDOLL	F	6 YY	56	SIBERIAN	MN	3 YY
22	RAGDOLL	M	1,5 YY	57	DSH	FS	1 YY
23	RAGDOLL	M	3 YY	58	DSH	FS	8 YY
24	RAGDOLL	MN	7 YY	59	DSH	M	1 YY
25	RAGDOLL	M	8 MM	60	DSH	M	7 YY
26	RAGDOLL	F	9 YY	61	DSH	M	1 YY
27	RAGDOLL	F	1,5 YY	62	DSH	MN	13 YY
28	RAGDOLL	MN	7,5 YY	63	BSH	M	4 MM
29	RAGDOLL	F	1,5 YY	64	SPHYNX	M	2 YY
30	RAGDOLL	F	1 YY	65	DSH	M	10 MM
31	RAGDOLL	MN	8 YY	66	DSH	MN	8 MM
32	RAGDOLL	F	1 YY	67	SPHYNX	M	2 YY
33	RAGDOLL	F	6 YY	68	DSH	M	1 YY
34	RAGDOLL	F	2 YY	69	DSH	M	10 YY
35	RAGDOLL	M	2,5 YY	70	nd	F	6 MM
				71	nd	nd	6 MM

Table 2.1. Signalment of the cats enrolled for the establishment of reference interval of PON-1 activity. DSH: domestic shorthair; BSH: british shorthair; M: male; MN: male neutered; F: female; FS: female spayed; MM: months; YY: years

The reference interval determined in clinically healthy cats spans from 57.8 to 153.7 U/mL. Details about the determination of reference interval and about data distribution are reported in **Table 2.2** and summarized in **Figure 2.1** in accordance with the ASVCP guidelines (Friedrichs et al., 2012).

	Mean ± S.D.	Median	Min – Max	Outlier	$Limit_{min}$ RI (90% CI)	$Limit_{max}$ RI (90% CI)	Distr.	Method
PON-1 U/mL (n =70)	91,4 ± 23,0	86,8	46,6 – 174,1	2 (S) 1 (R)	57,8 (46,6 - 64,7)	153,7 (132,3 - 174,1)	NG	NP

Table 2.2. Reference interval of Paraoxonase activity in a healthy cats population. PON-1: paraoxonasi-1; S.D.: standard deviation; RI: reference interval; CI: confidence interval; Distr.: distribution, NG: non gaussian, NP: non parametric

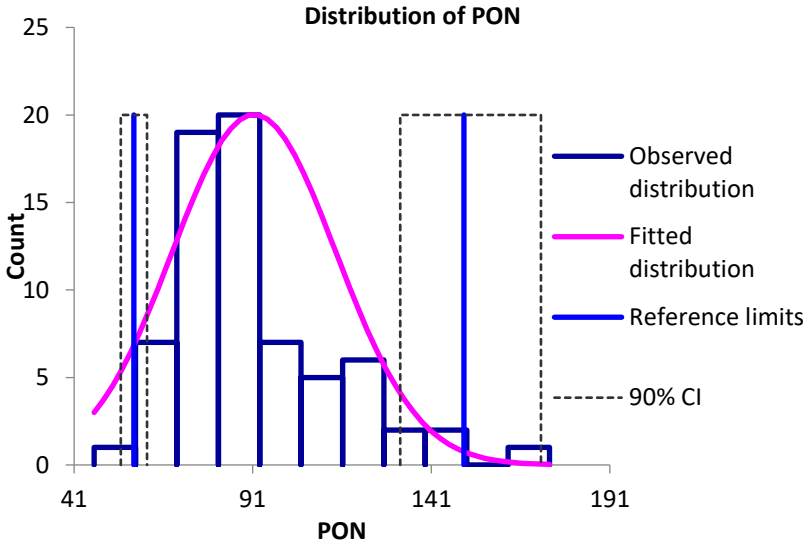

Figure 2.1. Data distribution for the reference interval of the paraoxonase-1 activity in a healthy cat population (n = 70).

No significant age-related difference in PON-1 activity was revealed by linear regression analyses (P =0.822). Conversely, PON-1 activity was significantly higher in male than in female cats (P =0.025) (**Table 2.3**).

PON-1 U/mL	Mean ± S.D.	Median	Min – Max	Outlier	$Limit_{min}$ (RI) (90% CI)	$Limit_{max}$ (RI) (90% CI)	Distr.	Method
F (n = 26)	84,6 ± 17,5	81,4	62 – 147,8	1 (S)	62,9 (59,5 67,6)	137,7 (110,2 - 210,9)	NG	NP
M (n = 42)	96,3 ± 24,3	91,3	61,1 – 174,1	1 (S)	61,3 (61,1 - 66,3)	171,6 (136,6 - 174,1)	NG	NP

Table 2.3. Reference interval of Paraoxonase activity in a healthy cats population, divided by gender. PON-1: paraoxonasi-1; F: female; M: male; S.D.: standard deviation; RI: reference interval; CI: confidence interval; Distr.: distribution, NG: non gaussian, NP: non parametric

However, the reference intervals calculated separately on male and female cats were largely overlapping to each other (61.3-171.6 U/mL in male, 62.9-137.7 U/mL in female) and, more importantly, the lower reference limit of male cats was very similar to that of female.

DISCUSSION

PON-1 hydrolyses a broad range of substrates, including esters, lactones, organophosphates, lipid peroxides and estrogen esters (Mackness et Mackness, 2015). Due to the lack of a "natural" substrate for PON-1, non-physiological substrates are used to measure PON-1 activity: paraoxon (paraoxonase activity), non-phosphorus arylesters (arylesterase activity) or lactones (lactonase activity) (Camps et al., 2009). The use of paraoxon as substrate was based on technical reasons as well as on previous publications. Phenylacetate (PA), p-nitrophenyl acetate (pNA) and 5-thiobutil butyrolactone (TBBL) have been previously validated in cats. However, despite paraoxon is more toxic than other substrates, it offers some advantages: the use of phenylacetate or TBBL is time consuming, since it can only be performed using manual methods or 96-well microplates and ultra-violet wavelength; on the other hand, even though the measurement with p-nitrophenyl acetate can be automated, results in healthy cats are very low (median 3.45 U/mL) and it may be difficult to appreciate the decreased values expected for a negative APP such as PON-1 (Tecles et al., 2015). Paraoxon-based method provides results higher than other substrates, so it may be more useful in practice.

Interestingly, reference intervals for PON-1 activity recorded in this study were lower than those reported for dogs (Rossi et al., 2014a) and humans (Tomas et al., 2000) and similar to those of cattle (Giordano et al., 2013) or horses (Ruggerone et al., 2018). The peculiar lipoprotein metabolism may explain the low PON-1 activity of cattle; however, it is surprising a similar finding in cats, metabolically more similar to dogs. The lack of significant age-related differences

seems to contrast with what reported in cattle (Giordano et al., 2013) and people (Furlong et al., 2016). Nevertheless, these latter studies were focused on PON-1 activity in newborns, in which the biosynthetic ability of the liver is still incomplete. In the current study the youngest animals were already weaned and can be considered as "young adults", with a PON-1 activity not significantly different from adults, as either in people or in horses (Furlong et al., 2016; Ruggerone et al., 2018). It is therefore possible that also newborn cats (not included in this study) may have a significantly lower PON-1 activity compared with adults. Conversely, the higher PON-1 activity recorded in male cats, contrasts with what reported in dogs, on which no significant sex-related differences were found (Rossi et al., 2014a), or in mice, cattle or horses, on which PON-1 activity is higher in females than in males (Bin al et al., 2003; Giordano et al., 2013; Ruggerone et al., 2018). The sex-related differences recorded in this study may suggest that different RIs should be used in male or female cats. However, the two RIs were largely overlapping and, more important, the lower reference limit, which in practice may correspond to the clinical decision limit, was very similar, with a difference quantitatively lower than the intrinsic imprecision of the method. Therefore, although the decision to use or not separate reference intervals should depend on statistical approaches (Harris et Boyd, 1990; Lathi et al., 2004), the current guidelines for the establishment of de novo reference intervals admit the use of non-statistical approaches based on practical considerations (Friedrichs et al., 2012). From this perspective, it would not be advisable to differentiate the RIs of male vs female cats, since it would likely not affect the clinical decisions. Many of the cats used to set out the reference interval belong to Ragdoll breed. It is unknown if this could have somehow influence out results, even if it is unlikely, since they does not seems to have different PON-1 activity compared to other breeds.

III. ROLE OF PARAOXONASE-1 (PON-1) AS A DIAGNOSTIC MARKER FOR FELINE INFECTIOUS PERITONITIS

MATERIALS AND METHODS

Animals/Caseload

All the animals included in this study were submitted for routine or diagnostic clinical examinations to the Veterinary Teaching Hospital of our Institution or to private practitioners. Both clinically healthy cats and cats with clinical signs consistent with FIP (regardless of the final diagnosis e.g. FIP or diseases other than FIP) were recruited.

Inclusion criteria for the clinically healthy cats included as a control group (HEALTHY) were the absence of any clinical signs or laboratory abnormalities consistent with disease or inflammation (e.g. leukocytosis, hyperprotidemia), in order to exclude any possible subclinical illness which could influence PON-1 activity. For sick cats the inclusion criteria were the presence of one or more clinical signs consistent with FIP, namely: effusions, hyperthermia, anorexia, weight loss, depression, jaundice, neurological or ocular affection.

The entire caseload had results of complete blood cell count and a biochemistry panel (including spectrophotometric evaluation of creatinine, urea, total protein, alanine aminotransferase, alkaline phosphatase, glucose at least) which should result inside the reference intervals for healthy cats. Sick cats had a definitive diagnosis of feline infectious peritonitis (FIP group) or disease other than FIP (NON FIP group) and were further grouped accordingly. In order to increase the number of FIP affected animals, cats with a strong suspicion for FIP were included as long as they have been humanly euthanized and had signalment, laboratory analyses and clinical signs consistent with the disease. For the sick cats, different analyses had been performed, depending on the clinician

request (e.g. electrophoresis, effusion analyses, ΔTNCC evaluation, effusion cytology, abdominal or cardiac ultrasound). After the diagnostic procedures, serum leftover (at least 150 µL) was transferred into a plain 1,5 mL Eppendorf tube and then frozen at -20°C.

The samples had been collected for diagnostic purposes by referring veterinarians according to standard veterinary procedures with a signed owner's consent. Therefore, according to the regulations of our institution, a formal approval of the Institutional Ethical Committee was not required (EC decision 29 Oct 2012, renewed with the protocol n° 02-2016).

Measurement of PON-1 activity

PON-1 activity was measured using a paraoxon based method, already described in Study II (see above)

Statistical Analysis

Statistical analysis were performed using Analyse-it for Microsoft Excel (Analyse-it Software Ltd, Leeds, United Kingdom). Statistical significance was set at $P < 0.05$. Specifically, differences in PON-1 activity among groups were evaluated using Kruskall-Wallis test, followed, when statistically relevant results were found, by a Wilxocon Mann-Whitney test.

Then, cats in the FIP group were further divided in a group of confirmed FIP (with IHC positive results) cats and a group of cats with suspected FIP (cats with clinical signs or laboratory data strongly consistent with the disease, but lack of a definitive diagnosis by IHC). Then, they were further divided based on the clinical form of the disease in dry and wet FIP affected cats. The same statistical tests, namely the Kruskal-Wallis test, followed by a Wilcoxon Mann-Whitney test, were performed to compare PON-1 activity among these subgroups and NON FIP and HEALTHY groups. The same analyses were performed to compare NON FIP subgroups (divided based on the

underlying disease in neoplasia, chylothorax, cardiopathy, infectious or other) with FIP and HEALTHY groups.

Finally, in order to evaluate the diagnostic power of PON-1 activity in discriminating FIP affected cats, a receiver operating characteristic curve (ROC) was built. The area under the curve (AUC) was calculated in order to evaluate the difference from the no-discrimination line. The ROC curve was used to determine clinically relevant threshold.

RESULTS

Firstly, 191 cats were enrolled in the study. After the application of the inclusion criteria, 159 cats were finally selected (32 cases were excluded from the study due to the lack of a definitive diagnosis, or to the absence of laboratory analyses). Among the included cats, 59 were female and 90 were male (in 10 cats the gender was not reported by the referring veterinarian). The most represented breed was the domestic shorthair (n =91). Age ranged from 3 months to 19 years old, with a median of 3 years of age. These animals were further divided into three groups.

The HEALTHY group included 71 cats (43 male, 24 female, two unknown) (see study II). All the animals had a normal physical examination and the absence of any laboratory abnormalities consistent with inflammation or disease.

The NON FIP group included 54 cats (26 female, 20 male, 8 unknown). 8 cats were younger than 2 years, 20 cats were aged between 2 and 10 years and 19 were older than 10 years. Effusion was present in 47 cats (19 peritoneal, 30 thoracic, 6 pericardial. 8 cats had bicavitary effusion). The remaining 7 cats had hyperthermia and lethargy (n = 4), neurological or ocular signs (n = 3). Most of the cats had a definitive diagnosis of neoplasia, followed by cardiogenic effusion, chylothorax, septic effusion, infectious diseases, immune-mediated haemolytic anaemia (IMHA), diabetes, intestinal occlusion, traumatism or hepatic lipidosis. Based on the definitive diagnosis, six

subgroups had been further compared: INFECTIVE (n = 4), NEOPLASIA (n = 21), CARDIOPATHY (n = 7), CHYLOTHORAX (n = 5), SEPTIC (n = 7) and OTHER (n = 10).

The FIP group was composed by 34 cats (12 female, 19 male, 3 unknown). 23 cats were younger than 1 year; nevertheless, 6 cats were adults (5 unknown). In 27 cats, effusions were present (21 peritoneal, 8 pleural, 2 pericardial. 4 cats showed bi or multi-cavitary effusion). 7 cats were affected by a dry form of the disease with neurological or ocular signs (4) or more unspecific signs such as hyperthermia, jaundice, lethargy, vomiting (3). In 19 cases, the definitive diagnosis was achieved through IHC on post-mortem biopsies. In the remaining 15 cases a strong suspicion for FIP was achieved due to signalment (young cats), clinical signs (such as fever, jaundice, ocular signs) and laboratory abnormalities (hyperprotidemia, typical electrophoretic pattern, effusion cytology, ΔTNC with SysmexXT-2000iV), together with the severe clinical course of the disease that always ended with the spontaneous death or human euthanasia.

Difference in PON-1 activity among HEALTHY, FIP and NON FIP

PON-1 activity was significantly different among the three groups (P <0.0001). Specifically, PON-1 activity was significantly lower in FIP cats than in HEALTHY and NON FIP cats (P <0.0001 in both cases). Cats belonging to NON FIP group had values of PON-1 activity significantly lower than the HEALTHY group (P <0.0001). (**Table 3.1** and **Figure 3.1**)

Group	Median	IQR I-III	Min – Max	90%CI
FIP (n = 34)	26,55	16,56 – 44,67	5,40 – 78,20	18,70 to 37,85
NON FIP (n = 54)	57,9	35,52 – 74,47	3,80 – 122,60	50,70 to 63,10
HEALTHY (n = 71)	87,5	74,52 – 103,02	46,60 – 215,50	83,10 to 91,50

Table 3.1. Value of PON-1 activity (U/mL) in cats belonging to group FIP, NON FIP and HEALTHY. For each group median, interquartil range, maximum and minimun value an 90% confidence interval has been reported.

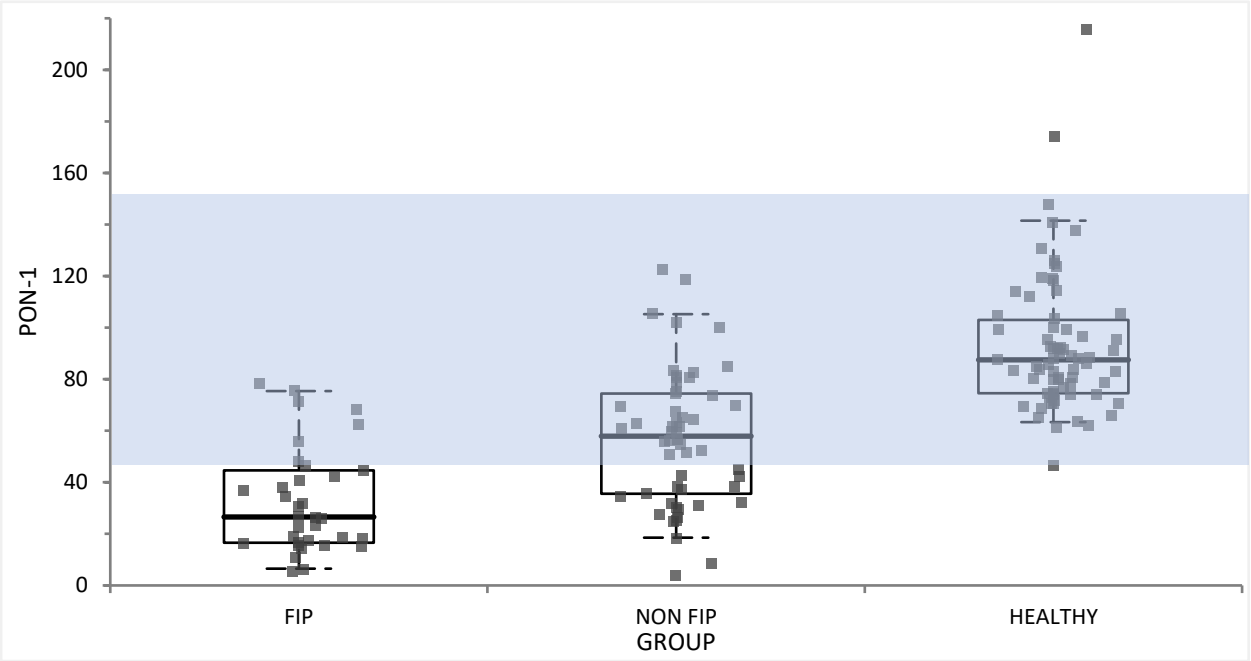

Figure 3.1. Comparison of the value of PON-1 (paraoxonase 1) among the groups FIP, NON FIP and HEALTHY. In light blue are reported the reference interval of PON-1 for healthy cats.

Comparison between suspected FIP and confirmed FIP

The SUSPECTED FIP subgroup was composed by cats with signalment, clinical signs and/or laboratory data consistent with the FIP (**Table 3.2**). The values recorded in the SUSPECTED FIP subgroup were not significantly different from the confirmed FIP subgroup (P =0.61). Conversely, both subgroups maintain significantly lower results compared with NON FIP and HEALTHY groups (P <0.0001). (**Table 3.3** and **Figure 3.2a - b**)

ID	BREED	SEX	AGE	CLINICAL SIGNS	LABORATORY DATA
126	DSH	MN	1 YY	Abdominal effusion	Anaemia, leucocytosis, lymphopenia, hypoalbuminemia, >α2; ΔTNC = 7,4; AGP = 5,81; cytology consistent; Ab anti-FCoV 1:1800; PCR on effusion +
127	DSH	MN	1 YY	Thoracic effusion	Anaemia, lymphopenia, hyperprotidemia, hypoalbuminemia, >β1, γ; ΔTNC = 49.6; AGP = 1,45; cytology consistent
128	DSH	MN	4 YY	Abdominal effusion, jaundice	Lymphopenia, thrombocytopenia, azotaemia, hypercreatininemia, hyperbilirubinemia, hypocalcaemia, > ALP, hypoalbuminemia, >γ; ΔTNC = 21.22; cytology: acellular
129	DSH	M	1 YY	Thoracic effusion	Leukopenia, lymphopenia, neutropenia, thrombocytopenia, hypoalbuminemia,; ΔTNC = 3.1
130	HB	FS	11 MM	Pericardial effusion, lethagy, anorexia	Mild azotaemia, hyperbilirubinemia, hyperprotidemia, , > ALT, hypoalbuminemia, >β2, γ; ΔTNC = 7.7; AGP = 2; cytology consistent; ICC on effusion +
131				Abdominal effusion	ΔTNC = 21,8; PCR on effusion +
132	DSH	F	9 YY	Thoracic and abdominal effusion	ΔTNC = 29,8; necroscopy consistent
134	DSH	M	6 MM	Abdominal effusion, neurological signs	Anaemia, monocytosis, > AST, hyperprotidemia, hyperphosphatemia, hypoalbuminemia, >γ; cytology consistent; Ab anti-FCoV 1:1800; PCR on CSF +
135	DSH	F	8 MM	Abdominal effusion, anorexia, fever	Anaemia, eosinopenia, thrombocytopenia, , > AST, hyperbilirubinemia, hyperlipemia, azotaemia, hyperphosphatemia, hypoalbuminemia; ΔTNC = 25; cytology consistent; PCR on effusion +
136	EX	F	4 MM	Abdominal effusion	Anaemia, eosinopenia, hyperlipemia, < ALT, ALP, hypoalbuminemia; cytology consistent; PCR on effusion +
137	DSH	MN		Tri-cavitary effusion, neurological signs, jaundice	Anaemia, leucocytosis, lymphopenia, thrombocytopenia, hyperprotidemia, hypoalbuminemia, >β1; ΔTNC = 12; cytology consistent; necroscopy consistent
138	DSH	MN		Abdominal effusion	Leucocytosis, lymphopenia, azotaemia, >β1; ΔTNC = 0.98; necroscopy consistent
139	SCO	M	6 MM	Abdominal effusion, fever	Anaemia, eosinopenia, hyperbilirubinemia, hypoalbuminemia; ΔTNC = 1.58; cytology consistent
140	SPH	MN	8 MM	Abdominal effusion, fever	Anaemia, leucocytosis, lymphopenia, hypoalbuminemia, >α2, β1 e γ; ΔTNC = 109.72; cytology consistent
141	BSH	MN	1 YY	Abdominal effusion	Anaemia, lymphopenia, hyperprotidemia, hyperbilirubinemia, hyperlipemia, hypoalbuminemia, >γ; necroscopy consistent

Table 3.2. Signalment, clinical signs and laboratory data of the cats belonging to the SUSPECTED FIP subgroup. DSH: domestic shorthair, HB: holy birman cat, EX: exotic, SCO: scottish fold, SPH: sphinx, BSH: british shorthair, F: female, FS: female spayed, M: male, MN: male neutered, YY: years, MM: months

Group	Median	IQR I-III	Min – Max	90%CI
CONFIRMED FIP (n = 19)	25,700	15,60 - 47,42	6,30 - 78,20	16,10 to 44,50
SUSPECTED FIP (n = 15)	30,60	19,67 - 41,93	5,40 - 68,10	19,10 to 42,20

Table 3.3. Value of PON-1 activity (U/mL) in cats belonging to subgroup CONFIRMED FIP and SUSPECTED FIP. For each group median, interquartil range, maximum and minimun value an 90% confidence interval has been reported.

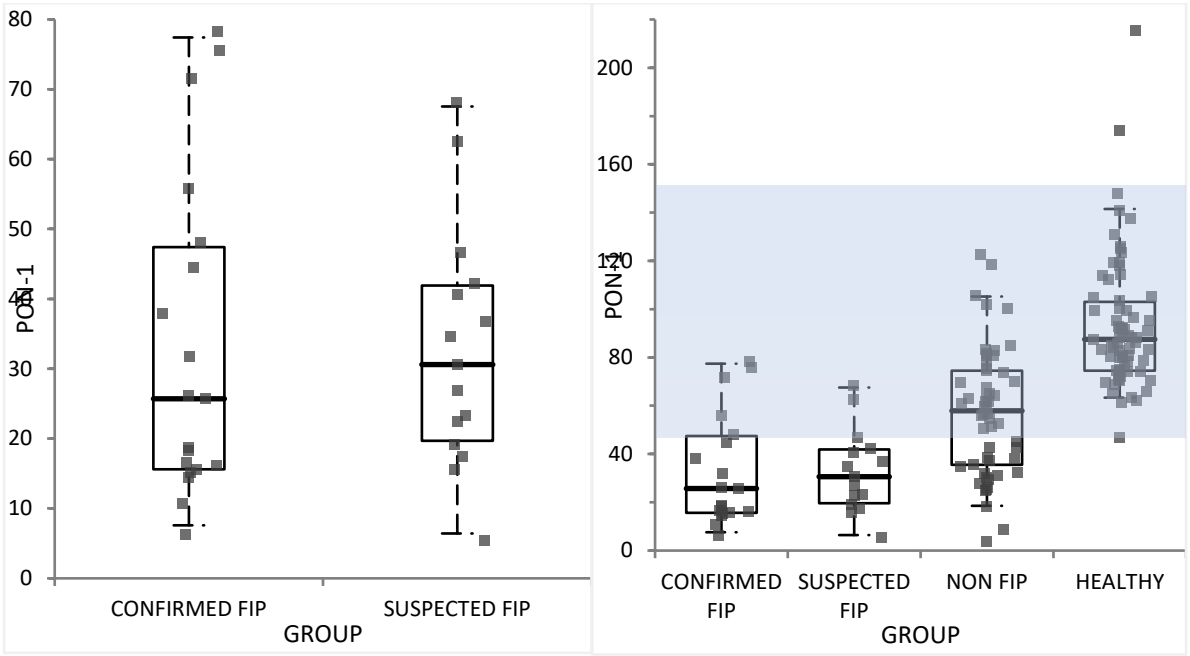

Figure 3.2a. Comparison of the value of PON-1 (paraoxonase 1) between the subgroup of CONFIRMED and SUPECTED FIP. (left) **3.2b.** Comparison of the value of PON-1 (paraoxonase 1) among the subgroup of CONFIRMED, SUPECTED FIP, NON FIP and HEALTHY. In light blue are reported the reference interval of PON-1 for healthy cats. (right)

Comparison between cats with wet FIP and cats with dry FIP

The cats with wet FIP had a lower PON-1 activity compared with cats with dry FIP (P = 0.035). Even though, cats with the wet form still maintained lower values of PON-1 compared to NON FIP and HEALTHY group (P <0.0001), whereas cats with the dry form of FIP showed significantly lower PON-1 compared with the HEALTHY group (P =0.0003), but not statistically lower compared to NON FIP group (P = 0.44). However, the power of this analyses could be strongly affected by the scarce number of dry FIP cases. (**Table 3.4** and **Figure 3.3a - b**)

Group	Median	IQR I-III	Min – Max	90%CI
DRY FIP (n = 7)	48,00	32,77 - 72,30	15,10 - 78,20	15,10 to 78,20
WET FIP (n = 27)	23,20	16,183 – 39,950	5,40 – 71,50	17,40 to 34,60

Table 3.4. Value of PON-1 activity (U/mL) in cats belonging to subgroup DRY and WET FIP. For each group median, interquartile range, maximum and minimum value an 90% confidence interval has been reported.

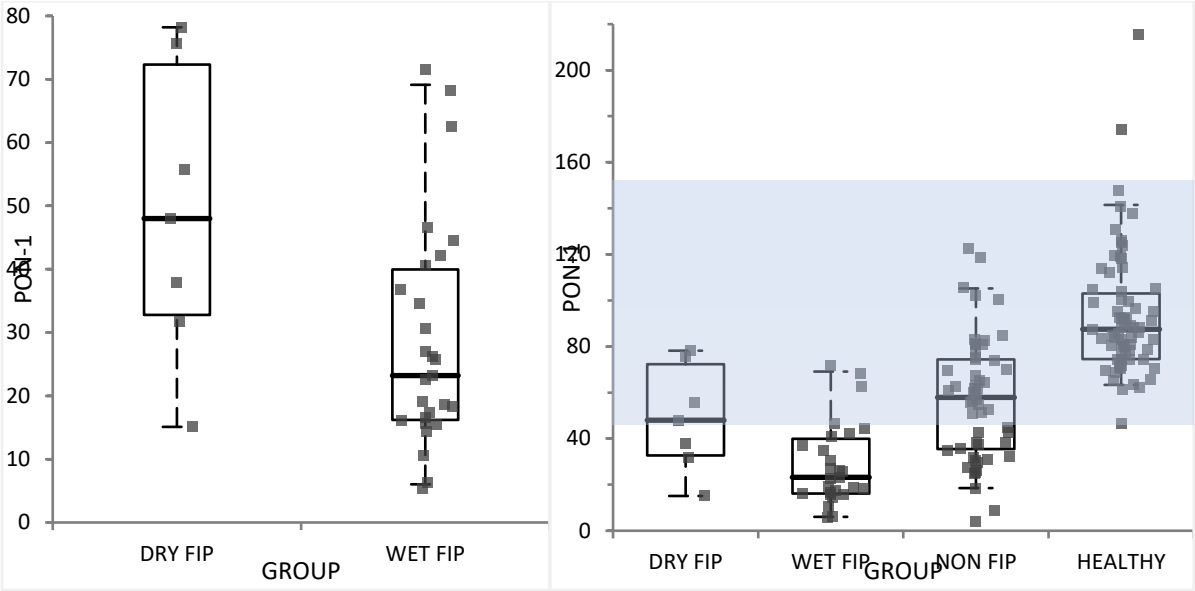

Figure 3.3a. Comparison of the value of PON-1 (paraoxonase 1) between the subgroup of DRY and WET FIP (left). **3.3b.** Comparison of the value of PON-1 (paraoxonase 1) among the subgroup of DRY, WET FIP, NON FIP and HEALTHY. In light blue are reported the reference interval of PON-1 for healthy cats.

Comparison among NON FIP subgroups

The NON FIP group, as reported above, has been divided into 6 subgroups based on the definitive diagnosis: neoplasia, septic, cardiopathy, chylothorax, infective or other. The comparison of PON-1 activity among groups did not result in any significant differences.

In all the subgroups PON-1 resulted significantly lower than HEALTHY group (P <0.0001), except for chylothorax (P =0.066). Similarly, all the subgroups except for the septic one (P =0.88) showed a higher value of PON-1 compared with FIP group (P <0.0001). (**Table 3.5** and **Figure 3.4**)

Group	Median	IQR I-III	Min – Max	90%CI
OTHER (n = 10)	57,450	41,32 - 74,92	24,90 - 105,40	26,20 to 80,60
CARDIOPATHY (n = 7)	73,800	40,45 - 82,43	35,60 - 84,80	35,60 to 84,80
CHYLOTHORAX (n = 5)	69,900	49,33 - 88,30	34,60 - 102,00	34,60 to 102,00
INFECTIVE (n = 4)	64,100	56,30 - 75,31	51,40 - 82,60	0 to 0
NEOPLASIA (n = 21)	54,700	31,80 - 63,27	25,30 - 118,60	38,30 to 61,80
SEPTIC (n = 7)	31,800	10,12 - 73,62	3,80 - 122,60	3,80 to 122,60

Table 3.5. Value of PON-1 activity (U/mL) in cats belonging to subgroup OTHER, CARDIOPATHY, CHYLOTHORAX, INFECTIVE, NEOPLASIA and SEPTIC. For each group median, interquartile range, maximum and minimum value an 90% confidence interval has been reported.

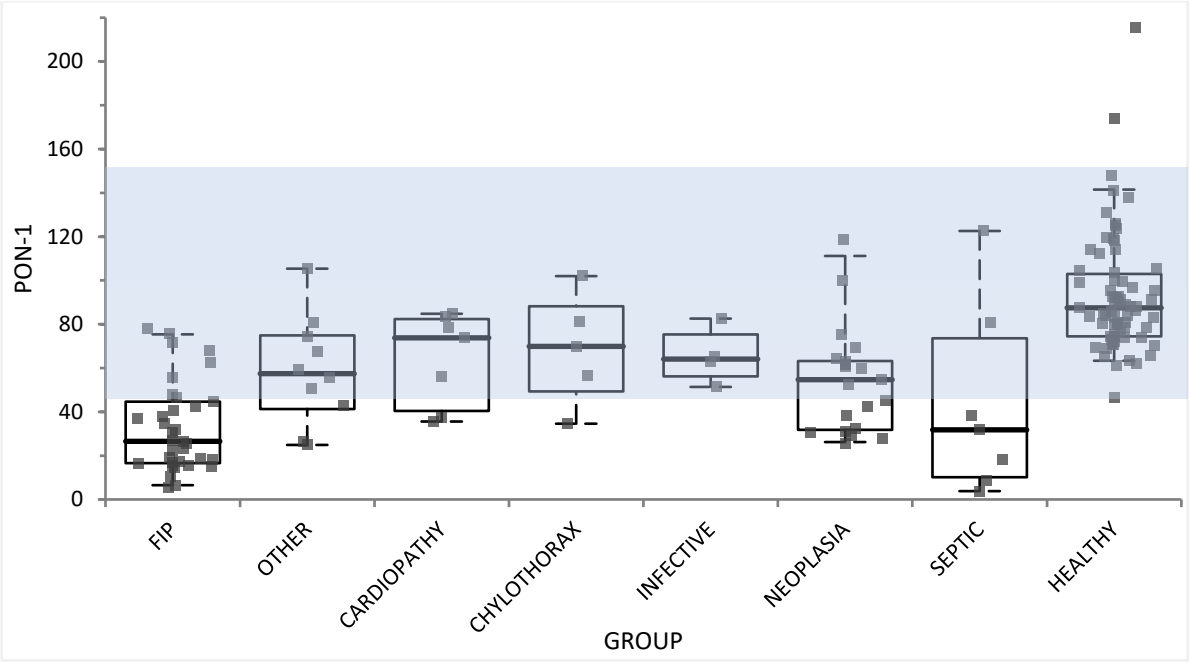

Figure 3.4. Comparison of the value of PON-1 (paraoxonase 1) among the group FIP, the subgroup OTHER, CARDIOPATHY, CHYLOTHORAX, INFECTIVE, NEOPLASIA, SEPTIC and the HEALTHY group. In light blue are reported the reference interval of PON-1 for healthy cats.

Diagnostic performance of PON-1

The analyses of ROC curve highlighted a statistical difference from the no-discrimination line (**P =0.02**) with an AUC equal to 88.7% (CI 95% = 0.83 to 0.94). Based on the ROC curve, the threshold that maximizes the diagnostic power of the test providing equal sensitivity and specificity corresponds to a PON-1 value activity of 51,40 U/mL (Se and Sp = 82%). However, depending on the clinical setting, it was possible to select a cut off threshold that maximized the specificity, in order to decrease the number of false positive results. In this study, this threshold is equal to a PON-1 value of 24,90 U/mL (positive Likelihood Ratio - LR+ = 18.38; Sp = 97.6%; Se = 44.1%). (**Figure 3.5**)

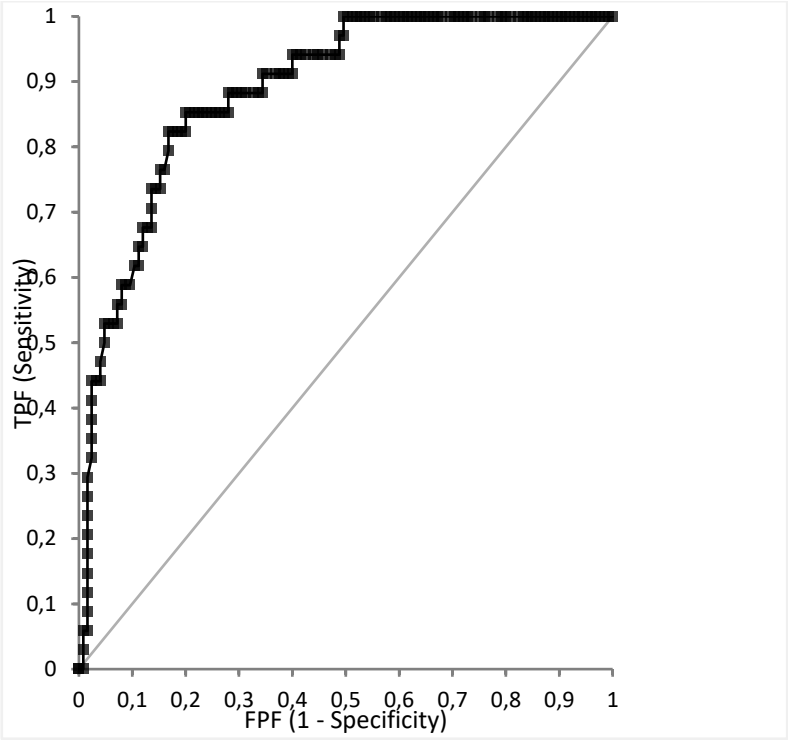

Figure 3.5. ROC curve of the values of PON-1 activity. The central line represents the no-discrimination line. In this case, NON FIP cats included both NON FIP and HEALTHY group.

DISCUSSION

PON-1 acts as a negative acute phase reactant in cats, similarly to other species (Giordano et al., 2013; Rossi et al., 2014). From this perspective, the results obtained from the comparison of the three groups was not surprising, especially due to the strong inflammatory response and oxidative stress that mounts in FIP cats (Regan et al., 2009; Tecles et al., 2015). Generally, during an inflammatory response, several mediators, including pro-inflammatory cytokines such as TNF-α and IL-1β, are produced (Montorfano et al., 2014). These, in turn, induce cellular oxidative processes that end in the production of reactive oxygen species (ROS). Moreover, ROS are also produced by leukocytes, specifically neutrophils and macrophages. So, the cellular damage caused by these reactive molecules go along with an inflammatory process, that recruited and induce activation of leukocytes. Due to the intimate relationship between oxidative stress and inflammation, it is easy to imagine its role in cats affected by FIP (Tecles et al., 2015). It has been

shown how, during this disease, the pro-inflammatory cytokines increase the releasing of the bone marrow neutrophils reservoir and decrease their apoptotic rate (Paltrinieri et al., 2008; Takano et al., 2009). That, in turn, enhance the oxidative stress, through the release of strong oxidant components. A previous study, based on a different substrate for the paraoxonase activity assessment and on a lower number of cats, had already highlighted how PON-1 may decrease in FIP affected cats (Tecles et al., 2015). This may happen both due to its role as a negative acute phase reactant and to an enzymatic consumption for its antioxidant properties. Indeed, PON-1 has a tendency to decrease significantly in those inflammatory processes that involve also a strong oxidative response. So, a low value of PON-1 activity was expected in FIP affected cats. In NON FIP group, PON-1 activity resulted to be higher compared to FIP cats, but still lower than in healthy cats. This result may depend on the high number of cats with a definitive diagnosis of neoplasia. It is interesting to note that one of the anti-tumoral mechanisms is represented by the macrophages production of ROS. Moreover, in some neoplasia, such as human renal carcinoma, are the neoplastic cells themselves that produce ROS, to enhance their survival (Toyokuni et al., 1995). These assumptions lead to the conclusion that the inflammatory microenvironment created by different neoplasia, could affect also the oxidative stress of the entire organism. Another hypothesis is that the decrease of PON-1 during neoplastic cachexia could be related both with a decrease of HDL particles (that usually bind PON-1) or with a hepatic lipidosis, that lead to a PON-1 production decrease. However, it is unlikely that neoplasia, especially when confined to a single organ, could cause the same systemic effect of a strong inflammation such as FIP.

A possible limitation of the study is that FIP was confirmed by post-mortem analyses only in some cases included in the FIP group. On one hand, however, FIP was the most likely diagnosis on all the cats belonging to this group, based on clinical and laboratory findings. On the other hand, PON-1 activity did not significantly differ in cats with confirmed FIP compared with cats on which FIP was highly probable based on in vivo findings. Moreover, both subgroups, maintained a PON-

1 activity level lower than both NON FIP and H groups. This could be a further support to the suspected diagnosis in those cats in which IHC was not performed. The suspicion of FIP was release after the cats died (both for natural causes or euthanasia) and was supported by signalment, history, clinical signs and laboratory results. All the cats belonging to the suspected FIP subgroup presented an effusive form of the disease. None of the findings could confirm the diagnosis alone. However, the presence of several data consistent with FIP increased the diagnostic suspicion, as already reported in literature (Stranieri et al., 2018).

In the effusive form of FIP PON-1 activity resulted to be lower with respect of the non-effusive form. This result is consistent with the major inflammatory component of the effusive FIP. Indeed, in the non-effusive form a partially protective immune response causes, through the cell mediated immunity, the destruction of infected monocytes and, in turn, a lower viral spread (Pedersen, 2014b). For this reason, usually the granulomas are more localized. Conversely, in the effusive form, pyogranulomas tend to be more disseminated. Moreover, the presence of neutrophils in the granulomas itself, it is typical of the effusive form (Pedersen, 2014b). Since it is known that neutrophils and macrophages are the major producer of ROS, it is possible to conclude that in the effusive form, a strong inflammation go together with marked oxidative stress, leading to an evident decrease in PON-1 activity. The localization of the granulomas could also influence the different results obtained in PON-1 measurement. As an example, one cat showed a value of PON-1 that falls inside the reference interval for healthy cats. In this particular case, the virus associated with the typical lesions had been found microscopically only in the cerebellum, which was also the only organ with histological lesions in this cat. The strict localization associated with the presence of the haemato-encephalic barrier could lead to a decrease in the strength and efficacy of the immune response (Pedersen, 2009). The same hypothesis could also explain the lack of significant differences between dry FIP subgroup and NON FIP group in which the oxidative

component was not so pivotal. However, this are only speculations, that need an increased number of cases to be confirmed.

The comparison among different NON FIP subgroups resulted in the absence of significant differences. This outcome could be influenced by the different (and in some cases scarce) sample size of the subgroups. Comparing the different subgroups with the H group, all of them showed a lower level of PON-1 activity except for the CHYLOTHORAX subgroup. This result could be explained by the low systemic inflammation caused by the presence of this type of effusion, but results should be interpreted cautiously due to the low number of cats in this subgroup (only 5 cases). The comparison between the NON FIP subgroups and FIP group resulted in higher value of PON-1 for all the subgroup except for the SEPSIS one. This was composed by 7 cats: 6 of them presented effusion, while one presented a septic spondylitis, complicated by toxoplasmosis and FeLV infection. This could justify the extremely low value of PON-1 in this latter cat. The presence of septic effusion could determine a strong inflammation, due to the high level of inflammatory cells, associated with strong oxidative stress (Draganov et al., 2010). On the other hand, since it is a localized problem, it is not always cause of severe systemic repercussion. Specifically, three cases presented anaemia due to the presence of inflammatory processes, possibly related with the action of the oxidative stress on erythrocytes (Ottenjann et al., 2006). Anaemia could be considered as a sign of systemic repercussion of a localized problem and could explain the low value of PON-1. In one case, the extremely low value on PON-1 could be related with the presence of *Nocardia* in the effusion. It is known that *Nocardia* causes a severe mixed unspecific serositis, similar to those observed in FIP (Hayward, 1968). Finally, in one case anaemia was not present, maybe due to a low systemic impact or an initial phase of the disease. As a result, the value of PON-1 was sensibly higher. This relationship between anaemia and PON-1 activity has already been reported in human medicine (Okuturlar et al., 2016).

Finally, the analyses of the ROC curve demonstrated that PON-1 may differentiate cats with FIP from cats without FIP. The value on PON-1 that maximizes the diagnostic power of the test is equal to 51.40 U/mL (both sensitivity and specificity are 82%). However, this threshold is not clinically relevant, because it may include several false positive and false negative results. It is possible to set out a value of PON-1 activity that maximize the sensitivity (specifically: PON-1 =78.30 U/mL; Se =100%; Sp =50.4%). The use of this threshold allows to exclude completely the disease. Nevertheless, this value fell inside the reference interval for PON-1 activity in the healthy population (57.8 – 157.3 U/mL). This means that there will be a certain number of healthy cats which can be mistaken for false positive. Conversely, due to the poor prognosis of a FIP diagnosis, it is desirable to decrease the number of false positive results, that could be obtained increasing the specificity of the test. A value of PON-1 equal to 24.90 U/mL could be more useful in a clinical setting (LR+ =18.38; Sp =97.6%; Se =44.1%). Indeed, a cat with a value of PON-1 lower than this threshold had 18 time the probability to be affected by FIP than not to be affected. However, even using this threshold, there were some false positive results. As already stated in literature, the result of a single test is not sufficient to confirm a diagnosis of FIP (Stranieri et al., 2018). The results of this study state how PON-1 measurement is not an exception. However, the association of more than one test could increase the suspicion of FIP (even if, in the end, it is always desirable to confirm the diagnosis through IHC on biopsies). Moreover, the method used in this study to measure PON-1 activity, based on the paraoxon as substrate, compared to other substrates is quite more analytically sensitive, allowing the evaluation on a wide range of values, thus permitting to differentiate cut-off values based on the purpose of the investigation (screening vs confirmatory studies).

IV. DIAGNOSTIC PERFORMANCE OF RT-PCR COMPARED TO IMMUNOHISTOCHEMISTRY FOR THE DIAGNOSIS OF FELINE INFECTIOUS PERITONITIS

MATERIAL AND METHODS

Animals/Caseload

Tissue samples were collected, post-mortem, at the Veterinary Teaching Hospital of Milan from cats deceased or euthanized with a suspicion of FIP or due to other systemic diseases or serious injuries and subjected to necropsy for diagnostic purposes. All the samplings were performed within routine diagnostic procedures and the owners signed an informed consent about the use of tissues and samples for research. Therefore, according to the Ethical Committee of the University of Milan (decision n° 2, 2016), residual aliquots of tissues can be used for research purposes without any additional formal request of authorization to the Ethical Committee.

The inclusion criteria were: (1) tissues sampling within six hours from death; (2) availability of signalment, history and clinical information (physical examination, clinical pathology, diagnostic imaging depending on the clinical presentation).

This information, along with the results of necropsy and histology, were used classify the cats in FIP or NON FIP. Specifically, FIP group included cats suspected for FIP based on history and/or laboratory alterations, on which diseases other than FIP were excluded, showing gross and histological lesions consistent with FIP in at least one tissue, including tissues sampled for diagnostic purposes and not included in this study (e.g. brain or cerebellum). The NON FIP group included cats on which, despite a clinical presentation potentially consistent with FIP, diseases

other than FIP were already diagnosed on a clinical basis (e.g. based on results of diagnostic imaging or cytology) and/or without any histological lesion consistent with FIP.

Tissues sampling

Samples from spleen, liver, mesenteric lymph node, kidney, large intestine, small intestine and lung, collected from the necropsied cats, were included in this study. Moreover, tissues from organs different from those listed above, with macroscopic lesions, as well as organs not visibly affected but likely involved (based on the patient history, e.g. brain and cerebellum from cats with neurological signs) were also collected during necropsy to perform routine histology and immunohistochemistry for diagnostic purposes. The information about these organs were used to achieve a correct classification as FIP or NON FIP.

From each sampled organ, a fragment was taken and sectioned, with a sterile scalpel, in two adjacent halves of approximately one-centimetre of diameter each. If macroscopic lesions were present, the two halves were obtained sectioning the sample approximately in the centre of the lesion (**Figure 4.1**). One half of the fragment was immediately frozen at -20°C in plain tubes for molecular biology, while the other half was collected into 10% neutral-buffered formalin to perform histology and immunohistochemistry.

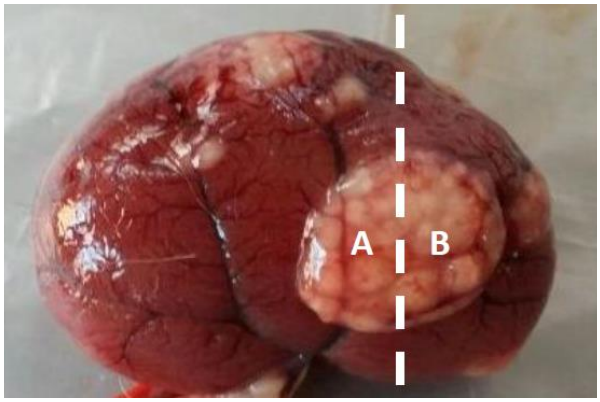

Figure 4.1. Representation of sampling obtained from organs with macroscopic lesions. The kidney in figure was divided in two section splitting in two halves the lesion (dashed line). The A section was used for immunohistochemistry, while the B section was used for molecular biology test.

Histology and immunohistochemistry

Formalin fixed samples were sent to the Department of Comparative Biomedicine and Food Science of the University of Padova. Sections (5 μm) obtained from formalin-fixed paraffin-embedded samples were stained with haematoxylin-eosin for histology with an automated stainer (Autostainer XL, Leica Biosystems, Wetzlar, Germany). For immunohistochemistry (IHC) 3 μm paraffin sections were placed on surface-coated slides (Superfrost Plus). Slides were then incubated at 37°C for 30 minutes before the immunostaining performed with an automatic immunostainer (Ventana Benchmark XT, Roche-Diagnostics), which uses a kit with a secondary antibody with a horseradish peroxidase (HRP)-conjugated polymer, that binds mouse and rabbit primary antibodies (ultraViews Universal DAB, Ventana Medical System). All reagents were dispensed automatically except for the primary antibody, which was manually dispensed. As primary antibody, a mouse monoclonal antibody against the feline coronavirus was used (clone FIPV3-70 Serotec, Oxford, UK). FIP positive tissues were used as positive controls. For negative controls, the antibody diluent (Ventana Medical Systems) was applied instead of the primary monoclonal antibodies. Immunohistochemistry was considered positive if FCoV antigen was detected within typical histological lesions above described (Pedersen, 2009; Kipar and Meli, 2014). In fact, the detection of FCoV antigen in scattered macrophages or epithelial cells (e.g. intestinal columnar cells, likely infected by FECV) only, was not considered as a positive result (Kipar et al., 2010).

Histological examination was performed under light microscopy and a detailed description was provided for each organ. For each organ, lesions were categorized as consistent with FIP when showing one or more typical lesions such as pyogranulomas on one or more serosal surfaces; granulomas with or without necrotic areas; lymphocytic and plasmacytic focal and perivascular infiltrates; granulomatous to necrotizing phlebitis and fibrinous serositis (Pedersen, 2009; Kipar et Meli, 2014). Conversely, if there are lesion consistent with diseases other than FIP, they were

considered as NON FIP. In addition, tissues were categorized as susplpected, when there are results from laboratory data or histology on other organs, which could be consistent with FIP.

FCoV RT-nPCR

From frozen-thawed samples, RNA was obtained using a NucleoSpin RNA kit (Macherey-Nagel, Bethlehem, PA). Twenty mg of tissues were thinly shredded on sterile plates using sterile scalpels and vigorously vortexed in RA1 lysis buffer until completely dissolved. All the further steps were performed according to the manufacturer's instructions. RNA samples were then frozen at -80°C or immediately used for PCR. A reverse transcription nested PCR (RT-nPCR) targeting a 177 bp product of the highly conserved 3′ untranslated region (3′ UTR) of the FCoV genome was used (Herrewegh et al., 1995). FCoV RT-nPCR positive RNA was used as positive control and RNAse-free water as negative control. PCR products were visualized under UV transilluminator on a 1.5 % agarose gel stained with ethidium bromide. PCR was considered positive if showing a 177bp band on agarose gel electrophoresis (Herrewegh et al., 1995).

Analyses of results

For IHC and RT-PCR, true-positive (results consistent with FIP in cats with FIP) and false-positive results (results consistent with FIP in cats without FIP) as well as true-negative (results not consistent with FIP in cats without FIP) and false-negative results (results not consistent with FIP in cats with FIP) were recorded. Sensitivity, specificity, accuracy, positive and negative predictive values as well as positive and negative likelihood ratios (LR+ and LR−, respectively) were then calculated for IHC and/or PCR.

The concordance between IHC and PCR was assessed using Cohen's k coefficient (Landis et Koch, 1977). Concordance was classified as none ($k < 0$); minimal ($0 < k < 0.20$); weak ($0.21 < k < 0.40$); moderate ($0.41 < k < 0.60$); strong ($0.61 < k < 0.80$) and almost perfect ($0.81 < k < 1$).

This test was performed both on the totality of the samples and considering the two groups (FIP and non FIP) separately to evaluate concordance in the diagnosis of FIP. Additionally, it was performed also on the results recorded for type of organ, to evaluate which organ might give a substantial number of concordant results and, consequently, might provide the same information independently on the used test.

RESULTS

A total of 26 cats were included in the study. Specifically, 14 cats were included in the FIP group, and 12 in the non FIP group (**Table 4.1**).

	ID	Breed	Age	Sex	Clinical suspicion	Final diagnosis	Tissues not collected
FIP	1	DSH	8 MM	F	FIP	Non-effusive FIP	Lu
	2	DSH	6 MM	M	FIP	Non-effusive FIP	
	3	DSH	10 MM	FS	FIP	Non-effusive FIP	SI
	4	DSH	1,5 YY	FS	FIP	Non-effusive FIP	
	5	DSH	Nd	F	FIP/Neoplasia	Non-effusive FIP	
	6	MC	9 YY	MN	FIP	Effusive FIP	
	7	ES	6 MM	M	FIP	Effusive FIP	
	8	DSH	5 YY	FS	FIP	Effusive FIP	SI and LI
	9	R	1 YY	MN	FIP	Effusive FIP	
	10	DSH	1 YY	FS	FIP	Effusive FIP	
	11	DSH	1 YY	MN	FIP	Effusive FIP	
	12	DSH	2 YY	MN	FIP/Septic	Effusive FIP	Ly
	13	DSH	Nd	MN	FIP	Effusive FIP	
	14	MC	1 YY	F	FIP/Septic	Effusive FIP	
Non FIP	15	DSH	15 DD	F	Trauma	Bite wounds	
	16	DSH	15 YY	FS	Neoplasia	Chronic pneumonia	
	17	DSH	1,5 MM	FS	Poisoning	Rodenticide poisoning	
	18	DSH	5 y	F	FIP	Diabetes mellitus	Ly
	19	DSH	Nd	M	Trauma	Multiple wounds	
	20	DSH	Nd	MN	FIP/Neoplasia	Pulmonary adenocarcinoma	
	21	DSH	2 MM	F	FPV	FPV	
	22	DSH	Nd	F	Trauma	Multiple wounds	

23	DSH	2 y	M	FIP	Heart failure
24	DSH	1,5 YY	FS	Heart failure	Heart failure
25	DSH	7 YY	MN	Heart failure	Heart failure
26	DSH	9 YY	MN	FIP/Neoplasia	Thymic carcinoma

Table 4.1. Signalment, clinical suspicion, final diagnosis and not collected tissues for the cats enrolled. DD: days; DSH: domestic shorthair; F: female; FPV: feline panleukopenia virus; FS: female spayed; LI: large intestine; Lu: lung; Ly: mesenteric lymph node; MM: months; M: male; MN: male neutered; nd: not determined; SI: small intestine; YY: years

As regards for the FIP group, five cats showed non-effusive forms of the disease, with primarily neurological (n°2, 3, 5) and ocular symptoms (n°1, 4), along with fever, lethargy and anorexia. The remaining nine cats exhibited effusive forms of FIP, with abdominal (n°6, 7, 8, 10, 12, 13, 14) and/or thoracic (n°9, 11, 12) effusions. When present, the effusion displayed typical features of FIP. The exceptions were represented by cats n° 12 and 14, whose effusions were initially consistent with FIP, while at necropsy had a purulent appearance, confirmed by cytologic evaluation, which revealed a septic component too.

Regarding the non FIP group, in four cases, FIP was considered as a possible differential diagnosis because of the presence of hyphaema (n°18), body cavity effusion and jaundice (n°20), anisocoria ad tremors (n°23), neurological symptoms and thoracic effusion (n°26). Laboratory findings, diagnostic imaging and post mortem analyses subsequently led to the diagnoses of unrestrained diabetes mellitus, pulmonary adenocarcinoma, end stage heart failure and thymic carcinoma respectively. In eight cats, FIP was not suspected and, specifically, death occurred for severe injuries (n°15, 19 and 22), rodenticide poisoning (n°17), inflammatory (n°16), infectious (n°21) and cardiac (n°24, 25) diseases, diagnosed based on history, laboratory and histological analyses.

Table 4.2 and **4.3** resume the results of histology, immunohistochemistry and RT-PCR that are discussed in detail in the next sections.

		Spleen			Liver			Lymph Node			Kidney			Small intestine			Large Intestine			Lung		
	ID	H	I	P	H	I	P	H	I	P	H	I	P	H	I	P	H	I	P	H	I	P
FIP	1	-	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	NC	NC	NC
	2	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-
	3	-	-	+	+	+	+	+	+	+	+	+	+	NC	NC	NC	+	+	+	+	+	+
	4	+	+	+	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
	6	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	-	-
	7	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	-	+	+
	8	+	+	+	+	+	+	+	+	+	+	+	+	NC	NC	NC	NC	NC	NC	+	+	+
	9	-	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+
	10	+	-	ND	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	11	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	+	-
	12	+	+	+	+	+	+	NC	NC	NC	-	-	+	-	-	+	-	-	+	+	+	+
	13	+	+	+	+	+	+	-	+	+	-	-	+	+	+	+	+	+	+	-	+	+
	14	+	+	+	-	-	+	-	-	+	+	+	+	+	+	+	+	-	+	-	-	+
NON FIP	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	NC	NC	NC	-	-	-	-	-	-	-	-	-	-	-	-
	19	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	20	-	-	+	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
	21	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
	22	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-
	23	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	25	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-
	26	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 4.2. Results of histological examination, immunohistochemistry and PCR obtained on each collected sample. H: histology; I: immunohistochemistry; NC: not collected; ND: not determined; P: PCR

	FIP			Non FIP		
	Histology	**IHC**	**PCR**	**Histology**	**IHC**	**PCR**
Spleen	8/14 (57.1%)	8/14 (57.1%)	10/13 (76.9%)	1/12 (8.3%)	0/12 (0.0%)	2/12 (16.7%)
Liver	8/14 (57.1%)	8/14 (57.1%)	9/14 (64.3%)	1/12 (8.3%)	0/12 (0.0%)	0/0 (0.0%)
Lymph node	6/13 (46.2%)	9/13 (69.2%)	11/13 (84.6%)	1/11 (9.1%)	0/11 (0.0%)	2/11 (18.2%)
Kidney	9/14 (54.3%)	8/14 (57.1%)	12/14 (85.7%)	2/12 (16.7%)	0/12 (0.0%)	1/12 (8.3%)
Small intestine	5/12 (41.7%)	6/12 (50.0%)	8/12 (66.7%)	0/12 (0.0%)	0/12 (0.0%)	1/12 (8.3%)
Large intestine	7/13 (53.8%)	6/13 (46.2%)	10/13 (76.9%)	0/12 (0.0%)	0/12 (0.0%)	0/12 (0.0%)
Lung	9/13 (69.2%)	10/13 (76.9%)	10/13 (76.9%)	1/12 (8.3%)	0/12 (0.0%)	0/12 (0.0%)

Table 4.3. Number and percentages of positive results at histology, IHC and PCR for each tissue collected from the FIP and non FIP groups.

Histology

Histological examination was performed on 93 tissue samples obtained from FIP cats and 83 obtained from NON FIP cats (**Table 4.2**). Overall, 52/93 tissues (55.9%) from FIP cats exhibited histological features consistent with the disease. Specifically, the most common findings were

pyogranulomatous and/or granulomatous inflammation, fibrinous inflammation, lymphoplasmacytic inflammation, necrosis, and perivascular and vascular inflammation. Those lesions were often simultaneously detected in the same animal and even within the same organ. It was not uncommon (41/93 tissues, 44.1%) that examined histological sections did not show either relevant lesions or lesions not consistent with FIP. In the FIP group, the tissues that most often showed the typical FIP histological lesions (table 3) were the lung (9/13, 69.2%), the liver and the spleen (8/14, 57.1%) followed by the kidney (9/14, 54.3%) and the large intestine (7/13, 53.8%), while the small intestine was the organ that less frequently showed lesions imputable to FIP (**Table 3**). In all the cases, except in cat n° 2, which presented neurological signs *in vivo*, FIP lesions were found at least in one of the examined organs. In cat n°2 histological findings of the CNS showed the presence of lesions consistent with FIP.

As regards for the NON FIP group, histological examination did not show lesions consistent with FIP in 78/84 samples (92.9%) and therefore lesions potentially consistent with FIP were found, at least in one tissue, only in 6/84 tissues (7.1%), collected from 3/12 cats (25.0%). The organs that most frequently showed lesions possibly consistent with FIP (**Table 4.3**) were kidney, lymph node, spleen and liver. Lesions consistent with FIP were never detected in the small and large intestine.

Immunohistochemistry

All the 12 cats (100%) assigned to the non FIP group had a negative IHC for FCoV antigen in all the examined organs, while 13/14 cats (92.8%) assigned to the FIP group showed positive results in at least one of the examined organs. The only cat with negative IHC in all the examined organs was the cat n°2 with lesions restricted on the SNC (which were either morphologically consistent with FIP and immunohistochemically positive). FCoV immunodetection was more frequent in

lung and lymph node, followed by spleen, liver and kidney, while small and large intestine were less often found positive (**Table 4.3**).

RT-PCR

As regards for the FIP group, all the cats showed a positive result in at least one of the examined tissues, and 70/92 samples (76.1%) resulted positive, conversely 22/92 tissues (23.9%) resulted negative. The organs which more often tested positive were kidney and mesenteric lymph node, followed by spleen, lung and large intestine, while liver and small intestine gave less frequently positive results (**Table 4.3**).

Considering the non FIP group, 5/13 cats (38.5%) exhibited a positive result in at least one tissue, and overall 6/83 samples (7.2%) were positive, while FCoV PCR was negative in 77/83 samples (92.8%). The organs displaying the most frequent detection of viral RNA resulted mesenteric lymph node and spleen. Kidney and small intestine were positive in only one case, while large intestine, liver and lung never tested positive (**Table 4.3**).

Agreement among histology, immunohistochemistry and PCR

The percentage of agreement (i.e. the percentage of cases on which both the methods classified as negative or positive the same sample) and the concordance expressed as Cohen's kappa coefficient, are reported in **Table 4.4**.

	Histo vs IHC	IHC vs PCR	Histo vs PCR
All tissues	90.3% 0.78 (0.68-0.88)	86.9% 0.72 (0.62-0.83)	80.0% 0.58 (0.46-0.70)
Spleen	88.5% 0.74 (0.46-1.00)	84.0% 0.68 (0.40-0.95)	76.0% 0.51 (0.19-0.83)
Liver	96.2% 0.91 (0.75-1.00)	96.2% 0.91 (0.75-1.00)	92.3% 0.83 (0.60-1.00)
Lymph node	83.3% 0.63 (0.30-0.95)	83.3% 0.67 (0.40-0.95)	66.7% 0.36 (0.03-0.68)
Kidney	88.5% 0.75 (0.50-1.00)	80.8% 0.62 (0.34-0.90)	76.9% 0.54 (0.22-0.86)
Small intestine	95.8% 0.88 (0.66-1.00)	87.5% 0.71 (0.42-1.00)	83.3% 0.61 (0.29-0.93)
Large intestine	96.0% 0.90 (0.70-1.00)	84.0% 0.64 (0.34-0.94)	88.0% 0.74 (0.47-1.00)
Lung	84.0% 0.67 (0.37-0.97)	92.0% 0.83 (0.61-1.00)	76.0% 0.50 (0.15-0.85)

Table 4.4. Percentage of agreement and concordance, expressed as Cohen's kappa coefficient, for all tissues considered together and for the single type of tissues.

The highest rate of agreement and concordance was found between histology and IHC either on the whole set of tissues and on each specific tissue. However, in the lung, the highest rate of agreement and concordance was found between IHC and PCR; while in liver and lymph nodes, IHC and PCR had the same rate of agreement and concordance than histology and IHC.

For what concern histology and IHC, tissues with the highest rate of agreement (close to or higher than 90%) and concordance (almost perfect, i.e. >0.8) were liver, small and large intestine, followed by spleen and kidney, while lung and lymph node had the lowest agreement (80-90%) and concordance (between 0.6 and 0.8, i.e. strong). The highest rate of agreement was found in tissues with a high prevalence of double negative results (i.e. where lesions were not commonly found). About the discordant results, the analyses of individual results (**Table 2**) revealed that the occurrence of negative IHC in tissues with lesions potentially consistent with FIP was more frequent than the contrary. More specifically, IHC was negative in all the 6 samples from the non FIP group and in 4 samples from the FIP group that were histologically consistent with FIP. Conversely, in the FIP group, immunohistochemistry was positive in 7 samples on which no

lesions histologically consistent with FIP were found. Among these, in 2 cases (spleen of cat n° 9 and small intestine of cat n° 7) no lesions at all were histologically detected in the section used for histology, while in the other 5 cases histology revealed lesions not consistent with FIP. It is important to highlight, that the presence of negative histology and positive immunohistochemistry was noted in cats on which almost all the other organs resulted positive for histology and immunohistochemistry, except for one case (cat n°1) in which only the kidney was positive for histology and immunohistochemistry.

As stated above, with the exception of liver, that had an almost absolute agreement and an almost perfect concordance, in all the other organs the agreement varied between 70 and 90% and concordance was strong (0.60-0.80) only for small and large intestine and moderate (0.40-0.60) for kidney and spleen. Lymph nodes had the lower rate of agreement and concordance was weak (<0.40). As regards the discordant results, positive PCR in tissues without lesions consistent with FIP, occurred more frequently than negative PCR in tissues with lesions consistent with FIP. Specifically, PCR was negative in all the 6 samples from the non FIP group and in 1 sample from the FIP group that had lesions histologically consistent with FIP. Conversely, PCR was positive in 21 tissues from FIP cats and in 6 tissues from the NON FIP group on which no lesions consistent with FIP were histologically found. 3/6 cases from the non FIP group did not have histological lesions at all, while in 3/6 cases lymphoid hyperplasia was found. In samples without lesions consistent with FIP, bur PCR-positive, in the FIP group, no lesions at all were detected in 9 cases, while 6 cases had lymphocytic hyperplasia, 2 cases had histiocytic infiltrates and 4 cases had lesions not consistent with FIP.

Finally, agreement between IHC and PCR was higher than 90% and concordance was almost perfect only for liver and lung. In all the other organs, agreement varied between 80 and 90% and concordance could be classified as strong (0.6-0.8). Overall double negative results (negative result in both RT-PCR and IHC) were more common than double positive results (positive result

in both RT-PCR and IHC). The discordant results highlighted how, in most of the cases, PCR was positive in tissues with negative IHC, with one exception (lung of cat n° 11). In particular, this occurred in the same 6 cats from the NON FIP group and in 14/21 tissues on which histology was negative and PCR was positive. Additionally, IHC was negative, with positive PCR in the kidney of cat n° 4 and in the large intestine of cat n° 14, that however, had histological lesions consistent with FIP.

Diagnostic accuracy of immunohistochemistry and RT-PCR

	IHC						PCR					
	Spec	**Sens**	**PPV**	**NPV**	**LR+**	**LR-**	**Spec**	**Sens**	**PPV**	**NPV**	**LR+**	**LR-**
Spleen	100,0	57,1	100,0	66,7	n.c.	0,43	83,3	76,9	83,3	76,9	4,62	0,28
Liver	100,0	57,1	100,0	66,7	n.c.	0,43	100,0	64,3	100,0	70,6	n.c.	0,36
Lymph node	100,0	69,2	100,0	73,3	n.c.	0,31	81,8	84,6	84,6	81,8	4,65	0,19
Kidney	100,0	57,1	100,0	66,7	n.c.	0,43	91,7	85,7	92,3	84,6	10,29	0,16
Small intestine	100,0	50,0	100,0	66,7	n.c.	0,50	91,7	66,7	88,9	73,3	8,00	0,36
Large intestine	100,0	46,2	100,0	63,2	n.c.	0,54	100,0	76,9	100,0	80,0	n.c.	0,23
Lung	100,0	76,9	100,0	80,0	n.c.	0,23	100,0	76,9	100,0	80,0	n.c.	0,23

Table 4.5. Specificity (Spec); sensitivity (Sens); positive predictive value (PPV), negative predictive value (NPV); positive likelihood ratio (LR+) and negative likelihood ratio (LR-) for IHC and PCR on each tissue used in the study.

Immunohistochemistry showed absolute specificity in all tissues and consequently a high positive predictive value. Conversely, the sensitivity was low, as well as the negative predictive value and the negative likelihood ratio, except for lung and lymph node for which, nevertheless, all the values were not satisfactory indicating that a negative immunohistochemistry does not exclude FIP.

Conversely, specificity and positive predictive value of PCR was absolute only for liver, lung and large intestine, although also for kidney and small intestine were very high. Only for spleen and lymph node specificity and PPV are not extremely high but the positive LR is still acceptable for clinical purposes. However, sensitivity and negative predictive values of PCR, although not absolute and probably not relevant for diagnostic purposes, are higher than for IHC. (**Table 4.5**)

DISCUSSION

The histological lesions most frequently detected in the FIP group agreed with the ones described in literature (Kipar et Meli, 2014). The absence of lesions in many histological sections is not unexpected and can be explained with the patchy distribution of FCoV lesions. The dissemination of lesions, in fact, is described to vary among different organs according to the clinical presentation (Kipar et al., 1998). The organs in which the typical histological lesions were less often observed resulted large and small intestine. This finding could be explained also by the rapid occurrence of autolytic changes. However, this occurrence, limited a proper histological evaluation only in three cases. Another possible explanation concerning two cats with exclusively thoracic effusion (n°9, 11), is that a greater number of lesions affected the organs of the thoracic, rather than abdominal, cavity, as demonstrated also by the histological examination of the lung. As regards the NON FIP group, in two cats, granulomatous inflammation potentially consistent with FIP was detected. In one case (kidney of cat n°21), this finding can be related with the onset of an inflammatory process secondary to ischaemic necrosis, caused by disruption of blood flow related with a pulmonary malignancy (Sykes, 2003). In the second case (spleen, liver and mesenteric lymph node of cat n°26), the finding could be caused by a reactive inflammation in response to the chronic formation of large amount of chylous effusion (Padrid, 2000). The presence of lymphoplasmacytic infiltrate was reported in many cases, especially in the kidney. This histological finding is frequently observed and regarded as consistent with FIP, but in order to be considered typical of FIP, it should be accompanied by granulomatous lesions, both macroscopically and microscopically. The presence of lymphoplasmacytic infiltrate in the kidney could be explained by the onset of inflammatory and degenerative changes typical of chronic kidney disease, histologically characterized by lymphoplasmacytic tubulointerstitial nephritis in the cat (Brown et al., 2016). Indeed, cats exhibiting this feature were adult or senior cats (n°17, 19, 23, 26) and in cat n°17 this finding was likely related with amyloid deposition. Moreover, the advanced age, as well as the

absence of suggestive lesions in other organs, make the diagnosis of FIP unlikely (Tasker, 2018). The detection of predominant lymphoplasmacytic infiltrate in small intestine of cat n°15 was due to Mycobacterium avium infection, although the aetiological agent had been observed only in histological sections from liver. Even if it is not possible to rule out the simultaneous presence of FIP and mycobacteriosis, the prompt recovery after specific mycobacteriosis treatment, along with a follow up period longer than a year, decrease the probability of FIP. In the remaining cases, the lymphoplasmacytic infiltrate could be due to the presence of centrilobular necrosis for liver of cat n°27, and of abdominal chylous effusion for kidney, small and large intestine of cat n°26. The less frequent immunohistochemical FCoV detection were in small and large intestine, in concordance with the less frequency of histological lesions. It is quite surprising given the predominantly enteric localisation of FCoV. Since FIP-related lesions tend to follow the course of cranial mesenteric artery and thus to locate mainly on the serosal surfaces of the abdominal organs, it would be logical to expect a higher frequency of immunohistochemical positivity in these organs (Pedersen, 2009). The only cat that did not show any immunohistochemical positivity in the examined organs (n°2) was affected by a non-effusive form of FIP and exhibited exclusively neurological symptoms and a positive immunohistochemistry in brain and cerebellum. A positive immunohistochemical result was interestingly recorded in the large intestine of cat n°22 from the NON FIP group, even if it was restricted to superficial epithelial cells. The presence of FCoV antigen within intestinal epithelial cells from asymptomatic carriers has already been described. Indeed, in healthy infected cats, colonic enterocytes are thought to be the main site of viral persistence in the gut (Kipar et al., 2010). However, since FCoV antigen was not found within a lesion, this finding was recorded as a negative result.

Considering diagnostic accuracy, regardless of the examined organ, the detection of immunohistochemical positivity is always diagnostic for FIP, since specificity and positive predictive value reached 100%. Therefore, the positive predictive value was not measurable. On

the contrary, a negative result does not always allow to exclude FIP. Nevertheless, it should be considered that performing immunohistochemistry on a wide range of organs, increases the probabilities to diagnose FIP. Indeed, considering as 'positive' those animals from which at least one of the sampled organs resulted positive, sensitivity and positive predictive value rise to almost 100%, and negative likelihood ratio almost reaches zero. When diagnostic accuracy was assessed considering as positives only samples which, together with immunohistochemical positivity, exhibited also histological lesions typical of FIP, results were unchanged considering the presence of at least one positive tissue, while a decreased sensitivity was recorded for spleen, small intestine and lung. This means that a positive immunohistochemical result is often, but not always, accompanied by the presence of typical histological lesions, possibly due to the variable distribution of the lesions, as already mentioned. In light of these observations, clearly FIP can not be rule out based only on negative histology and in case of a strong clinical suspicion it is advisable to obtain many sections of the same tissue and perform immunohistochemistry, especially when the targeted organs are spleen, small intestine and lung.

As previously reported, RT-PCR showed an overall higher sensitivity, but lower specificity compared to IHC (Barker et al., 2017). In the FIP group, RT-PCR resulted less frequently positive in liver, in which, however, numerous histological lesions consistent with FIP and immunohistochemical positivity were found. A possible explanation to this discrepancy could be that the viral load present in the tissue section subjected to RT-PCR was too low to be amplified, due to the eneve distribution of lesions and, in turn, of the virus within, as explained above. The detection of viral RNA in NON FIP cats was a predictable finding and has already been described in literature. Indeed, it has been demonstrated that FECV is able to cross the intestinal tract, causing recurrent viremia, and persist in different sites of the organism without causing a disease (Herrewegh et al., 1995; Kipar et al., 2010). In particular, the common evidence of FCoV RNA in mesenteric lymph node and spleen is in line with what previously described in literature about

naturally and experimentally infected cats, which seem to harbour high viral loads in these organs, without developing FIP. Conversely, the absence of RT-PCR positivity in liver and lung of NON FIP cats is partly surprising and in disagreement with those studies which identified these organs as probable FCoV persistence sites in healthy cats. Indeed, liver drains blood coming from the gut and lung can be considered as a 'filter' organ, and both harbour resident macrophages which can guarantee viral persistence (Kipar et al., 2010).

Considering the diagnostic accuracy results, regardless of the examined organ, a positive RT-PCR result cannot be considered diagnostic of FIP. The only two organs which exhibited absolute specificity were liver and lung, for which the positive likelihood ratio was not measurable. At the same time, liver showed the lowest sensitivity, with a negative likelihood ratio too high to rule out FIP in presence in case of a negative result. Conversely, diagnostic accuracy of the kidney was the highest, as demonstrated by the high positive likelihood ratio. Surprisingly, large intestine showed low sensitivity (recorded also with immunohistochemistry) and high specificity. RT-PCR low sensitivity on large intestine can be explained by a decreased viral faecal excretion, which has been demonstrated to occur in FIP affected cats, maybe due to the impaired tropism of mutated FCoV for intestinal epithelial cells (Chang et al., 2012). The organs which showed the lowest specificity resulted mesenteric lymph node (81.8%) and spleen (83.3%). This is a remarkable data, since those organs are frequently sampled for diagnostic purposes. These results demonstrate that a RT-PCR positivity in spleen and lymph node aspirates cannot be considered as diagnostic for FIP. Overall, a low specificity was recorded for RT-PCR in the diagnosis of FIP. This means that it is mandatory to confirm the diagnosis through histology and IHC However, based on results obtained in this work, RT-PCR positivity on liver and lung seems to be highly specific for FIP, and the simultaneous sampling from many organs led to an absolute sensitivity.

As regards of the concordance, in all cases except one, discordant samples resulted negative to immunohistochemistry and positive to RT-PCR. This is not a surprising finding, since RT-PCR has a higher sensitivity than the immunohistochemistry (Kipar et al., 2010; Barker et al., 2017).

Compared to the total, concordant positive samples were obviously more numerous within the FIP group. This does not seem to have affected the concordance between the examined methods, confirming that level of concordance should not be dependent on the composition of groups. As regards of the NON FIP group, the presence of viral RNA in those organs which more commonly gave discordant results is not surprising and agrees with what previously described. In spleen and mesenteric lymph node, indeed, is likely to detect viral RNA in absence of a positive immunohistochemistry. Finally, concordance rate between different organs emerged similar. Conversely, considering the concordance on positive or negative results separately, concordance rate resulted very variable depending on the examined organ. This point out a higher probability to diagnose FIP when information obtained by sampling several organs are combined, or, in alternative, when those organs which revealed a higher concordance are individually sampled. In particular, these organs are firstly liver and lung and then spleen and mesenteric lymph node. Provided that it is always advisable to confirm possible positive or negative RT-PCR results with immunohistochemical examination, data concerning concordance suggest that, when both tests cannot be performed, it would be preferable to examine one of these organs. In fact, the probability to obtain accurate information, even using a single diagnostic method, results good. Instead, when kidney or intestine are sampled, it is always advisable to perform immunohistochemistry, since the concordance of the two methods is not sufficiently high.

V. CANINE GUT MICROBIOTA COMPOSITION IN ASSOCIATION WITH LEISHMANIA INFANTUM

MATERIALS AND METHODS

Animals/Caseload

Dogs enrolled for this study were selected among those referred both to the Veterinary Teaching Hospital of our Institution or to external clinic for routine or diagnostic purpose. Therefore, according to the decision of the Ethical Committee of the University of Milan, residual aliquots of samples or tissues collected under informed consent of the owners can be used for research purposes without any additional formal request of authorization to the Ethical Committee (EC decision 29 Oct 2012, renewed with the protocol n° 02-2016).

Inclusion criteria were: (1) the absence of any antibiotic treatment for a period of at least three months, (2) no probiotic assumption for a period of at least three months, (3) absence of any gastroenteric clinical signs for at least two weeks, (4) negative result at the microscopic fecal exam, (5) negative result for the serologic rapid test 4Dx (evaluating *Dirofilaria immitis* antigen and antibodies against *Anaplasma phagocytophilium*, *Anaplasma platys*, *Borrelia burgdoferi*, *Erlichia canis* and *Erlichia ewingii*).

Based on the screening analyses, dogs were divided into three groups: healthy, asymptomatic but exposed to *Leishmania* and infected symptomatic for canine leishmaniasis. The healthy group (H) included clinically healthy dogs with any clinicopathological alteration, negative IFAT and PCR for *Leishmania*. Exposed asymptomatic group (E_A) was composed by dogs with no clinical signs consistent with both leishmaniasis or other diseases, nor clinicopathological abnormalities. IFAT in this case could be negative or low positive. The history of these animals must include an endemic zone living origin or a past history of IFAT high positive or clinical manifestation of

canine leishmaniosis with a positive outcome. Finally, the infected symptomatic group (S) included sick dogs with clinical signs and clinicopathological abnormalities consistent with *Leishmania,* positive result for IFAT and for one direct test for the evaluation of the presence of the parasite (PCR or cytology).

Sample collection

At admission, 2 mL of whole blood was collected from each dog by venipuncture of the jugular, cephalic od saphenous vein: 1 mL was transferred into an EDTA tube and 1 mL into a plain tube (Venoject, Terumo Italia Srl, Rome, Italy). According to the standard operating procedures of our laboratory whole blood in EDTA was analyzed for a complete blood cell count within 12-18 hours from the sampling. Of the leftover, 200 μL were stored into a 1.5 mL Eppendorf at -20° for subsequent PCR analyses. The blood placed in a plain tube was centrifuged at 2500 x *g* for 5 minutes and the serum was subsequently separated into a 1.5 mL Eppendorf. From each animal, a fresh fecal sample (at least 15 g) was collected and divided into two aliquots: one to use fresh for microscopic fecal examination and one immediately stored frozen at -20° for the microbiota analyses. Finally, a urine sample, taken by cystocentesis or spontaneous micturition, following the good veterinary procedures, and the sample was placed into a 5 mL plain tubes and centrifuged at 1250 x *g* for 5 minutes. Subsequently, the supernatant was separated into a 1.5 mL Eppendorf.

Screening analyses

Screening analyses had the aim to correctly classify the dogs. In cases in which not already available, a complete blood cell count was performed using the automated haematology analyser Sysmex XT-2000iV (Sysmex corporation, Kobe, Japan). On the serum samples a routine basic panel, including at least glucose, creatinine, urea, total protein, albumin, alanine aminotransferase,

and alkaline phosphatase was performed using the automated spectrophotometer BT3500 (Biotecnica instruments S.p.a, Roma, Italia). On urine supernatant, using the same instrument, the urinary protein / urinary creatinine ratio was assessed. An agarose gel serum protein electrophoresis was performed using the semiautomated instrument Hydrasis (Sebia Italia S.r.l., Bagno a Ripoli, Florence, Italy) as described in literature (Giordano et Paltrinieri, 2010). The antibody titer against *Leishmania* was measured through IFAT using the commercial kit MegaFLUO® Leish (Diagnostik Megacor, Hörbranz, Austria). Finally, on whole blood a qPCR for the identification of *Leishmania infantum* was performed. The extraction was done using a commercial kit (NucleoSpin® Blood, Macherey-Nagel, Germania) and following the instruction of the manufacturer. The qPCR was a modified protocol of what reported in literature for mix containing SYBR Green (Francino et al., 2006) using the instrument QuantStudio3 (Applied Biosystem). The positive control was represented by a sample of a dogs previously resulted positive for *Leishmania infantum*, while the negative control was water. Serology for *Dirofilaria immitis* antigen and antibodies against *Anaplasma phagocytophilium*, *Anaplasma platys*, *Borrelia burgdoferi*, *Erlichia canis* and *Erlichia ewingii* was performed using a rapid immunochromatographic test (SNAP 4Dx, IDEXX laboratories).

Microbiota analyses

All the microbiota analyses were performed in an external laboratory. Investigation of microbial communities (Metabarcoding analyses) by amplicon sequencing using hypervariable genomic region (V3-V4 region 16SrRNA gene amplification), was performed with an NGS approach on Illumina Platform. Total DNA was extracted for 180-220 mg of each stool sample using the QIAamp DNA Stool Mini Kit (QIAGEN S.r.l., Milan, Italy) following the manufacturer instruction. Indexed NGS libraries were set up following the Illumina 16srRNA protocol (**Figure 5.1**).

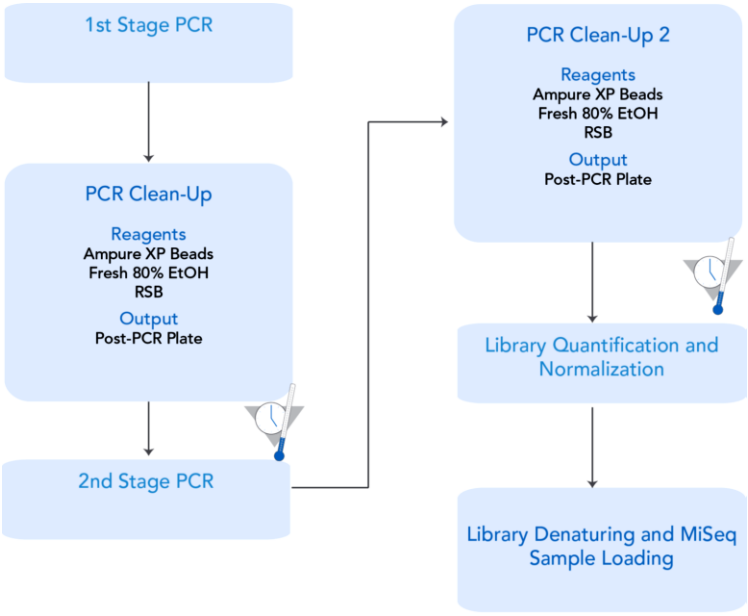

Figure 5.1. 16S library preparation workflow

The first step used PCR to amplify template out of a DNA sample using specific denaturated primers targeting the 16S V3 and V4 region with overhang adapters attached. 1 µL of the PCR product was used on a Bioanalyzer DNA 1000 chip (2100 Bioanalyzer with DNA 1000 Kit, Agilent, Santa Clara, CA, USA) to verify the size (expected size ~550 bp). After the first step of AMPure XP bead clean-up from free primers and primers dimers (A63880I; Beckman Coulter Inc., Brea, CA, USA) a second PCR was performed to attach dual index and Illumina sequencing adapters using the Nextera XT Index kit (Illumina, San Diego, CA, USA). A second AMPure XP bead clean-up step was performed in order to clean up the final library before quantification. 1 µL of a 1:50 dilution of the final library was run on the same Bioanalyzer DNA 1000 chip named above to verify the sample size (expected size ~630 bp). Calculate DNA concentration in nM, based on the size of DNA amplicons, was used to pool the obtained 60 libraries in equimolar concentration. Pooled library was sequenced using the Illumina Miseq technology in 2x300bp run with 20% of PhiX library as control.

Statistical Analyses

Previous standard workflows depended on clustering all 16s rRNA sequences (generated by next generation amplicon sequencing) that occur within a 97% similarity and then assigning these to 'OTUs' from reference trees. These approaches did not incorporate all the data, specifically, sequence quality and statistical information were not incorporated into the results. In contrast, the de novo read counts used here have been constructed through the incorporation of both the quality scores and sequence frequencies in a probabilistic noise model for nucleotide transitions. After filtering the sequences and removing the chimerae, the data were compared to a standard database of bacteria and labeled. The key step in the sequence analysis is the manner in which reads were denoised and assembled into groups called RSVs (Ribosomal Sequence Variants) instead of the traditional OTUs (Operational Taxonomic Units). The workflow was based on software packages from the open-source Bioconductor project (Callahan et al., 2016). The first step included filtering out low-quality sequencing reads and trimming the reads to a consistent length. Then, sequence variants were inferred and a sequence table that is a higher-resolution analogue of the common "OTU table" was produced. These steps were performed using the DADA2 method that relies on a parameterized model of substitution errors to distinguish sequencing errors from real biological variation. A naive Bayesian classifier method was used to assign taxonomy. This classifier compares sequence variants to a training set of classified sequences (GreenGenes V13 gg_13_8_train_set_97). Alpha diversity was assessed using Observed and Shannon metrics. Rarefaction Species curve was produce using "rarecurve" function of Vegan Package (vegan: Community Ecology Package. R package version 2.5-4.). The extent of alpha diversity considering the three groups was evaluated using Analyse-it for Microsoft excel. Specifically, a Kruskal-Wallis test was performed to evaluate the presence of possible differences, followed by a post hoc test in case of significant results. Beta diversity (between sample diversity comparison) was assessed with Non-metric multidimensional scaling (NMDS), principal coordinate decomposition (also called

classical scaling) of a distance matrix (PCOA) chosen to represent the dissimilarity between samples in a low-dimensional space based on Bray-Curtis dissimilarity matrix and Jaccard dissimilarity matrix. The phyloseq-format microbiome data was converted into a DESeqDataSet for Differential Abundance OTU call (McMurdie et Holmes, 2014). DESeq2 performs for each OTU a hypothesis test to see whether evidence is sufficient to decide against the null hypothesis that there is no effect of the A and that the observed difference between A and B was merely caused by experimental variability. Significance level was set at $P < 0.05$.

RESULTS

Screening analyses

A total of 54 dogs were firstly enrolled. After the screening analyses 15 dogs were rejected because they do not comply with inclusion criteria. So, 39 dogs were finally included in this study. Of this, 25 were male and 16 female. 12 dogs were younger than 3 years, while the remaining 28 were older (spans from 3 to 14 years). The most represented breed was the half-breed (26 dogs), while the other 13 were of different breeds (**Table 5.1**).

According to clinical examination and screening analyses results, these dogs were grouped as healthy (14 dogs), exposed asymptomatic (13 dogs) and infected symptomatic (12 dogs).

ID	GROUP	BREED	AGE	GENDER
1	Healthy	Half-breed	1 YY 10 MM	MN
2	Healthy	Medium Schnautzer	1YY 3 MM	FS
3	Healthy	Poodle	1,5 YY	MN
4	Healthy	Half-breed	7 YY	FS
5	Healthy	Half-breed	1 YY	MN
6	Healthy	CKCS	11 YY	MN
7	Healthy	Golden Retriever	5,5 YY	MN
8	Healthy	Half-breed	1,5 YY	FS
9	Healthy	Australia shepherd	7 YY	FS
10	Healthy	Half-breed	14 YY	FS
11	Healthy	Border Collie	5,5 YY	FS
12	Healthy	Half-breed	2YY 6 MM	MN
13	Healthy	West Highland White terrier	4YY 11MM	FS
14	Healthy	Border Collie	9YY	FS
15	Asymptomatic	Half-breed	8 YY	MN
16	Asymptomatic	Half-breed	6 YY	MN
17	Asymptomatic	Half-breed	6 YY	MN
18	Asymptomatic	Half-breed	4 YY	MN
19	Asymptomatic	Half-breed	14 YY	MN
20	Asymptomatic	Half-breed	4 YY	MN
21	Asymptomatic	Half-breed	1 YY	MN
22	Asymptomatic	Half-breed	1 YY	FS
23	Asymptomatic	Half-breed	1 YY	FS
24	Asymptomatic	Half-breed	1 YY	FS
25	Asymptomatic	Half-breed	10 YY 6 MM	MN
26	Asymptomatic	Rhodesian Ridgeback	10 YY	MN
27	Asymptomatic	Half-breed	8 YY	MN
28	Symptomatic	Beagle	6 YY	FS
29	Symptomatic	Half-breed	6 YY	FS
30	Symptomatic	Half-breed	7 YY	FS
31	Symptomatic	Golden Retriever	11 YY	M
32	Symptomatic	Breton	4 YY	M
33	Symptomatic	English Setter	3 YY	M
34	Symptomatic	Half-breed	3,6 YY	F
35	Symptomatic	Bloodhound	7 YY 8 MM	M
36	Symptomatic	Labrador	7 YY	M
37	Symptomatic	Half-breed	8 YY	F
38	Symptomatic	Half-breed	2 YY	M
39	Symptomatic	Half-breed	7 YY	M

Table 5.1. Signalment and groups of the dogs enrolled in this study. YY: years, MM: months, F: female, FS: female spayed, M: male, MN: male neutered.

All the dogs included in the infected symptomatic group had clinical signs consistent with leishmaniasis and one of the direct tests (namely PCR or cytology) resulted positive (**Table 5.2**)

ID	CLINICAL SIGNS	SPE	IFAT	DIRECT TESTS	LAB DATA
28	periocular bilateral alopecia, pinnae bilateral crust	> α2	1:1280	PCR	mild leukopenia with LGL, ↓alb, ↓A/G,
29	alopecia	> γ	1:1280	PCR, CYTOLOGY (spleen, BM)	Severe anaemia normocytic normochromic and thrombocytopenia, mild leukocytosis with mature neutrophilia, ↓alb, ↓A/G,
30	Lymph node enlargment	N	1:160	PCR, CYTOLOGY (BM)	N
31	PU/PD, depression	> α2, β2, γ	1:640	PCR, CYTOLOGY (BM)	Mild normocytic normochromic anaemia, severe proteinuria
32	hyperthermia, lymph nodes enlargment, weight loss	> γ	>1:1280	CYTOLOGY (LN)	Mild macrocytic hypochromic anaemia, LGL, ↑PT, ↓A/G,
33	lymph nodes enlargment, hyperthermia, polyarthritis	> β2, γ	>1:1280	PCR	Moderate macrocytic hypochromic anaemia, lymphopenia, ↑PT, ↓alb, ↓A/G
34	Lameness and poliarthritis	> γ	1:5120	PCR	Severe hypochromic anemia, ↑↑PT, ↓alb, ↓A/G, severe proteinuria
35	Lymph node enlargment anorexia, chachexia, pale mucous membranes.	> β2, γ	1:320	PCR, CYTOLOGY (LN)	Severe normocytic normochromic anaemia, leukopenia, neutropenia, ↓alb, ↓A/G, ↑UREA, ↑CREA
36	Monolateral epistaxis	> α2, β2, γ	1:640	PCR	↑PT, ↓A/G
37	mild depression	> α2, β2, γ	1:160	PCR, CYTOLOGY (LN)	Mild normocytic normochromic anemia, ↑PT, ↓A/G, mild proteinuria
38	Convulsion, bilateral nephropaty, urinary and bladder sediment.	> α2, γ	1:1280	PCR	Severe thrombocytopenia, ↑↑UREA, ↑↑CREA, ↑PT, ↓alb, ↓A/G, ↑ALT
39	dermathitis, PU/PD	>β2, γ	1:160	PCR	leukocytosis with eosinophilia, LGL, ↑PT, ↓A/G,

Table 5.2. Clinical signs and laboratory results of the infected symptomatic dogs. N: normal, BM: bone marrow.

Microbiota analysis

A total of 10,137,881 sequences, with an average of 259,946 sequences/sample (median 273,190, range 69,776 - 273,190) were of adequate quality and were subsequently analysed.

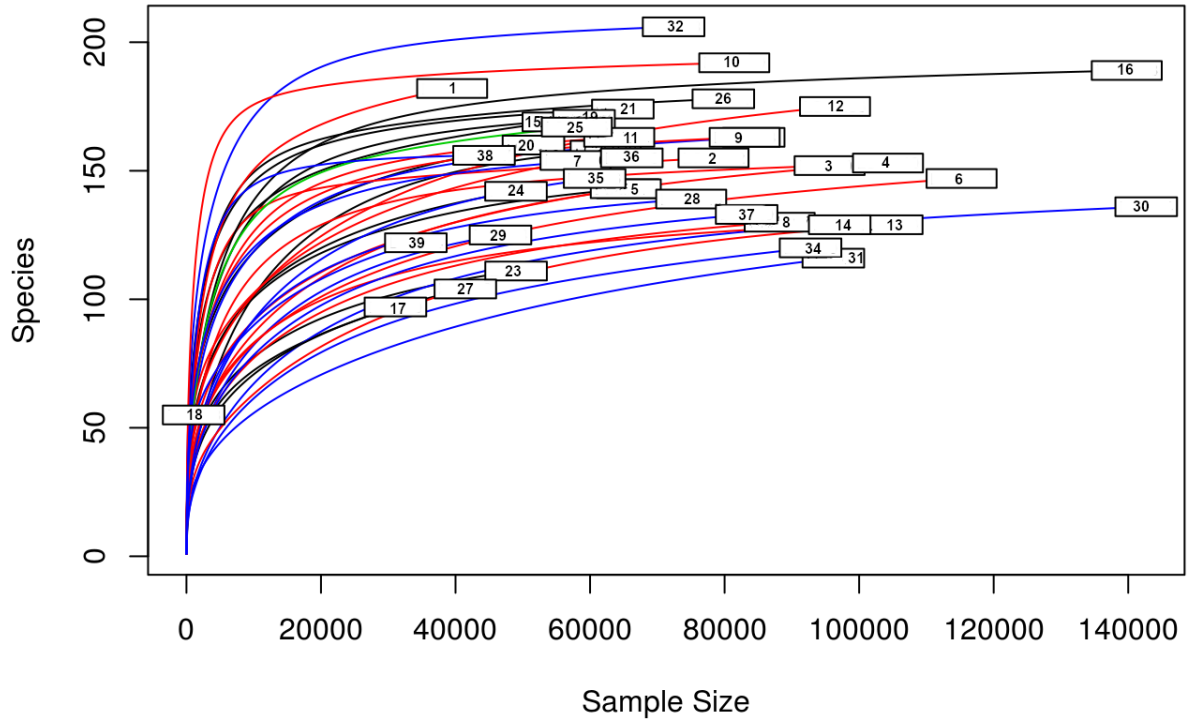

Figure 5.1. Alpha – diversity for the entire caseload. Each line represents one sample: in blue those belonging to S group, in red the H, in black the E_A. The green line represents one sample that was subsequently discarded. On the x-axis the number of sequences is found, while on the y-axis the number of different species is represented.

The alpha-diversity rarefaction curves confirmed the good quality of the caseload, since most of the sample curves grew very quickly and some of them reached a proper plateau, showing a high richness of bacterial species in each of the sample, even in those from which a lower number of sequences was extracted (**Figure 5.1**). In the beta-diversity graph (**Figure 5.2**), the group of symptomatic dogs (in red), seemed to be clustered differently with respect of the other two groups. Indeed, blue and light blue, referred to E_A and H respectively were apparently closer to each other, and this could mean that the microbial communities were similar in composition.

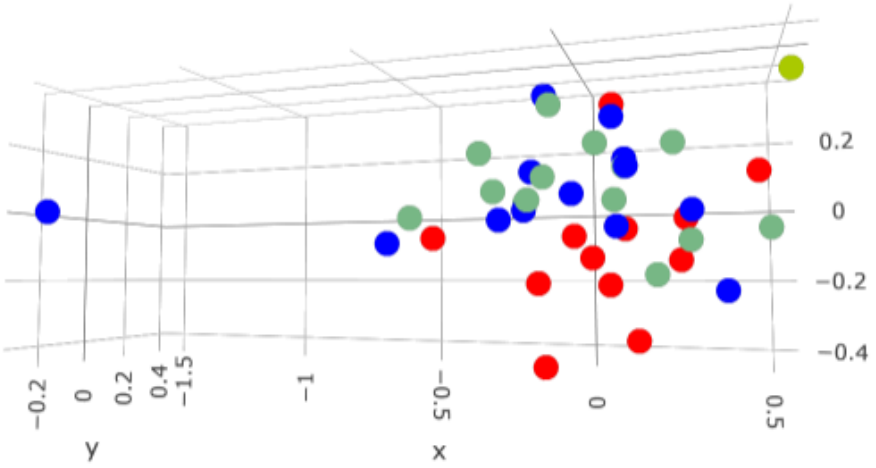

Figure 5.2. Beta-diversity of the whole caseload. Each dot represents one sample, in blue the exposed asymptomatic, in light blue the healthy dogs, in red the symptomatic dogs. The green dot is referred to a sample excluded from the study (N=null). On each axis is reported the variance.

For what concern phyla, *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Cyanobacteria* were the most represented phyla in all the caseload (**Figure 5.3**). The statistical analysis highlighted some significant differences. *Firmicutes* resulted more represented in the healthy (H) group, compared to the both the asymptomatic (E_A) and symptomatic (S) group (P =0.007 and =0.046 respectively). Proteobacteria was more abundant in S, compared to the H (P =0.004). Moreover, some differences were found also in less abundant phyla: [*Thermi*] was present in lower proportion in asymptomatic dogs than in healthy (P =0.049); *Spirochaetes* were less abundant in symptomatic dogs compared to both the other two groups (P =0.02 vs H and =0.001 vs E_A). Finally, *Deferribacteres* and *Tenericutes* were less abundant in symptomatic dogs compared to the asymptomatic ones (P =0.016 and =0.02 respectively).

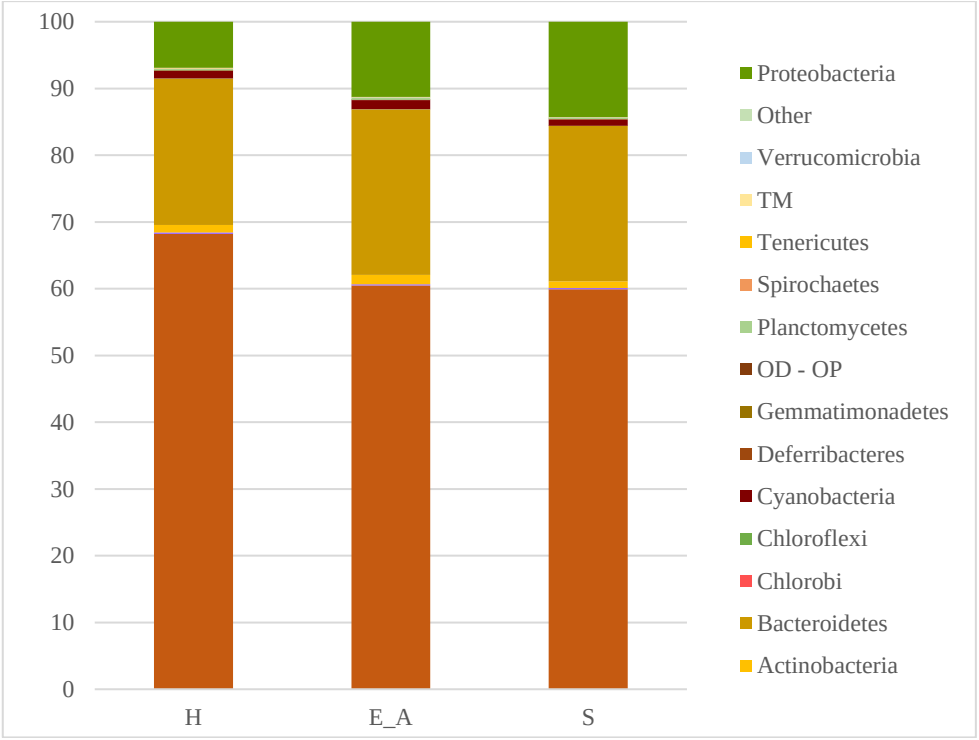

Figure 5.3. Bar plot representation of phylum relative abundance in the three groups.

For what concern class comparison, Clostridia was present in higher abundance in the healthy group, compared to the asymptomatic dogs (P =0.03), as well as Erysipelotrichi, compared to the symptomatic dogs (P =0.048). On the contrary, Gammaproteobacteria was less represented in H, compared to both the other groups (P =0.043 in E_A and =0.0003 in S). Again, even for rarest classes, significant differences were highlighted. Deinococci was less represented in asymptomatic than in healthy dogs (P =0.045), while [Brachyspirae], Deferribacteres and Mollicutes were more abundant in asymptomatic than in symptomatic dogs (P =0.007, =0.02 and =0.044 respectively) (**Figure 5.4**).

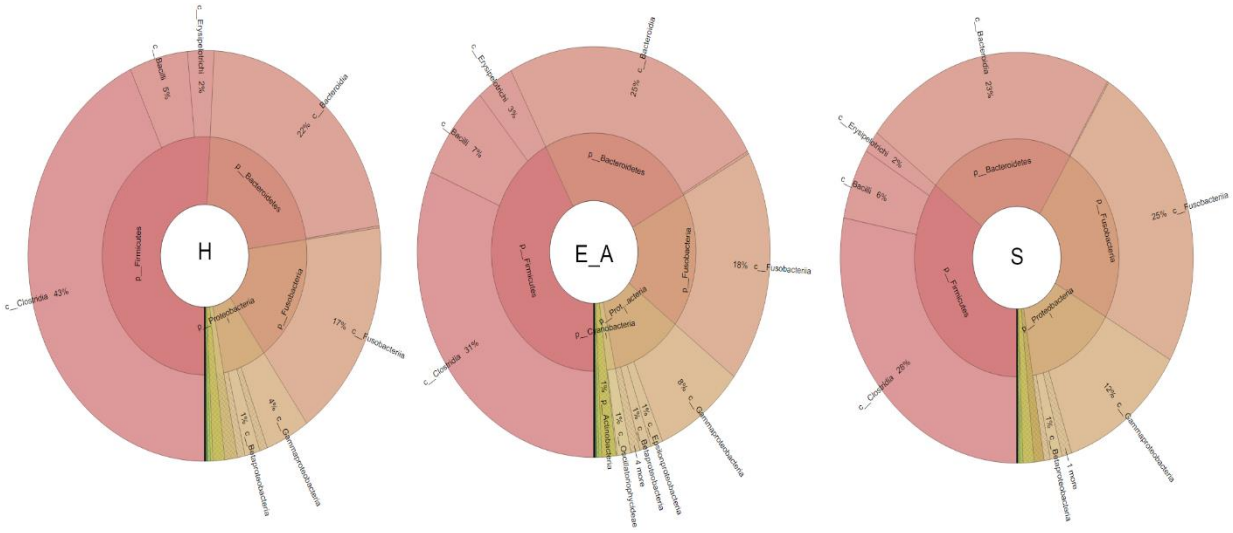

Figure 5.4. Microbiota composition of the group of healthy (H), exposed asymptomatic (E_A) and symptomatic (S) dogs represented through a Krona chart that illustrate phyla and classes of the bacteria sequenced.

Moreover, significant differences were reported when the analysis was extended to order, family and genus (**Table 5.3 - Table 5.5)**

ORDER	**H**	**E_A**	**S**
[Brachyspirales]	0,019	0,020^{a*}	0,003^{a*}
Aeromonadales	0,844^{a*}	0,959^{b*}	0,114^{a*,b*}
Anaeroplasmatales	0,042	0,0245^{a}	0,010^{a}
Bifidobacteriales	0,026^{a**}	0,007	0,011^{a**}
Deferribacterales	0,044^{a*}	0,034b	0,074^{a*,b}
Enterobacteriales	2,722^{a**,b**}	6,668^{a**}	11,243^{b**}
Erysipelotrichales	2,423	3,388^{a}	1,704^{a}
Lactobacillales	2,362^{a}	4,897^{a}	4,825
Pasteurellales	0,006^{a}	0,030	0,021^{a}
Streptophyta	0,010^{a}	0,092^{a}	0,018

Table 5.3. Relative abundances of the classes that resulted significantly different among the three groups. For each class, the paired letters mean that a significant difference was found between groups. When no other symbol is present P <0.05, * = P <0.01 and ** = P <0.001.

FAMILY	**H**	**E_A**	**S**
[o__Streptophyta]	0,010^{a*}	0,092^{a*}	0,018
[o__Bacteroidales]	0,042	0,037^{a}	0,023^{a}
[Tissierellaceae]	0,127^{a}	0,155	0,143^{a}
Actinomycetaceae	0,006^{a}	0,016^{b}	0,072^{a,b}
Bifidobacteriaceae	0,026^{a*}	0,007	0,011^{a*}
Brachyspiraceae	0,019	0,020^{a*}	0,003^{a*}
Clostridiaceae	14,061^{a**}	2,688^{a**,b*}	6,974^{a*}
Deferribacteraceae	0,044^{a*}	0,034^{b}	0,074^{a*,b}
Enterobacteriaceae	2,722^{a**,b**}	6,668^{a**}	11,243^{b**}
Enterococcaceae	0,021^{a*}	1,091	0,874^{a*}

Erysipelotrichaceae	2,423	3,388[a]	1,704[a]
Flavobacteriaceae	0,030[a]	0,067[a]	0,0324
Hyphomicrobiaceae	0,171	0,209[a]	0,130[a]
Micrococcaceae	0,024[a]	0,015	0,006[a]
Pasteurellaceae	0,006[a]	0,030[a]	0,0210
Porphyromonadaceae	0,114[a**,b**]	0,684[a**]	1,613[b**]
Prevotellaceae	5,403[a]	10,224[b*]	0,557[a,b*]
Succinivibrionaceae	0,844[a*]	0,959[b]	0,114[a*,b]
Turicibacteraceae	2,846[a]	2,433	0,657[a]
Veillonellaceae	2,810[a*]	1,961[b*]	0,809[a*,b*]

Table 5.4. Relative abundances of the families that resulted significantly different among the three groups. For each family, the paired letters mean that a significant difference was found between groups. When no other symbol is present P <0.05, * = P <0.01 and ** = P <0.001.

GENUS	**H**	**E_A**	**S**
Actinomyces	0.006[a*]	0.016[b]	0.068[a*,b]
Adlercreutzia	0.058[a]	0.136[b**]	0.0243[a,b**]
Allobaculum	0.414	1.067[a]	0.276[a]
Anaerobiospirillum	0.732	0.924[a]	0.103[a]
Anaerofilum	0.009[a]	0.009	0.109[a]
Brachyspira	0.019	0.020[a*]	0.003[a*]
Catenibacterium	0.628[a*]	0.680[b*]	0.0618[a*,b*]
Clostridium	13.549[a,b**]	2.414[a,c*]	6.603[b**,c*]
Dorea	1.624[a*]	2.034[b*]	3.659[a*,b*]
Enterococcus	0.020a[**,b*]	1.091[a**]	0.874[b*]
Escherichia	2.674[a,b*]	6.6285[a]	10.142[b*]
Fusobacterium	2.4635[a]	3.454	5.584[a]
Holdemania	0.023[a]	0.024	0.001[a]
Megamonas	2.214[a**]	1.222[b*]	0.189[a**,b*]
Mesorhizobium	0[a*]	0.006[a*]	0.002
Mucispirillum	0.044[a*]	0.034[b]	0.074[a*,b]
Parabacteroides	0.096[a**]	0.557[a**]	0.115
Peptostreptococcus	0.001[a*]	0.006	0.024[a*]
Phenylobacterium	0.011[a]	0.001[a]	0.003
Porphyromonas	0.0005[a,b**]	0.108[a]	1.48[b**]
Prevotella	5.403[a]	10.224[b*]	0.557[a,b*]
Proteus	0.0002[a*]	0.001[b]	1.078[a*,b]
Roseburia	0.086[a]	0.029[b**]	0.276[a,b**]
Ruminococcus	0.103	0.096[a]	0.202[a]
Streptococcus	2.12	3.6228[a]	3.512[a]
Turicibacter	2.846[a*]	2.433	0.657[a*]

Table 5.5. Relative abundances of the genus that resulted significantly different among the three groups. For each genus, the paired letters mean that a significant difference was found between groups. When no other symbol is present P <0.05, * = P <0.01 and ** = P <0.001.

DISCUSSION

The study of microbiota composition aroused a lot of interest in human medicine and it is a new research field in veterinary medicine too. The most represented phyla reported in previous studies in healthy dogs were *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Proteobacteria*, and this was confirmed also by the results of this thesis (Handl et al., 2011). The lower abundance of *Firmicutes* in the diseased animals, found in this study, is in agreement with what reported in literature, where this phylum is usually more represented in healthy animals (Suchodolski, 2016). The same consideration could be applied on *Clostridia* and *Erysipelotrichi* classes, which resulted more abundant in the microbiota of healthy dogs. *Proteobacteria* phylum is of particular concern in veterinary medicine, since it includes several gastrointestinal pathogens such as, for example, Escherichia coli. It is interesting to note that this phylum was more represented in symptomatic dogs, compared to the healthy ones. This finding has already been reported in literature (Zheng et al., 2018; Suchodolski et al., 2012b) and it could suggest the presence of dysbiosis, even in absence of overt gastrointestinal signs of disease. Considering the main class belonging to *Proteobacteria* phylum, meaning the Gammaproteobacteria, a significant higher abundance was found not only in the symptomatic, but also in the asymptomatic dogs, compared to the healthy ones and the same significant differences were maintained when the analyses were deepened until the genus level (*Enterococcus* and *Proteus*).

For what concerns results at genus level, a lower proportion of *Mucispirillum* (Fam. Deferribacteraceae) was found in symptomatic dogs compared to both the other two groups. *Mucispirillum* has been reported in mice gut microbiota and one study highlighted its protective role against Salmonella infection (Herp et al., 2019). Even though the same protective mechanism has not been proven in dogs, the lower proportion recorded in symptomatic dogs could be a sign of dysbiosis or of increase susceptibility to gastrointestinal disorder.

Interestingly, most of the family and genus less represented in symptomatic dogs (Veillonellaceae, *Megamonas*, *Prevotella*, *Catenibacterium* and *Clostridium*) belong to the phyla *Firmicutes* and *Bacteroidetes* and had a role in the production of short chain fatty acids (SCFAs). These metabolites (namely acetate, propionate and butyrate) derived from the fermentation of simple charbohydrates had a pivotal role in the nutrition of enterocytes, but also some immunomodulatory effects. Indeed, it is described the stimulation of anti-inflammatory cytokines production (e.g. IL-10 and TGFβ) and the decrease of proinflammatory cytokines (e.g. IL-6, IL-8 and TNFα) (Minamoto et al., 2019; Macfarlane et Macfarlane, 2012). Even though a metabolomic approach was not the aim of this study, it could be interesting to evaluate a possible decrease of SCFAs in symptomatic dogs that could possibly be in relationship with the presence of clinical disease.

Differences in symptomatic and asymptomatic compared to the healthy dogs were expected, since it is already reported the presence of an altered microbiota composition in dogs with disease other than gastrointestinal ones (e.g. multicentric lymphoma, diabetes mellitus) (Jergens et al, 2019; Gavazza et al., 2018). However, results in literature about microbiota composition in healthy or diseased dogs are not always concordant. Moreover, it should not be forgotten that factors other than the presence of disease could alter microbiota composition. In literature, even if several studies have been reported about canine gut microbiota, there is still a lack of investigation on a large caseload of healthy animals, which have been deeply investigated in people. This fact could possibly prevent reliable consideration about differences in gut microbiota composition between healthy and sick animals. All the dogs enrolled in this study were fed only on commercial food in order to reduce at least this possible confounding factor. However, the type of commercial food was not the same for every dog, hence it is not known the extent of the diet influence on our caseload. Moreover, environment, age and body condition score could affect the microbiota composition, not mentioning the individual variability also present in the healthy population. Thus, a larger caseload could increase the probability that significant difference found in this study were

specifically linked to the presence or absence of a clinical disease or infection. Hence further studies, possibly on a higher number of animals, are needed to better define the extent of changes in microbiota composition and possibly their correlation with the immune response evaluated also by the measurement of cytokine expression, both local and systemic.

VI. CORRELATION BETWEEN CANINE GUT MICROBIOTA COMPOSITION AND LEUKOCYTES CLASSES ASSESSED THROUGH FLOW CYTOMETRY IN ASSOCIATION WITH LEISHMANIA INFANTUM INFECTION

MATERIALS AND METHODS

Animal/Caseload and Microbiota Analyses

Inclusion criteria and sampling methods were the same as in study V. Results from screening and microbiota analyses of study V were used in this further study.

Flow Cytometry Analyses

Whole blood collected into an EDTA tube was analysed in a maximum of 48 hours, according to the standard operating procedures of our laboratory. Flow cytometry was used to access the leukocytes population of CD4+, CD8+, CD5+, CD14+, CD11b, CD21 and CD4+/CD8+ using the flow cytometer BryCyte E6 (Mindray, Schengen, China) and the results were analysed through a specific software (MRFlow, Mindray, Schengen, China). Specifically, three cytoflow tubes were prepared for each sample, containing a whole blood volume decided based on the formula 500/WBC, so that each tube contained exactly $500x10^3$ cells. Then, 25 µL of RPMI solution were added in order to reduce the unspecific antibodies binding. In each tube 50 µL of the specific antibody dilution was added, following this scheme (see **Table 6.1**):

- First tube: none (negative control)
- Second tube: CD5, CD8, CD4

- Third tube: CD14, CD11b, CD21

Antibody	Clone	Fluorocrome	Specificity	Producer	Dilution
CD5	YKIX322.3	FITC	T lymphocytes	Serotec, Oxford, UK	1:400
CD8	YCATE55.9	PE	T cytotoxic lymphocytes	Serotec, Oxford, UK	1:35
CD4	YKIX302.9	AF-647	T helper lymphocytes, neutrophils	Serotec, Oxford, UK	1:50
CD14	TUK4	PE	Monocyte	Serotec, Oxford, UK	1:25
CD11b	M1/70	PE-cy5	Neutrophils and monocytes	eBioscience, San Diego, CA, USA	1:500
CD21	CA2.1D6	AF-647	B lymphocytes	Serotec, Oxford, UK	1:200

Table 6.1. List of the antibodies in use and their dilution is shown.

Each tube incubates for at least 15 minutes at room temperature. The erythrocytes were lysed using an 8% ammonium chloride lysant solution and then washed through centrifugation for 1250 rpm for 8 minutes. The supernatant was discarded and the remaining pellet was resuspended in 500 µL of PBS 1x and read by the flow cytometer. The cells were classified in a dot plot based on cellular size (Forward Scatter, FSC) and complexity (Side Scatter, SSC). On this graph a gate was placed in order to exclude platelets and cellular debris from the analyses. Subsequently, cell populations were analysed based on complexity and fluorescence. Then, for each sample the percentage of CD5, CD8, CD4 (only with low complexity, in order to include only lymphocyte), CD14, CD11b, CD21 positive cells was recorded. The percentage of neutrophils was obtained using the formula: CD11b-CD14.

All the percentages obtained were then related to the white blood cells total number, in order to calculate the absolute number for each leukocyte subset.

Statistical Analysis

Statistical analysis was performed using Analyse-it for Microsoft Excel (Analyse-it Software Ltd, Leeds, United Kingdom). Statistical significance was set at $P < 0.05$. Specifically, differences in each leukocytes subset among the three different group were evaluated using Kruskal-Wallis test, followed, when statistically relevant results were found, by a Wilcoxon Mann-Whitney test. These comparisons have been performed for both the percentages and the absolute number of leukocytes classes. Correlation between microbiota phylum and absolute number of leukocytes classes was performed using the Spearmann's test.

RESULTS

For what concern the composition of the three groups, see the results of the study V above. For dogs 11, 17 and 36 it was not possible to collect enough EDTA whole blood sample to perform the flow cytometric evaluation. On the other hand, two additional dogs, 40 and 41, belonging respectively to asymptomatic and symptomatic groups, have been included in this part of the study. For these two dogs it was not possible to collect a proper faecal sample for the microbiota evaluation.

Flow cytometry evaluation was finally performed on 38 dogs. For 36 out of 38, microbiota composition results were available for the correlation (**Table 6.2**).

ID	GROUP	BREED	AGE	GENDER	MICROBIOTA
1	Healthy	Half-breed	1 YY 10 MM	MN	YES
2	Healthy	Medium Schnautzer	1YY 3 MM	FS	YES
3	Healthy	Poodle	1,5 YY	MN	YES
4	Healthy	Half-breed	7 YY	FS	YES
5	Healthy	Half-breed	1 YY	MN	YES
6	Healthy	CKCS	11 YY	MN	YES
7	Healthy	Golden Retriever	5,5 YY	MN	YES
8	Healthy	Half-breed	1,5 YY	FS	YES
9	Healthy	Australia shepherd	7 YY	FS	YES

10	Healthy	Half-breed	14 YY	FS	YES
12	Healthy	Half-breed	2YY 6 MM	MN	YES
13	Healthy	West Highland White terrier	4YY 11MM	FS	YES
14	Healthy	Border Collie	9YY	FS	YES
15	Asymptomatic	Half-breed	8 YY	MN	YES
16	Asymptomatic	Half-breed	6 YY	MN	YES
18	Asymptomatic	Half-breed	4 YY	MN	YES
19	Asymptomatic	Half-breed	14 YY	MN	YES
20	Asymptomatic	Half-breed	4 YY	MN	YES
21	Asymptomatic	Half-breed	1 YY	MN	YES
22	Asymptomatic	Half-breed	1 YY	FS	YES
23	Asymptomatic	Half-breed	1 YY	FS	YES
24	Asymptomatic	Half-breed	1 YY	FS	YES
25	Asymptomatic	Half-breed	10 YY 6 MM	MN	YES
26	Asymptomatic	Rhodesian Ridgeback	10 YY	MN	YES
27	Asymptomatic	Half-breed	8 YY	MN	YES
28	Symptomatic	Beagle	6 YY	FS	YES
29	Symptomatic	Half-breed	6 YY	FS	YES
30	Symptomatic	Half-breed	7 YY	FS	YES
31	Symptomatic	Golden Retriever	11 YY	M	YES
32	Symptomatic	Half-Breed	3 YY	M	YES
33	Symptomatic	Half-breed	5 YY	F	YES
34	Symptomatic	Half-breed	3,6 YY	F	YES
35	Symptomatic	Bloodhound	7 YY 8 MM	M	YES
37	Symptomatic	Half-breed	8 YY	F	YES
38	Symptomatic	Half-breed	2 YY	M	YES
39	Symptomatic	Half-breed	7 YY	M	YES
40	Asymptomatic	Half-breed	1 YY 6 MM	M	NO
41	Symptomatic	Half-breed	6,7 YY	M	NO

Table 6.2. Signalment, groups and availability of microbiota composition for the dogs enrolled in this study. YY: years, MM: months, F: female, FS: female spayed, M: male, MN: male neutered.

Comparison of percentages of leukocytes classes among three groups

The comparison of the percentages of leukocyte classes resulted in the absence of any statistical significance when considering CD14+ (corresponding with monocytes, P =0.63) and CD4:CD8 ratio (P =0.34) (**Figure 6.1**).

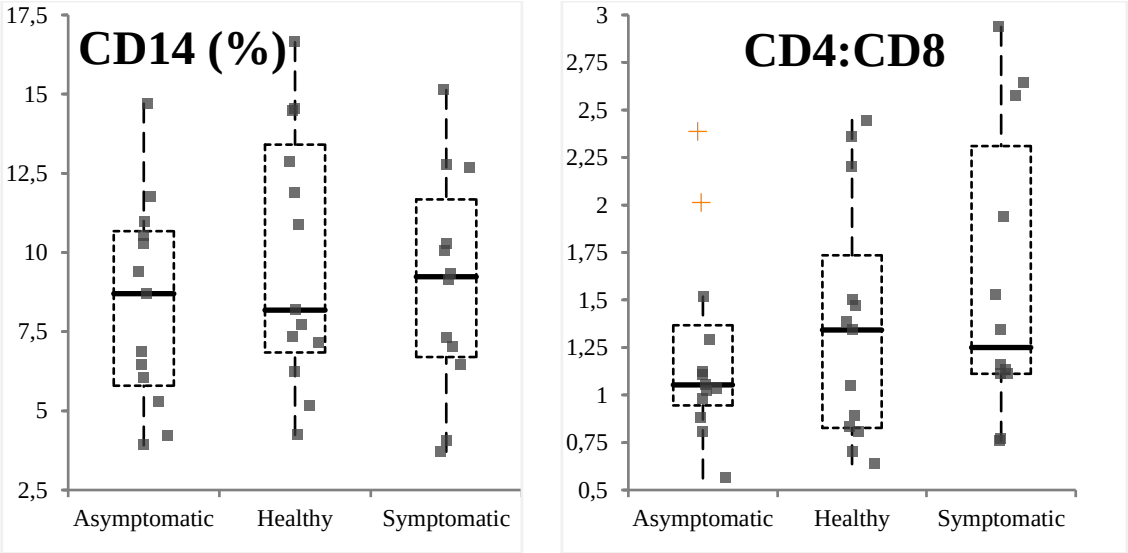

Figure 6.1. Comparison of the percentage of CD14+ cells (left) and CD4:CD8 ratio (right) among the group ASYMPTOMATIC, HEALTHY and SYMPTOMATIC. The orange crosses represent the near outliers.

For what concern all the other classes, a significant difference has been observed for CD11b+ (P =0.0015), Neutrophils (P =0.011), CD5+ (P =0.0045), CD4+ (P =0.022), CD8+ (P =0.017) and CD21+ (P =0.002). Specifically, CD11b and Neutrophils were lower in the HEALTHY group compared both to the ASYMPTOMATIC (P =0.022 and P =0.026 respectively) and SYMPTOMATIC group (P =0.0009 and P =0.0077 respectively). The comparison between SYMPTOMATIC and ASYMPTOMATIC did not result in significant differences (**Table 6.3** and **Figure 6.2**).

	Group	Median	IQR	95% CI		Min	Max
CD11b+ (%)	**Asymptomatic**	79,91	8,36	73,09	to 81,76	65,76	90,72
	Healthy	72,82	5,88	69,68	to 75,63	65,30	83,62
	Symptomatic	83,32	15,31	76,35	to 92,37	71,79	94,42
Neutrophils (%)	**Asymptomatic**	70,49	13,94	61,50	to 77,01	56,37	84,68
	Healthy	62,82	11,29	56,83	to 68,79	52,43	75,44
	Symptomatic	74,07	18,86	67,02	to 86,48	59,11	90,71

Table 6.3. Percentages of CD11b+ cells and neutrophils in dogs belonging to ASYMPTOMATIC, HEALTHY and SYMPTOMATIC group. For each group median, interquartile range, 95% confidence interval and maximum and minimum value has been reported.

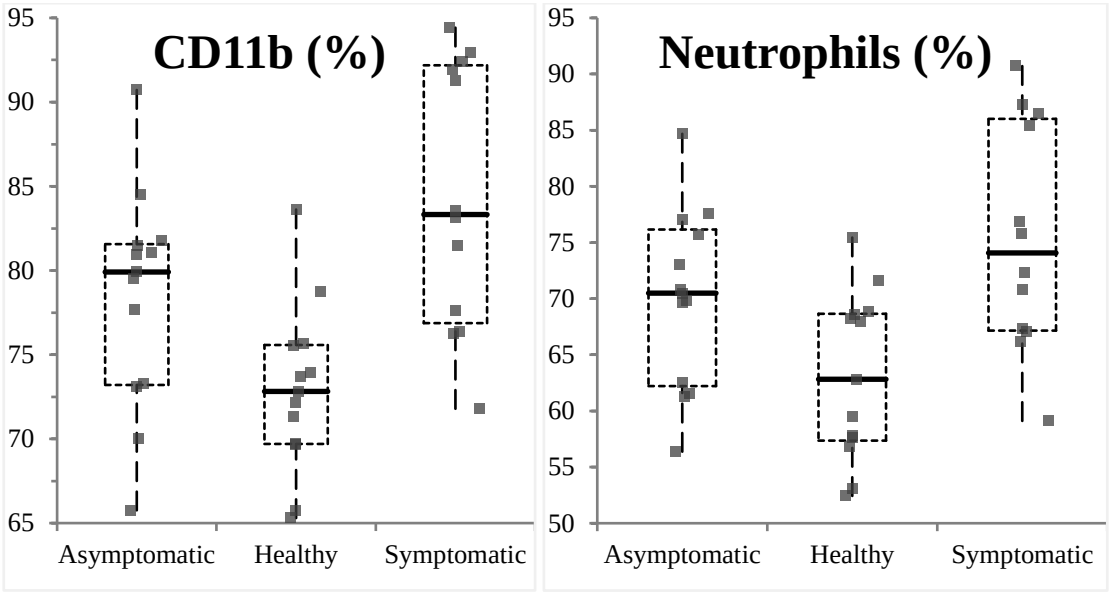

Figure 6.2. Comparison of the percentage of CD11b+ cells (left) and Neutrophils (right) among the group ASYMPTOMATIC, HEALTHY and SYMPTOMATIC.

For what concern the lymphocytes classes, CD21+ and CD8+ cells were significantly lower in the SYMPTOMATIC group compared to both ASYMPTOMATIC (P =0.0019 and 0.0039 respectively) and HEALTHY (P =0.0028 and P =0.0077), while no differences were recorded in the comparison between these latter two groups. On the other hand, CD4+ and CD5+ were significantly lower in the SYMPTOMATIC group, compared to the HEALTHY one (P =0.0055 and P =0.0023 respectively), but not to dogs belonging to the ASYMPTOMATIC group (**Table 6.4** and **Figure 6.3**)

	Group	Median	IQR	95% CI		Min	Max
CD21+ (%)	**Asymptomatic**	5,81	2,59	4,49	to 7,67	2,57	15,15
	Healthy	5,62	4,64	3,43	to 10,43	0,83	11,26
	Symptomatic	1,30	1,70	0,54	to 2,58	0,19	8,43
CD8+ (%)	**Asymptomatic**	5,77	2,80	3,61	to 6,54	2,29	7,72
	Healthy	5,61	2,92	3,84	to 7,93	3,47	9,89
	Symptomatic	3,04	3,52	1,55	to 5,13	0,64	7,27
CD4+ (%)	**Asymptomatic**	5,01	4,53	3,74	to 8,76	2,57	9,67
	Healthy	7,08	2,84	5,60	to 8,49	3,83	12,70
	Symptomatic	3,99	3,69	2,08	to 5,94	1,88	10,43
CD5+ (%)	**Asymptomatic**	13,09	5,74	8,91	to 16,96	6,68	21,64
	Healthy	14,91	5,73	13,43	to 19,71	10,24	29,50
	Symptomatic	9,35	9,34	4,26	to 13,81	3,01	17,46

Table 6.4. Percentages of CD21+ cells and neutrophils in dogs belonging to ASYMPTOMATIC, HEALTHY and SYMPTOMATIC group. For each group median, interquartile range, 95% confidence interval and maximum and minimum value has been reported.

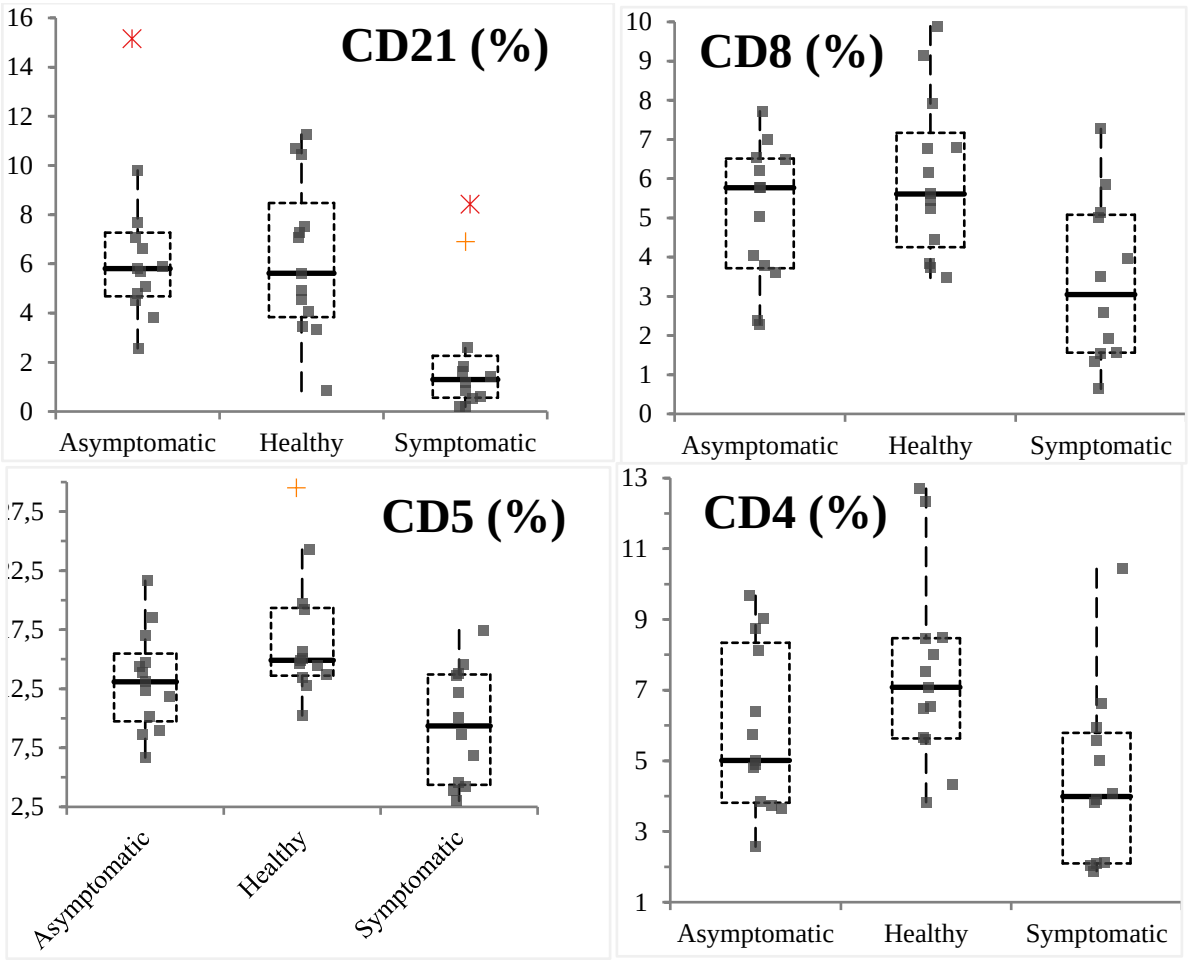

Figure 6.3. Comparison of the percentage of CD21+ cells (upper left), CD8+ cells (upper right), CD5+ cells (lower left) and CD4+ cells (lower right) among the group ASYMPTOMATIC, HEALTHY and SYMPTOMATIC. The orange crosses represent the near outliers, while the red stars represent the outliers.

Comparison of the absolute number of leukocytes classes among three groups

The comparison of the absolute number of leukocyte classes resulted in the absence of any statistical significance when considering CD14+ (P =0.51), CD11b+ (P =0.44) and Neutrophils (P =0.43) among the three groups considered (**Figure 6.4**).

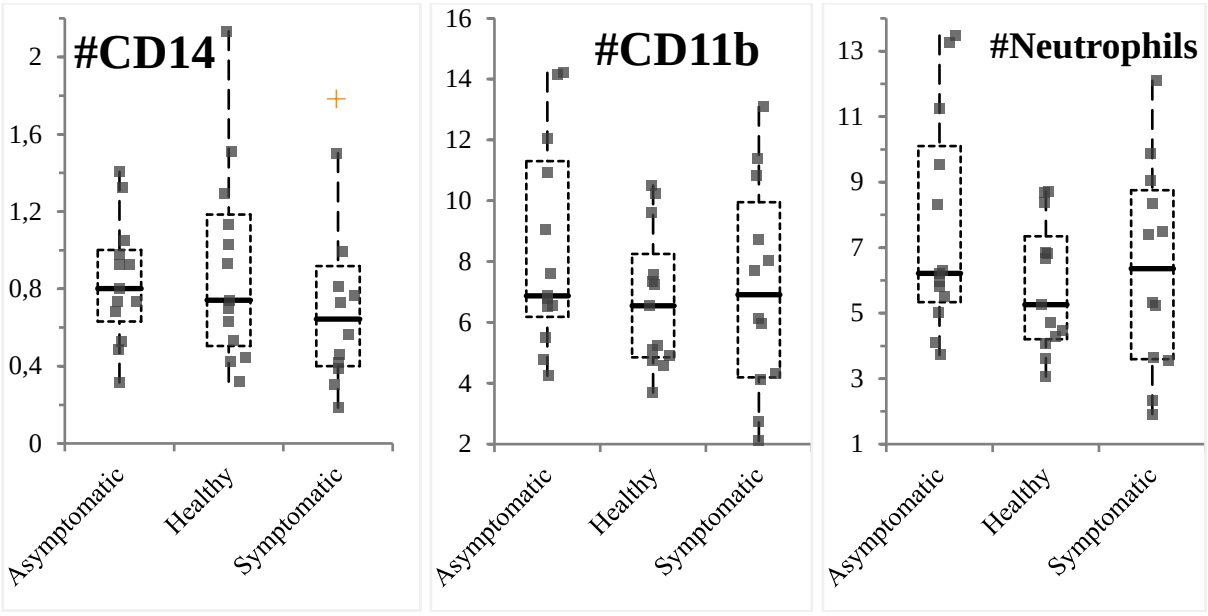

Figure 6.4. Comparison of the absolute value (expressed as x10³/µL) of CD14+ cells (left), CD11b+ cells (centre) and neutrophils (right) among the group ASYMPTOMATIC, HEALTHY and SYMPTOMATIC. The orange crosses represent the near outliers.

For what concern lymphocytes classes, a significant difference has been observed in CD5+ (P =0.0042), CD4+ (P =0.0018), CD8+ (P =0.0043) and CD21+ (P =0.0004) among the three groups (**Table 6.5**).

		Median	IQR	95% CI	Max	Min
CD5+ (x10³/µL)	Asymptomatic	1,37	0,61	0,91 to 1,66	2,05	0,61
	Healthy	1,68	0,98	0,90 to 1,90	2,96	0,83
	Symptomatic	0,51	0,73	0,33 to 1,09	2,03	0,31
CD4+ (x10³/µL)	Asymptomatic	0,63	0,30	0,35 to 0,70	0,81	0,34
	Healthy	0,70	0,46	0,39 to 0,91	1,27	0,27
	Symptomatic	0,28	0,30	0,16 to 0,52	0,63	0,13
CD8+ (x10³/µL)	Asymptomatic	0,50	0,32	0,30 to 0,69	1,13	0,17
	Healthy	0,49	0,45	0,30 to 0,79	0,10	0,21
	Symptomatic	0,14	0,26	0,11 to 0,46	0,75	0,07
CD21+ (x10³/µL)	Asymptomatic	0,66	0,38	0,34 to 0,80	1,10	0,22
	Healthy	0,55	0,67	0,28 to 1,04	1,56	0,05
	Symptomatic	0,10	0,20	0,03 to 0,27	0,66	0,01

Table 6.5. Absolute values expressed as x10³/µL of CD5+, CD4+, CD8+ and CD21+ cells in dogs belonging to ASYMPTOMATIC, HEALTHY and SYMPTOMATIC group. For each group median, interquartile range, 95% confidence interval and maximum and minimum value has been reported.

Specifically, in all those cases, lymphocyte belonging to classes of CD5+, CD4+, CD8+ and CD 21+ were lower in the SYMPTOMATIC group compared to both ASYMPTOMATIC (P =0.0047, 0.0028, 0.0039 and 0.0016 respectively) and HEALTHY (P =0.0047, 0.0028, 0.0047 and 0.0003

respectively). On the other hand, the comparison between HEALTHY and ASYMPTOMATIC did not result in significant differences, in any lymphocyte class (**Figure 6.5**).

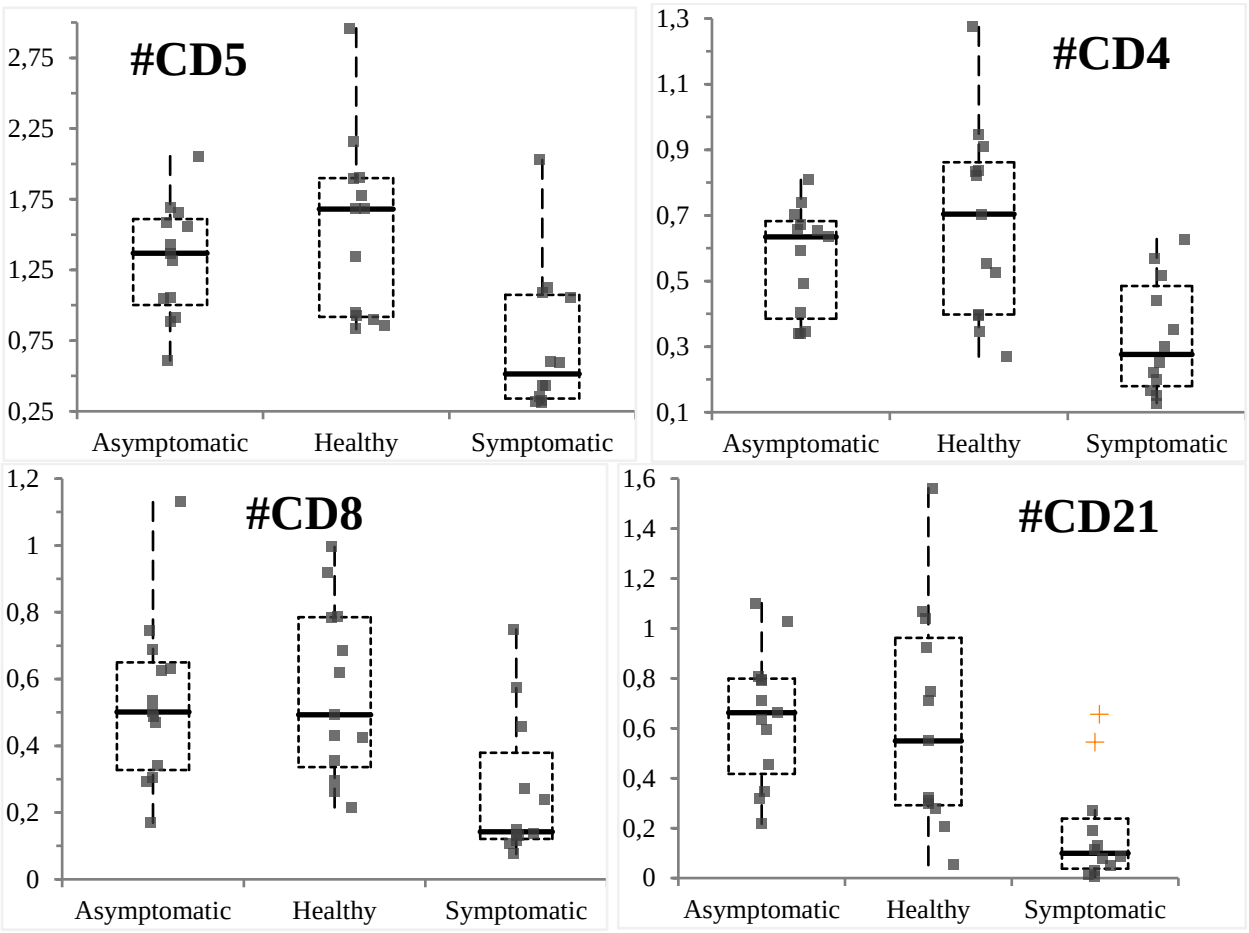

Figure 6.5. Comparison of the absolute value (expressed as x10³/µL) of CD5+ cells (upper left), CD4+ cells (upper right), CD8+ (lower let) and CD21+ (lower right) cells among the group ASYMPTOMATIC, HEALTHY and SYMPTOMATIC. The orange crosses represent the near outliers.

Correlation between microbiota phyla and leukocyte classes

The statistical analyses of correlation between different phyla found in the gut microbiota (see Study V) and the absolute number of leukocyte classes results in few significant results.

Specifically, a mild positive correlation was found between *Actinobacteria* and CD11b (r =0.35) and *Fusobacteria* and CD4:CD8 (r =0.33). A mild negative correlation was found between *Fusobacteria* and CD11b (r =-0.35) and Neutrophils (r =-0.39) (**Figure 6.6**).

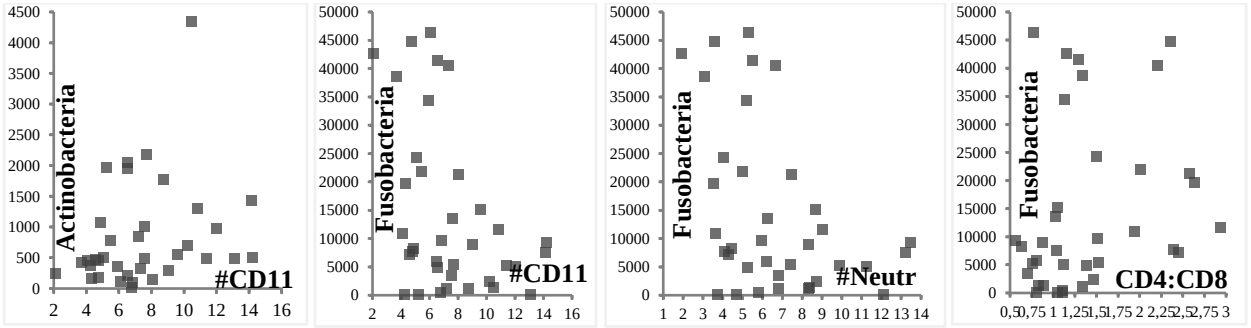

Figure 6.6. Significant correlation among Actinobacteria and CD11b (first graph), Fusobacteria and CD11b (second graph), neutrophils (third graph) and CD4:CD8 (fourth graph)

DISCUSSION

It has been demonstrated that the immune system plays a pivotal role in the development and outcome of *Leishmania* spp. infection in dogs. Results from the present study were in agreement with some studies present in the literature. In the murine model of cutaneous leishmaniasis a Th1 response has been correlated with resistance and a Th2 response to susceptibility to the disease. However, this is not true for human and murine visceral leishmaniasis, in which usually a mixed response has been found (Miralles et al., 1994). In canine *Leishmania* spp. infection, even though not completely understood, the Th1-like immunity seems to be protective as well (Solano-Gallego et al., 2000). In our study all the lymphocyte classes were significantly decreased in the SYMPTOMATIC group compared with both the exposed ASYMPTOMATIC and the HEALTHY dogs. CD21+ is a B-cells antigen and even though B-cells are involved in the subsequent stimulation of plasma cells and immunoglobulin production, responsible for most of the clinical signs of canine leishmaniasis, this process usually occurs in lymphoid organs or in bone marrow. For this reason, it is not surprising to find a low proportion (both in percentage and absolute number) of CD21+ cells in peripheral blood of dogs affected by clinical leishmaniasis, as already reported in literature (Bourdoiseau et al., 1997; Reis et al., 2006). The significant decrease in the number of CD5+, CD4+ cells in SYMPTOMATIC dogs is not surprising as well, because the lack of T lymphocytes and the related Th1 response is associated with the susceptibility to the disease,

as already stated above (Bourdoiseau et al., 1997; Reis et al., 2006). The use of CD4:CD8 ratio in canine leishmaniasis is still controversial. Indeed, even though our results agreed with some studies, others reported a decrease of that ratio in symptomatic dogs (Miranda et al., 2007; Coura-Vital et al., 2011). In our study there is not a significant difference in CD4:CD8 among the three groups examined and this could be due to a lower concentration of CD8+ together with CD4+ cells in the SYMPTOMATIC group that lead to a lack of any change in the ratio between the two lymphocytes populations. The evaluation of the related box plot, even though not significant, suggest that, in this study, CD4:CD8 seems to be lower in the asymptomatic group. This evidence is in agreement with what reported in the study of Reis and colleagues (Reis et al., 2006) in which an increase in CD8+ determine an inversion on CD4:CD8 ratio and is associated with low parasite density in bone marrow. These results are reflection of the complexity of canine leishmaniasis thus it could be interesting to further investigate the role of Th1/Th2 response through the use of cytokine expression measurement. The results of correlation, even though statistically significant, were not high enough to be relevant. An evaluation on a higher number of animals is suggested to confirm this correlation. Moreover, this is the first report, to our knowledge, of this kind of evaluation, so there are no data to hypothesize the effective role of this phyla on the immune system. However, this could be a starting point for new investigation on the role of microbiota in shaping the immune system.

CONCLUSIONS

This PhD project was aimed to investigate the presence of possible correlations between gut microbiota and the presence of systemic infectious diseases in dogs and cats. Based on the peculiar pathogenesis, feline infectious peritonitis and canine leishmaniasis have been identified as the ideal disease model to investigate this correlation, since clinical presentation in both the diseases relies not only on the pathogenic effect of the etiologic agents but also on the type of immune response mounted by the host, which can be efficient in eliminating the pathogen or, on the contrary, in enhancing its systemic spread and/or causing multi-organic dysfunctions and frequently the death of affected animals. This approach in the investigation of these diseases is, in our knowledge, the first studies ever accomplished on this topic.

Moreover, for FIP, due to difficulties in diagnosing the disease *in vivo,* that lead to a delay in the enrolment of clinical cases, the performances of new biomarker have been evaluated. Specifically, reference interval and diagnostic performance of PON-1 have been measured, as well as the concordance of PCR with the IHC, which is the gold standard for FIP diagnosis.

Specifically, the most relevant findings of this thesis are the following:

- Preliminary results about microbiota composition in cats affected by FIP and healthy coronavirus positive cats have been provided. The absence of significant differences among the three groups could be related with the low number of samples, caused mainly by the strict inclusion criteria that have been applied in order to reduce all the factors that could influence microbiota composition (for example outdoor access or contact with other species). Nevertheless, in our study, the cats positive to FCoV but resistant to FIP group showed a lower *Bacteroidetes* to *Firmicutes* ratio compared to the other groups, possibly indicating that the presence of the FCoV in the enterocytes. may alter microbiota stability, even in absence of overt clinical signs. Investigation of the causes and clinical significance of these microbiota changes on a larger number of animals are required to better understand

the pathogenesis and possibly provide some input for treatment of this fatal condition. The correlation of the data collected here, together with feature concerning the immune asset of the intestinal mucosa in these cats would represent an interesting starting point for future investigations.

- Reference interval for PON-1 activity in healthy cats has been calculated and spans from 57.8 to 153.7 U/mL. No partitioning for age of gender are required. However, it could be interesting the measurement of PON-1 activity in newborns, which are not included in the study and could have low values of PON-1 due to a liver immaturity.
- PON-1 seems to have a usefulness as a biomarker in the diagnosis of FIP, even if the best results have been provided during the effusive form of the disease. The paraoxon-based method is a less expensive test that provide results in a short time. As already stated in literature, the association of PON-1 results with other tests, especially in dubious cases, could increase the positive predictive value of the disease. Further studies on other diseases with a strong inflammatory (panleukopenia, sepsis or triadis) or oxidative stress (neoplasia) component are required to better characterized the diagnostic potential of this negative acute phase reactant.
- Even though the immunohistochemistry is considered the gold standard for the diagnosis of FIP, its sensitivity is not optimal, especially in those cases in which lesions are localized in few organs. For this reason, it is useful to associate information regarding different organs and, when not possible, preferring mesenteric lymph node and lung biopsies, instead of intestine ones. For what concern the RT-PCR, it shows a low specificity, as already reported in literature, but with liver and lung sample that show maximum specificity. On the other hand, RT-PCR on renal samples gave the highest sensitivity. However, it is not possible to use RT-PCR to confirm the diagnosis of FIP because of the high number of false positive results. The good agreement between these two methods

suggests the possible use of an association of both techniques in order to minimize diagnostic errors.

- Results of microbiota composition among dogs with or without *Leishmania* (both in presence of clinical signs or not) highlight some significant differences, specifically in the *Firmicutes* and *Proteobacteria* phyla, that are in agreement with what reported in literature. Moreover, an interesting finding about lower abundance of *Mucispirillum* and SCFAs producing genus were found in symptomatic dogs. An evaluation on a higher number of cases, and eventually the chance to sample infected asymptomatic, instead of exposed ones (chosen for ethical reasons), could lead to different results. This is the first study that investigate the gut microbiota composition in dogs with leishmaniasis and gave new insight on this disease.
- Results from flow cytometric evaluation are partially in agreement with what reported in literature. Immune response could be further investigated through the measurement of cytokine expression. Some correlations have been found between microbiota phyla and leukocyte classes. However, these preliminary results need an investigation on a higher number of dogs. Moreover, in literature there are no data about this correlation, so it is quite difficult to biologically explain it. Nevertheless, this could be a starting point for the researcher.

In conclusion, the investigation of possible correlations between the intestinal microbiota composition and the immune asset in systemic infectious diseases of dogs and cats would require a big amount of data to be adequately investigated, as well as a deepen knowledge about the exact relationship that allows microbiota to shape immunity. However, data obtained in this PhD project could be a starting point to better investigate this subject and eventually highlight new aspects of the pathogenesis of investigated disease.

REFERENCES

Abt MC, Osborne LC, Monticelli LC, Doering TA, Alenghat T, Sonnenberg GF, Paley MA, Antenus M, Williams KL, Erikson J., Wherry EJ., Artis D. Immunity (2012) 37(1):158-170 Commensal Bacteria Calibrate the Activation Threshold of Innate Antiviral Immunity

Adak A, Khan MR. Cellular and Molecolar Life Science (2019) 76(3):473-493 An insight into gut microbiota and its functionalities.

Addie D, Belák S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, Hartmann K, Hosie MJ, Lloret A, Lutz H, Marsilio F, Pennisi MG, Radford AD, Thiry E, Truyen U, Horzinek MC. Journal of Feline Medicine and Surgery (2009) 11(7):594-604 Feline infectious peritonitis. ABCD guidelines on prevention and management.

Addie DD, Jarrett O. Veterinary Record (2001) 148(21):649-53 Use of a reverse-transcriptase polymerase chain reaction for monitoring the shedding of feline coronavirus by healthy cats.

Alessandri G, Milani C, Mancabelli L, Mangifesta M, Lugli GA, Viappiani A, Duranti S, Turroni F, Ossiprandi MC, van Sinderen D, Ventura M. Environmental Microbiology (2019) 21(4):1331-1343 Metagenomic dissection of the canine gut microbiota: insights into taxonomic, metabolic and nutritional features.

Alkanani AK, Hara N, Gottlieb PA, Ir D, Robertson CE, Wagner BD, Frank DN, Zipris D. Diabetes (2015) 64(10):3510-20 Alterations in Intestinal Microbiota Correlate With Susceptibility to Type 1 Diabetes.

Almeida AB, Sousa VR, Gasparetto ND, da Silva GF, Figueiredo FB, Dutra V, Nakazato L, Madeira MF. Diagnostic Microbiology and Infectious Disease (2013) 76(3):321-4 Canine visceral leishmaniasis: diagnostic approaches based on polymerase chain reaction employing different biological samples.

Almeida MA, Jesus EE, Sousa-Atta ML, Alves LC, Berne ME, Atta AM. Veterinary Immunology and Immunopathology (2005) 106(1-2):151-8 Antileishmanial antibody profile in dogs naturally infected with Leishmania chagasi.

Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Science (2005) 307(5717):1915-20 Host-bacterial mutualism in the human intestine.

Baneth G. Leishmaniasis. In: Greene GE, ed. Infectious Diseases of the Dog and Cat, 3rd edn. Philadelphia, W.B. Saunders (2005) 685–95

Barker EN, Stranieri A, Helps CR, Porter EL, Davidson AD, Day MJ, Knowles T, Kipar A, Tasker S. Veterinary Research (2017) 48(1):60 Limitations of using feline coronavirus spike protein gene mutations to diagnose feline infectious peritonitis.

Bin Ali A, Zhang Q, Lim YK, Fang D, Retnam L, Lim SK. Free Radical Biology&Medicine (2003) 34(7):824-9 Expression of major HDL-associated antioxidant PON-1 is gender dependent and regulated during inflammation.

Bokulich NA, Chung J, Battaglia T, Henderson N, Jay M, Li H, D Lieber A, Wu F, Perez-Perez GI, Chen Y, Schweizer W, Zheng X, Contreras M, Dominguez-Bello MG, Blaser MJ. Science Translational Medicine (2016) 8(343):343ra82 Antibiotics, birth mode, and diet shape microbiome maturation during early life.

Bosch BJ, Van der Zee R, de Haan CA, Rottier PJ. Journal of Virology (2003) 77(16):8801-11 The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex.

Bourdoiseau G, Bonnefont C, Magnol JP, Saint-André I, Chabanne L. Veterinary Immunology and Immunopathology (1997) 56(3-4):345-51 Lymphocyte subset abnormalities in canine leishmaniasis.

Brandonisio O, Panunzio M, Faliero SM, Ceci L, Fasanella A, Puccini V. Veterinary Immunology and Immunopathology (1996) 53(1-2):95-103 Evaluation of polymorphonuclear cell and monocyte functions in Leishmania infantum-infected dogs.

Brown CA, Elliott J, Schmiedt CW, Brown SA. Veterinary pathology (2016) 53(2):309-326 Chronic kidney disease in aged cats: clinical features, morphology, and proposed pathogeneses.

Callahan BJ, Sankaran K, Fukuyama JA, McMurdie PJ, Holmes SP. F1000Research (2016) 5:1492 Bioconductor Workflow for Microbiome Data Analysis: from raw reads to community analyses.

Camps J, Marsillach J, Joven J. Critical Reviews in Clinical Laboratory Science (2009) 46:83-106 The paraoxonases: role in human diseases and methodological difficulties in measurement.

Cardoso L, Schallig HD, Cordeiro-da-Silva A, Cabral M, Alunda JM, Rodrigues M. Veterinary Immunology and Immunopathology (2007) 117(1-2):35-41 Anti-Leishmania humoral and cellular immune responses in naturally infected symptomatic and asymptomatic dogs.

Chang HW, Egberink HF, Halpin R, Spiro DJ, Rottier PJM. Emerging Infectious Diseases (2012) 18(7):1089-1095 Spike protein fusion peptide and feline coronavirus virulence.

Ciaramella P, Oliva G, Luna RD, Gradoni L, Ambrosio R, Cortese L, Scalone A, Persechino A. Veterinary Record (1997) 141(21):539-43 A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by *Leishmania infantum*.

Cornelissen E, Dewerchin HL, Van Hamme E, Nauwynck HJ. Virus Research (2009) 144(1-2):285-9. Absence of antibody-dependent, complement-mediated lysis of feline infectious peritonitis virus-infected cells.

Corrêa AP, Dossi AC, de Oliveira Vasconcelos R, Munari DP, de Lima VM. Veterinary Parasitology (2007) 143(3-4):267-74 Evaluation of transformation growth factor beta1, interleukin-10, and interferon-gamma in male symptomatic and asymptomatic dogs naturally infected by Leishmania (Leishmania) chagasi.

Costa M, Weese JS. The Veterinary Journal (2019) 249:10-15 Methods and basic concepts for microbiota assessment.

Coura-Vital W, Marques MJ, Giunchetti RC, Teixeira-Carvalho A, Moreira ND, Vitoriano-Souza J, Vieira PM, Carneiro CM, Corrêa-Oliveira R, Martins-Filho OA, Carneiro M, Reis AB. The Veterinary Journal (2011) 190(2):e43-7 Humoral and cellular immune responses in dogs with inapparent natural Leishmania infantum infection.

Crawford AH, Stoll AL, Sanchez-Masian D, Shea A, Michaels J, Fraser AR, Beltran E. Journal of Veterinary Internal Medicine (2017) 31(5):1477-1486 Clinicopathologic Features and Magnetic Resonance Imaging Findings in 24 Cats With Histopathologically Confirmed Neurologic Feline Infectious Peritonitis.

da Costa-Val AP, Cavalcanti RR, de Figueiredo Gontijo N, Michalick MS, Alexander B, Williams P, Melo MN. The Veterinary Journal (2007) 174(3):636-43 Canine visceral leishmaniasis: relationships between clinical status, humoral immune response, haematology and Lutzomyia (Lutzomyia) longipalpis infectivity.

de Groot-Mijnes JD, van Dun JM, van der Most RG, de Groot RJ. Journal of Virology (2005) 79(2):1036-44 Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease.

de Lima VM, Peiro JR, de Oliveira Vasconcelos R. Veterinary Immunology and Immunopathology (2007) 115(1-2):189-93 IL-6 and TNF-alpha production during active canine visceral leishmaniasis.

de Menezes JP, Saraiva EM, da Rocha-Azevedo B. Parasites & Vectors (2016) 9: 264. The site of the bite: Leishmania interaction with macrophages, neutrophils and the extracellular matrix in the dermis.

de Souza HSP, Fiocchi C, Iliopoulos D Nature Reviews. Gastroenterology & Hepatology (2017) 14(12):739-749 The IBD interactome: an integrated view of aetiology, pathogenesis and therapy.

Dejea CM, Fathi P, Craig JM, Boleij A, Taddese R, Geis AL, Wu X, DeStefano Shields CE, Hechenbleikner EM, Huso DL, Anders RA, Giardiello FM, Wick EC, Wang H, Wu S,

Pardoll DM, Housseau F, Sears CL Science (2018) 359(6375):592-597 Patients with familial adenomatous polyposis harbor colonic biofilms containing tumorigenic bacteria.

Desai AR, Musil KM, Carr AP, Hill JE. Veterinary Microbiology (2009) 137 (1–2), 120–128 Characterization and quantification of feline fecal microbiota using cpn60 sequence-based methods and investigation of animal-to-animal variation in microbial population structure.

Di Muccio T, Scalone A, Bruno A, Marangi M, Grande R, Armignacco O, Gradoni L, Gramiccia M. PLoS One (2015) 10(6):e0129418 Epidemiology of Imported Leishmaniasis in Italy: Implications for a European Endemic Country.

Draganov D, Teiber J, Watson C, Bisgaier C, Nemzek J, Remick D, Standiford T, La Du B. Advances in Experimental Medicine and Biology (2010) 660:89-97 PON1 and oxidative stress in human sepsis and an animal model of sepsis.

Drechsler Y, Alcaraz A, Bossong FJ, Collisson EW, Diniz PP. The Veterinary Clinics of North America. Small Animal Practice (2011) 41(6):1133-69 Feline coronavirus in multicat environments.

Duthie S, Eckersall PD, Addie DD, Lawrence CE, Jarrett O. Veterinary Record (1997) 141(12):299-303 Value of alpha 1-acid glycoprotein in the diagnosis of feline infectious peritonitis.

Duvallet C, Gibbons SM, Gurry T, Irizarry RA, Alm EJ. Nature Communications (2017) 8(1):1784 Meta-analysis of gut microbiome studies identifies disease-specific and shared responses.

Engwerda CR, Ato M, Kaye PM. Trends in Parasitology (2004) 20(11):524-30 Macrophages, pathology and parasite persistence in experimental visceral leishmaniasis.

Feingold KR, Memon RA, Moser AH, Grunfeld C. Atherosclerosis (1998) 139:307-315 Paraoxonase activity in the serum and hepatic mRNA levels decrease during the acute phase response.

Felten S, Leutenegger CM, Balzer HJ, Pantchev N, Matiasek K, Wess G, Egberink H, Hartmann K. BMC Veterinary Research (2017b) 13(1):228 Sensitivity and specificity of a real-time reverse transcriptase polymerase chain reaction detecting feline coronavirus mutations in effusion and serum/plasma of cats to diagnose feline infectious peritonitis.

Felten S, Matiasek K, Gruendl S, Sangl L, Wess G, Hartmann K. Journal of Feline Medicine and Surgery (2017a) 19(4):410-418 Investigation into the utility of an immunocytochemical assay in body cavity effusions for diagnosis of feline infectious peritonitis.

Fischer Y, Weber K, Sauter-Louis C, Hartmann K. Tierarztliche Praxis. Ausgabe K, Kleintiere/Heimtiere (2013) 41(5):297-303 The Rivalta's test as a diagnostic variable in feline effusions--evaluation of optimum reaction and storage conditions.

Font A, Closa JM, Mascort J. Journal of Veterinary Internal Medicine (1994) 8(3):233-5 Monoclonal gammopathy in a dog with visceral leishmaniasis.

Francino O, Altet L, Sánchez-Robert E, Rodriguez A, Solano-Gallego L, Alberola J, Ferrer L, Sánchez A, Roura X. Veterinary Parasitology (2006) 137(3-4):214-21 Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis.

Friedrichs KR, Harr KE, Freeman KP, Szladovits B, Walton RM, Barnhart KF, Blanco-Chavez J; American Society for Veterinary Clinical Pathology. Veterinary Clinical Pathology (2012) 41(4):441-53 ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics.

Furlong CE, Marsillach J, Jarvik GP, Costa L.G. Chemico-Biological Interactions (2016) 259 (Pt B): 51–62 Paraoxonases-1, -2 and -3: What are their Functions?

Gavazza A, Rossi G, Lubas G, Cerquetella M, Minamoto Y, Suchodolski JS. Veterinary Comparative Oncology (2018) 16(1):E169-E175 Faecal microbiota in dogs with multicentric lymphoma.

Geffré A, Concordet D, Braun JP, Trumel C. Veterinary Clinical Pathology (2011) 40(1):107-12 Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel.

Giordano A, Paltrinieri S. Veterinary Clinical Pathology (2010) 39(4):464-73 Interpretation of capillary zone electrophoresis compared with cellulose acetate and agarose gel electrophoresis: reference intervals and diagnostic efficiency in dogs and cats.

Giordano A, Stranieri A, Rossi G, Paltrinieri S. Veterinary Clinical Pathology (2015) 44(2):295-302 High diagnostic accuracy of the Sysmex XT-2000iV delta total nucleated cells on effusions for feline infectious peritonitis.

Giordano A, Veronesi MC, Rossi G, Pezzia F, Probo M, Giori L, Paltrinieri S. The Veterinary Journal (2013) 197:499-501 Serum paraoxonase-1 activity in neonatal calves: age related variations and comparison between healthy and sick animals.

Giori L, Giordano A, Giudice C, Grieco V, Paltrinieri S. Journal of Small Animal Practice (2011) 52(3):152-7 Performances of different diagnostic tests for feline infectious peritonitis in challenging clinical cases.

Giudice E, Passantino A. Acta Veterinaria Hungarica (2011) 59(2):205-13 Detection of Leishmania amastigotes in peripheral blood from four dogs--Short communication.

Goedert J. EBioMedicine (2016) 5:14–15 Effect of HIV, immune deficiency, and confounding on the distal gut microbiota.

Gosalbes MJ, Durbán A, Pignatelli M, Abellan JJ, Jiménez-Hernández N, Pérez-Cobas AE, Latorre A, Moya A. PLoS One (2011) 6(3):e17447 Metatranscriptomic approach to analyze the functional human gut microbiota.

Handl S, Dowd SE, Garcia-Mazcorro JF, Steiner JM, Suchodolski JS. FEMS Microbiology Ecology (2011) 76(2):301-10 Massive parallel 16S rRNA gene pyrosequencing reveals highly diverse fecal bacterial and fungal communities in healthy dogs and cats.

Harris EK, Boyd JC. Clinical Chemistry (1990) 36(2):265-70 On dividing reference data into subgroups to produce separate reference ranges.

Hayward AHS. Journal of Small Animal Practice (1968) 9:75-82 Thoracic effusion in the cat.

Hazuchova K, Held S, Neiger R. Journal of Feline Medicine and Surgery (2017) 19(8):809-816 Usefulness of acute phase proteins in differentiating between feline infectious peritonitis and other diseases in cats with body cavity effusions.

Hentges DJ, Maier BR, Burton GC, Flynn MA, Tsutakawa RK Cancer Research (1977) 37(2):568–571 Effect of a high-beef diet on the fecal bacterial flora of humans.

Herp S, Brugiroux S, Garzetti D, Ring D, Jochum LM, Beutler M, Eberl C, Hussain S, Walter S, Gerlach RG, Ruscheweyh HJ, Huson D, Sellin ME, Slack E, Hanson B, Loy A, Baines JF, Rausch P, Basic M, Bleich A, Berry D, Stecher B. Cell Host&Microbe (2019) 25(5):681-694.e8 Mucispirillum schaedleri Antagonizes Salmonella Virulence to Protect Mice against Colitis.

Herrewegh AA, de Groot RJ, Cepica A, Egberink HF, Horzinek MC, Rottier PJ. Journal of Clinical Microbiology (1995) 33(3):684-9 Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR.

Herrewegh AA, Smeenk I, Horzinek MC, Rottier PJ, de Groot RJ. Journal of Virology (1998) 72(5):4508-14 Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus.

Hooda S, Minamoto Y, Suchodolski JS, Swanson KS. Animal Health Research Reviews (2012) 13(1):78-88 Current state of knowledge: the canine gastrointestinal microbiome.

Ibba F, Rossi G, Meazzi S, Giordano A, Paltrinieri S. Research in Veterinary Science (2015) 98:89-91 Serum concentration of high density lipoproteins (HDLs) in leishmaniotic dogs.

IRIS Canine GN Study Group Diagnosis Subgroup, Littman MP, Daminet S, Grauer GF, Lees GE, van Dongen AM. Journal of Veterinary Internal Medicine (2013) 27 Suppl 1:S19-26 Consensus recommendations for the diagnostic investigation of dogs with suspected glomerular disease.

Ito Y, Nisiyama Y, Shimokata K, Kimura Y, Nagata I (1976) Infection and Immunity 13(2): 332-336 Interferon producing capacity of germfree mice

Ives EJ, Vanhaesebrouck AE, Cian F. Journal of Feline Medicine and Surgery (2013) 15(12):1149-53 Immunocytochemical demonstration of feline infectious peritonitis virus within cerebrospinal fluid macrophages.

Jacob F, Polzin DJ, Osborne CA, Neaton JD, Kirk CA, Allen TA, Swanson LL. Journal of American Veterinary Medicine Association (2005) 226(3):393-400 Evaluation of the association between initial proteinuria and morbidity rate or death in dogs with naturally occurring chronic renal failure.

Jakobsson HE, Rodríguez-Piñeiro AM, Schütte A, Ermund A, Boysen P, Bemark M, Sommer F, Bäckhed F, Hansson GC, Johansson ME. EMBO Reports (2015) 16(2):164-77 The composition of the gut microbiota shapes the colon mucus barrier.

Jergens AE, Guard BC, Redfern A, Rossi G, Mochel JP, Pilla R, Chandra L, Seo YJ, Steiner JM, Lidbury J, Allenspach K, Suchodolski J. Frontiers in Veterinary Science (2019) 6:199 Microbiota-Related Changes in Unconjugated Fecal Bile Acids Are Associated With Naturally Occurring, Insulin-Dependent Diabetes Mellitus in Dogs.

Kaakoush NO, Day AS, Huinao KD, Leach ST, Lemberg DA, Dowd SE, Mitchell HM. Journal of Clinical Microbiology (2012) 50 (10), 3258–3266 Microbial dysbiosis in pediatric patients with Crohn's disease.

Kamada N, Seo SU, Chen GY, Núñez G. Natural Reviews. Immunology (2013) 13(5):321-35 Role of the gut microbiota in immunity and inflammatory disease.

Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C. Journal of Lipid Research (2004) 45: 1169-1196 Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host.

Kipar A, Baptiste K, Barth A, Reinacher M. Journal of Feline Medicine and Surgery (2006a) 8(1):69-72 Natural FCoV infection: cats with FIP exhibit significantly higher viral loads than healthy infected cats.

Kipar A, Bellmann S, Kremendahl J, Köhler K, Reinacher M. Veterinary Immunology and Immunopathology (1998) 65:243-257 Cellular composition, coronavirus antigen expression and production of specific antibodies in lesions in feline infectious peritonitis.

Kipar A, May H, Menger S, Weber M, Leukert W, Reinacher M. Veterinary Pathology (2005) 42(3):321-30 Morphologic features and development of granulomatous vasculitis in feline infectious peritonitis.

Kipar A, Meli ML, Baptiste KE, Bowker LJ, Lutz H. Journal of General Virology (2010) 91(Pt 7):1698-707 Sites of feline coronavirus persistence in healthy cats.

Kipar A, Meli ML, Failing K, Euler T, Gomes-Keller MA, Schwartz D, Lutz H, Reinacher M. Veterinary Immunology and Immunopathology (2006b) 112(3-4):141-55 Natural feline coronavirus infection: differences in cytokine patterns in association with the outcome of infection.

Kipar A, Meli ML. Veterinary Pathology (2014) 51(2):505-26 Feline infectious peritonitis: still an enigma?

Koh H, Kim M, Lee J, Kim H, Park S. Microbes Environment (2015) 30 (3), 284–287 Changes in the swine gut microbiota in response to porcine epidemic Diarrhea infection.

Kostic AD, Gevers D, Siljander H, Vatanen T, Hyötyläinen T, Hämäläinen AM, Peet A, Tillmann V, Pöhö P, Mattila I, Lähdesmäki H, Franzosa EA, Vaarala O, de Goffau M, Harmsen H, Ilonen J, Virtanen SM, Clish CB, Orešič M, Huttenhower C, Knip M; DIABIMMUNE Study Group, Xavier RJ Cell Host & Microbe (2015) 17(2):260-73 The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes.

Koutinas AF, Koutinas CK. Veterinary Pathology (2014) 51(2):527-38 Pathologic mechanisms underlying the clinical findings in canine leishmaniasis due to Leishmania infantum/chagasi.

Kristensen K, Henriksen L Journal of Allergy and Clinical Immunology (2016) 137(2):587-90 Cesarean section and disease associated with immune function.

Lahti A, Petersen PH, Boyd JC, Rustad P, Laake P, Solberg HE. Clinical Chemistry (2004) 50 (5):891–900 Partitioning of nongaussian-distributed.

Landis JR, Koch GG. Biometrics (1997) 33(1):159-74 The measurement of observer agreement for categorical data.

Lee YK, Mazmanian SK. Science (2010) 330(6012):1768-73 Has the microbiota played a critical role in the evolution of the adaptive immune system?

Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Nature (2006) 444(7122):1022-3 Microbial ecology: human gut microbes associated with obesity.

Liu S, Zhao L, Zhai Z, Zhao W, Ding J, Dai R, Sun T, Meng H, Current in Microbiology (2015) 71 (6), 643–649 Porcine epidemic Diarrhea virus infection induced the unbalance of gut microbiota in piglets.

Locksley RM, Heinzel FP, Sadick MD, Holaday BJ, Gardner KD Jr. Annales de l'Institut Pasteur. Immunology (1987) 138(5):744-9 Murine cutaneous leishmaniasis: susceptibility correlates with differential expansion of helper T-cell subsets.

Lopes M, Carneiro M, Dos Santos L, Vieira L. Parasite immunology (2016 38(1):37-44 Indigenous microbiota and Leishmaniasis.

Lopez R, Lucena R, Novales M, Ginel PJ, Martin E, Molleda JM. Zentralblatt fur Veterinarmedizin. Reihe B. (1996) 43(8):469-74 Circulating immune complexes and renal function in canine leishmaniasis.

Macfarlane GT, Macfarlane S. Journal of AOAC International (2012) 95(1):50-60 Bacteria, colonic fermentation, and gastrointestinal health.

Mackness M, Mackness B. Gene (2015) 567:12-21 Human paraoxonase-1 (PON1): Gene structure and expression, promiscuous activities and multiple physiological roles.

Manna L, Reale S, Viola E, Vitale F, Foglia Manzillo V, Pavone LM, Caracappa S, Gravino AE. Veterinary Parasitology (2006) 142(3-4):271-80 Leishmania DNA load and cytokine expression levels in asymptomatic naturally infected dogs.

Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM, Thomas LV, Zoetendal EG, Hart A. Gut (2016) 65(2):330-9 The gut microbiota and host health: a new clinical frontier.

Maroli M, Feliciangeli MD, Bichaud L, Charrel RN, Gradoni L. Medical and Veterinary Entomology (2013) 27(2):123-47 Phlebotomine sandflies and the spreading of leishmaniases and other diseases of public health concern.

Martínez-Moreno A, Martínez-Cruz MS, Blanco A, Hernández-Rodríguez S. Veterinary Parasitology (1993) 51(1-2):49-59 Immunological and histological study of T- and B-lymphocyte activity in canine visceral leishmaniosis.

Martínez-Subiela S1, Tecles F, Eckersall PD, Cerón JJ. Veterinary Record (2002) 150(8):241-4 Serum concentrations of acute phase proteins in dogs with leishmaniasis.

McMurdie PJ, Holmes S. PLoS One (2013) 8(4), e61217 Phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data.

McMurdie PJ, Holmes S. PLoS Computational Biology (2014) 10(4):e1003531 Waste not, want not: why rarefying microbiome data is inadmissible.

McNeil NI. American Journal of Clinical Nutrition (1984) 39(2):338-42 The contribution of the large intestine to energy supplies in man.

Meli M, Kipar A, Müller C, Jenal K, Gönczi E, Borel N, Gunn-Moore D, Chalmers S, Lin F, Reinacher M, Lutz H. Journal of Feline Medicine and Surgery (2004) 6(2), 69-81 High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats.

Metzker ML. Genome Research (2005) 15(12):1767-76 Emerging technologies in DNA sequencing.

Minamoto Y, Minamoto T, Isaiah A, Sattasathuchana P, Buono A, Rangachari VR, McNeely IH, Lidbury J, Steiner JM, Suchodolski JS. Journal of Veterinary Internal Medicine (2019) 33(4):1608-1618 Fecal short-chain fatty acid concentrations and dysbiosis in dogs with chronic enteropathy.

Miralles GD, Stoeckle MY, McDermott DF, Finkelman FD, Murray HW. Infection and Immunity (1994) 62(3):1058-63 Th1 and Th2 cell-associated cytokines in experimental visceral leishmaniasis.

Miranda S, Martorell S, Costa M, Ferrer L, Ramis A. Veterinary Parasitology (2007) 144(3-4):251-60 Characterization of circulating lymphocyte subpopulations in canine leishmaniasis throughout treatment with antimonials and allopurinol.

Modi S, Collins J, Relman D. Journal of Clinical Investigations (2014) 124 (10), 4212–4218 Antibiotics and the gut microbiota.

Montorfano I, Becerra A, Cerro R, Echeverria C, Saez E, Morales MG, Fernandez R, Cabello-Verrugio C, Simon F. Laboratory Investigation (2014) 94, 1068-1082 Oxidative stress mediates the conversion of endothelial cells into myofibroblasts via a TGF-b1 and TGF-b2-dependent pathway.

Murphy EA, Velazquez KT, Herbert KM. Current Opinion in Clinical Nutritional and Metabolic Care (2015) 18(5):515-20 Influence of high-fat diet on gut microbiota: a driving force for chronic disease risk.

Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, Queipo-Ortuño MI. BMC Medicine (2013) 11:46 Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study.

Mylonakis ME, Papaioannou N, Saridomichelakis MN, Koutinas AF, Billinis C, Kontos VI. Veterinary Clinical Pathology (2005) 34(3):243-7 Cytologic patterns of lymphadenopathy in dogs infected with Leishmania infantum.

Neish AS. Gastroenterology (2009) 136(1):65-80 Microbes in gastrointestinal health and disease.

Nicolato Rde C, de Abreu RT, Roatt BM, Aguiar-Soares RD, Reis LE, Carvalho Md, Carneiro CM, Giunchetti RC, Bouillet LE, Lemos DS, Coura-Vital W, Reis AB. PLoS One (2013) 8(12):e82947 Clinical forms of canine visceral Leishmaniasis in naturally Leishmania infantum-infected dogs and related myelogram and hemogram changes.

Nie P, Li Z, Wang Y, Zhang Y, Zhao M, Luo J, Du S, Deng Z, Chen J, Wang Y, Chen S, Wang L. Medicinal Research Review (2019) [Epub ahead of print]. Gut microbiome interventions in human health and diseases.

Norris JM, Bosward KL, White JD, Baral RM, Catt MJ, Malik R. Australian Veterinary Journal (2005) 83(11):666-73 Clinicopathological findings associated with feline infectious peritonitis in Sydney, Australia: 42 cases (1990-2002).

Okuturlar Y, Gedikbasi A, Akalin N, Gunaldi M, Yilmaz D, Mert M, Harmankaya O, Soylu A, Karakaya P, Kumbasar A. Archives of medical science (2016) 12(4):697-703 Serum paraoxonase 1 activity in patients with iron deficiency anemia.

Ottenjann M, Weingart C, Arndt G, Kohn B. Journal of Veterinary Internal Medicine (2006) 20:1143–1150 Characterization of the Anemia of Inflammatory Disease in Cats with Abscesses, Pyothorax, or Fat Necrosis.

Padrid P. Veterinary Clinics: Small Animal Practice (2000) 30(6):1295-1308 Canine and feline pleural disease.

Palm NW, de Zoete MR, Cullen TW, Barry NA, Stefanowski J, Hao L, Degnan PH, Hu J, Peter I, Zhang W, Ruggiero E, Cho JH, Goodman AL, Flavell RA. Cell (2014) 158(5):1000-1010 Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease.

Paltrinieri S, Giordano A, Tranquillo V, Guazzetti S. Journal of Veterinary Diagnostic Investigation (2007) 19(3):266-72 Critical assessment of the diagnostic value of feline alpha1-acid glycoprotein for feline infectious peritonitis using the likelihood ratios approach.

Paltrinieri S, Gradoni L, Roura X, Zatelli A, Zini E. Veterinary Clinical Pathology (2016) 45(4):552-578 Laboratory tests for diagnosing and monitoring canine leishmaniasis.

Paltrinieri S. The Veterinary Journal (2008) 177:26–35 The acute phase reaction.

Pedersen NC, Black JW, Boyle JF, Evermann JF, McKeirnan AJ, Ott RL. Advances in Experimental Medicine and Biology (1984) 173:365-80 Pathogenic differences between various feline coronavirus isolates.

Pedersen NC, Boyle JF. American Journal of Veterinary Research (1980) 41(6):868-76 Immunologic phenomena in the effusive form of feline infectious peritonitis.

Pedersen NC. Journal of Feline Medicine and Surgery (2009) 11(4):225-58 A review of feline infectious peritonitis virus infection: 1963-2008.

Pedersen NC. The Veterinary Journal (2014a) 201(2):133-41 An update on feline infectious peritonitis: diagnostics and therapeutics.

Pedersen NC. The Veterinary Journal (2014b) 201(2):123-32 An update on feline infectious peritonitis: virology and immunopathogenesis.

Pesteanu-Somogyi LD, Radzai C, Pressler BM. Journal of Feline Medicine and Surgery (2006) 8(1):1-5 Prevalence of feline infectious peritonitis in specific cat breeds.

Pietro SD, Bosco VR, Crinò C, Francaviglia F, Giudice E. Veterinary World (2016) 9(6):633-7. Prevalence, type, and prognosis of ocular lesions in shelter and owned-client dogs naturally infected by Leishmania infantum.

Pinelli E, Boog CJ, Rutten VP, van Dijk B, Bernadina WE, Ruitenberg EJ. Tissue Antigens (1994) 43(3):189-92 A canine CD8+ cytotoxic T-cell line specific for Leishmania infantum-infected macrophages.

Pinelli E, Gonzalo RM, Boog CJ, Rutten VP, Gebhard D, del Real G, Ruitenberg EJ. European Journal of Immunology (1995) 25(6):1594-600 Leishmania infantum-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner.

Płoneczka-Janeczko K, Bania J, Bierowiec K, Kiełbowicz M, Kiełbowicz Z. Biomedical Research International (2017) 3710404 Bacterial diversity in feline conjunctiva based on 16S rRNA gene sequence analysis: a pilot study.

Ready PD. Annual Review of Entomology (2013) 58:227-50 Biology of phlebotomine sand flies as vectors of disease agents.

Ready PD. Revue Scientifique et Technique (2008) 27(2):399-412 Leishmaniasis emergence and climate change.

Regan AD, Cohen RD, Whittaker GR. Virology (2009) 384(1):135-43 Activation of p38 MAPK by feline infectious peritonitis virus regulates pro-inflammatory cytokine production in primary blood-derived feline mononuclear cells.

Reis AB, Teixeira-Carvalho A, Giunchetti RC, Guerra LL, Carvalho MG, Mayrink W, Genaro O, Corrêa-Oliveira R, Martins-Filho OA. Clinical and Experimental Immunology (2006) 146(2):303-11 Phenotypic features of circulating leucocytes as immunological markers for clinical status and bone marrow parasite density in dogs naturally infected by Leishmania chagasi.

Reis LE, Coura-Vital W, Roatt BM, Bouillet LÉ, Ker HG, Fortes de Brito RC, Resende Dde M, Carneiro M, Giunchetti RC, Marques MJ, Carneiro CM, Reis AB. Veterinary Parasitology (2013) 197(3-4):498-503 Molecular diagnosis of canine visceral leishmaniasis: a comparative study of three methods using skin and spleen from dogs with natural Leishmania infantum infection.

Riemer F, Kuehner KA, Ritz S, Sauter-Louis C, Hartmann K. Journal of Feline Medicine and Surgery (2016) 18(4):348-56 Clinical and laboratory features of cats with feline infectious peritonitis--a retrospective study of 231 confirmed cases (2000-2010).

Robinson M, Smyth G. Biostatistics (2008) 9 (2), 321–332 Small-sample estimation of negative binomial dispersion, with applications to SAGE data.

Rossi G, Giordano A, Costarelli E, Moretti P, Paltrinieri S. ACVP and ASVCP annual meeting, Atlanta, Nov 8th-12th, 2014. Published in: Veterinary Clinical Pathology (2014b) 43:E18 Analytical validation of a paraoxon based method to measure the activity of paraoxonase-1 in feline serum.

Rossi G, Giordano A, Pezzia F, Kjelgaard-Hansen M, Paltrinieri S. Veterinary Clinical Pathology (2013) 42: 329–341 Serum paraoxonase 1 activity in dogs: preanalytical and analytical factors and correlation with C-reactive protein and alpha-2-globulin.

Rossi G, Ibba F, Meazzi S, Giordano A, Paltrinieri S. The Veterinary Journal (2014a) 199(1):143-9 Paraoxonase activity as a tool for clinical monitoring of dogs treated for canine leishmaniasis.

Round JL, Mazmanian SK. Nature Reviews. Immunology (2009) 9(5):313-23 The gut microbiota shapes intestinal immune responses during health and disease.

Ruggerone B, Bonelli F, Nocera I, Paltrinieri S, Giordano A, Sgorbini M. Veterinary Clinical Pathology (2018) 47:69-77 Validation of a paraoxon-based method for measurement of paraoxonase (PON-1) activity and establishment of RIs in horses.

Santos-Gomes GM, Rosa R, Leandro C, Cortes S, Romão P, Silveira H. Veterinary Immunology and Immunopathology (2002) 88(1-2):21-30 Cytokine expression during the outcome of canine experimental infection by Leishmania infantum.

Sarangi AN, Goel A, Aggarwal R. Journal of Clinical and Experimental Hepatology (2019) 9(1):62-73 Methods for Studying Gut Microbiota: A Primer for Physicians.

Saridomichelakis MN, Koutinas AF. Veterinary Dermatology (2014) 25(2):61-71 Cutaneous involvement in canine leishmaniosis due to Leishmania infantum (syn. L. chagasi).

Saridomichelakis MN. Veterinary Dermatology (2009) 20(5-6):471-89 Advances in the pathogenesis of canine leishmaniosis: epidemiologic and diagnostic implications.

Sato, J., Kanazawa, A., Ikeda, F., Yoshihara, T., Goto, H., Abe, H., Komiya, K., Kawaguchi, M., Shimizu, T., Ogihara, T., Tamura, Y., Sakurai, Y., Yamamoto, R., Mita, T., Fujitani Y, Fukuda H, Nomoto K, Takahashi T, Asahara T, Hirose T, Nagata S, Yamashiro Y, Watada H. Diabetes Care (2014) 37 (8), 2343–2350 Gut dysbiosis and detection of “live gut bacteria” in blood of Japanese patients with type 2 diabetes.

Schmid SM, Suchodolski JS, Price JM, Tolbert MK. Frontiers in Veterinary Science (2018) 16 (5), 79 Omeprazole minimally alters the Fecal microbial Community in six Cats: a pilot study.

Schmidt M, Unterer S, Suchodolski JS, Honneffer JB, Guard BC, Lidbury JA, Steiner JM, Fritz J, Kölle P. PLoS One (2018) 13(8):e0201279 The fecal microbiome and metabolome differs between dogs fed Bones and Raw Food (BARF) diets and dogs fed commercial diets.

Shin NR, Whon TW, Bae JW. Trends in Biotechnology (2015) 33 (9), 496–503 Proteobacteria: microbial signature of dysbiosis in gut microbiota.

Shunmoogam N, Naidoo P, Chilton R. Vascular Health and Risk Management (2018) 14: 137–143 Paraoxonase (PON)-1: a brief overview on genetics, structure, polymorphisms and clinical relevance.

Solano-Gallego L, Koutinas A, Miró G, Cardoso L, Pennisi MG, Ferrer L, Bourdeau P, Oliva G, Baneth G. Veterinary Parasitology (2009) 165(1-2):1-18 Directions for the diagnosis, clinical staging, treatment and prevention of canine leishmaniosis.

Solano-Gallego L, Llull J, Ramos G, Riera C, Arboix M, Alberola J, Ferrer L. Veterinary Parasitology (2000) 10;90(1-2):37-45 The Ibizian hound presents a predominantly cellular immune response against natural Leishmania infection.

Solano-Gallego L, Villanueva-Saz S, Carbonell M, Trotta M, Furlanello T, Natale A. Parasites & Vectors (2014) 7:111 Serological diagnosis of canine leishmaniosis: comparison of three commercial ELISA tests (Leiscan, ID Screen and Leishmania 96), a rapid test (Speed Leish K) and an in-house IFAT.

Stranieri A, Giordano A, Bo S, Braghiroli C, Paltrinieri S. Journal of Feline Medicine and Surgery (2017) 19(8):880-887 Frequency of electrophoretic changes consistent with feline infectious peritonitis in two different time periods (2004-2009 vs 2013-2014).

Stranieri A, Giordano A, Paltrinieri S, Giudice C, Cannito V, Lauzi S. Journal of Veterinary Diagnostic Investigation (2018) 30(3):459-463 Comparison of the performance of laboratory tests in the diagnosis of feline infectious peritonitis.

Stranieri A, Paltrinieri S, Giordano A. Journal of Veterinary Diagnostic and Investigation. (2017b) 29(3):321-324 Diagnosing feline infectious peritonitis using the Sysmex XT-2000iV based on frozen supernatants from cavitary effusions.

Suchodolski JS, Dowd SE, Wilke V, Steiner JM, Jergens AE. PLoS One. (2012b) 7(6):e39333 16S rRNA gene pyrosequencing reveals bacterial dysbiosis in the duodenum of dogs with idiopathic inflammatory bowel disease.

Suchodolski JS, Markel ME, Garcia-Mazcorro JF, Unterer S, Heilmann RM, Dowd SE, Kachroo P, Ivanov I, Minamoto Y, Dillman EM, Steiner JM, Cook AK, Toresson L. PLoS One (2012a) 7(12):e51907 The fecal microbiome in dogs with acute diarrhea and idiopathic inflammatory bowel disease.

Suchodolski JS. The Veterinary Journal (2016) 215:30-7 Diagnosis and interpretation of intestinal dysbiosis in dogs and cats.

Swiatecka D, Narbad A, Ridgway KP, Kostyra H (2011) 145(1):267–272 The study on the impact of glycated pea proteins on human intestinal bacteria. International Journal of Food Microbiology

Sykes JE. Journal of feline medicine and surgery (2003) 5(6):353-356 Ischemic neuromyopathy due to peripheral arterial embolization of an adenocarcinoma in a cat.

Takano T, Azuma N, Satoh M, Toda A, Hashida Y, Satoh R, Hohdatsu T. Archive of Virology (2009) 154:775-781 Neutrophil survival factors (TNF-alpha, GM-CSF, and G-CSF) produced by macrophages in cats infected with feline infectious peritonitis virus contribute to the pathogenesis of granulomatous lesions.

Tasker S. Journal of Feline Medicine and Surgery (2018) 20(3):228-243 Diagnosis of feline infectious peritonitis: Update on evidence supporting available tests.

Tecles F, Caldín M, Tvarijonaviciute A, Escribano D, Martínez-Subiela S, Cerón JJ. Research in Veterinary Science (2015) 100:12-17 Serum biomarkers of oxidative stress in cats with feline infectious peritonitis.

The Human Microbiome Project Consortium. Nature (2012) 486:207-214 Structure, function and diversity of the healthy human microbiome

Tomás M, Sentí M, García-Faria F, Vila J, Torrents A, Covas M, Marrugat J. Arteriosclerosis, Thrombosis, and Vascular Biology (2000) 20:2113-2119 Effect of Simvastatin Therapy on Paraoxonase Activity and Related Lipoproteins in Familial Hypercholesterolemic Patients.

Toyokuni S, Okamoto K, Yodoi J, Hiai H FEBS letters (1995) 358 1-3 Persistent oxidative stress in cancer.

Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE, Sogin ML, Jones WJ, Roe BA, Affourtit JP, Egholm M, Henrissat B, Heath AC, Knight R, Gordon JI. Nature (2009) 457(7228):480-4 A core gut microbiome in obese and lean twins.

Vermeulen BL, Devriendt B, Olyslaegers DA, Dedeurwaerder A, Desmarets LM, Favoreel HW, Dewerchin HL, Nauwynck HJ. Veterinary Microbiology (2013) 164(1-2):46-59 Suppression of NK cells and regulatory T lymphocytes in cats naturally infected with feline infectious peritonitis virus.

Vogel L, Van der Lubben M, te Lintelo EG, Bekker CP, Geerts T, Schuijff LS, Grinwis GC, Egberink HF, Rottier PJ. Veterinary Research (2010) 41(5):71 Pathogenic characteristics of persistent feline enteric coronavirus infection in cats.

Vouldoukis I, Drapier JC, Nüssler AK, Tselentis Y, Da Silva OA, Gentilini M, Mossalayi DM, Monjour L, Dugas B. Antimicrobial Agents and Chemotherapy (1996) 40(1):253-6

Canine visceral leishmaniasis: successful chemotherapy induces macrophage antileishmanial activity via the L-arginine nitric oxide pathway.

Wang T, Cai G, Qiu Y, Fei N, Zhang M, Pang X, Jia W, Cai S, Zhao L. The ISME Journal. (2012) 6(2):320-9 Structural segregation of gut microbiota between colorectal cancer patients and healthy volunteers.

Weese J, Nichols J, Jalali M, Litster A. Veterinary Microbiology (2015) 180, 96–102 The rectal microbiota of cats infected with feline immunodeficiency virus infection and uninfected controls.

Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, Stonebraker AC, Hu C, Wong FS, Szot GL, Bluestone JA, Gordon JI, Chervonsky AV. Nature (2008) 455(7216):1109-13 Innate immunity and intestinal microbiota in the development of Type 1 diabetes.

Williams A, Probert C, Stepankova R, Tlaskalova-Hogenova H, Phillips A, Bland P. Immunology (2006) 119(4):470-8 Effects of microflora on the neonatal development of gut mucosal T cells and myeloid cells in the mouse.

Winkler ES, Thackray LB Current Opinion in Virology (2019) 37:44-51 A long-distance relationship: the commensal gut microbiota and systemic viruses.

Zatelli A, Borgarelli M, Santilli R, Bonfanti U, Nigrisoli E, Zanatta R, Tarducci A, Guarraci A. American Journal of Veterinary Research (2003) 64(5):558-61 Glomerular lesions in dogs infected with Leishmania organisms.

Zheng Y, Hao X, Lin X, Zheng Q, Zhang W, Zhou P, Li S. Microbial Pathogenesis (2018) 121:70-76 Bacterial diversity in the feces of dogs with CPV infection.

Zini E, Nolli S, Ferri F, Massari F, Gerardi G, Nicoli S, Romanelli G, Montinaro V, Trez D, Cavicchioli L, Ferro S. Veterinary Pathology (2018) 56 (3), 358–368 Pheochromocytoma in dogs undergoing adrenalectomy.

PUBLICATIONS

INTERNATIONAL PEER REVIEWED PAPERS

- Rossi G, Meazzi S, Giordano A, Paltrinieri S. Journal of Veterinary Diagnostic Investigation [Submitted, Under Review]. Paraoxonase-1 (PON-1) activity in cats: analytical validation, reference intervals and correlation with serum amyloid A and alpha-1-acid glycoprotein
- Groppetti D, Meazzi S, Filipe J, Colombani C, Panseri S, Palestrini C, Cannas S, Giordano A, Pecile A. [Manuscript in preparation] Maternal and neonatal cortisol link during perinatal period: preliminary results in German shepherd dogs.
- Meazzi S, Paltrinieri S, Brentali I, Ferriani R, Giordano A. [Manuscript in preparation] Role of paraoxonase-1 (PON-1) as a diagnostic marker for feline infectious peritonitis (FIP)
- Meazzi S, Stranieri A, Lauzi S, Bonsembiante F, Ferro S, Paltrinieri S, Giordano A. Research in Veterinary Science 2019. 125:272-278. Feline gut microbiota composition in association with feline coronavirus infection: A pilot study.
- Stranieri A, Probo M, Pisu MC, Fioletti A, Meazzi S, Gelain ME, Bonsembiante F, Lauzi S, Paltrinieri S. Journal of Feline Medicine and Surgery 2019. [Epub ahead of print] Preliminary investigation on feline coronavirus presence in the reproductive tract of the tom cat as a potential route of viral transmission.

ABSTRACTS

- Groppetti D, Meazzi S, Palestrini C, Giordano A, Pizzi G, de Vera d'Aragona I, Pecile A. 2019. Cortisol impact on reproductive and behavioral parameters throughout the perinatal

period: preliminary results in dogs. 22nd European Veterinary Society for Small Animal Reproduction (EVSSAR) Congress, June 28-29th 2019, Berlin, Germany

- Bonsembiante F, Giordano A, Moretti P, Meazzi S, Mazzariol S, Gelain ME. 2019 Post-mortem biochemistry: aqueous humor as an alternative matrix for biochemical analyses in dogs. 73th SISVET Congress, June 19-22th 2019, Olbia, Italy.
- Meazzi S, Stranieri A, Lauzi S, Martino P, Paltrinieri S, Giordano A. 2018 Gut microbiome and feline infectious peritonitis. 72th SISVET Congress, June 20-22th 2018, Turin, Italy.
- Meazzi S, Ferriani R, Paltrinieri S, Giordano A. 2018.Paraoxonase-1(PON-1) as a diagnostic marker for feline infectious peritonitis (FIP). 18th International Society of Animal Clinical Pathology (ISACP) Congress, August 5-8th 2018, Tokyo, Japan.

ORAL PRESENTATION

- Meazzi S, Ferriani R, Paltrinieri S, Giordano A. 2018.Paraoxonase-1(PON-1) as a diagnostic marker for feline infectious peritonitis (FIP). 18th International Society of Animal Clinical Pathology (ISACP) Congress, August 5-8th 2018, Tokyo, Japan.
- Meazzi S, Ferriani R, Verde D, Rossi S, Barbè F. 2017 A dog with a gastric foreign body and leishmaniasis. 19th European Society of Veterinary Clinical Pathology (ESVCP) Annual Meeting. September 6-9th 2017, London, United Kingdom

CO-SUPERVISION OF DEGREE THESIS

- Enrico Bellini: CONFRONTO TRA DUE METODI STRUMENTALI PER LA CONTA CELLULARE IN VERSAMENTI DI GATTO. Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Sara Meazzi. October 2018

- Irene Brentali: UTILIZZO DELLA PARAOXONASI-1 (PON-1) NELLA DIAGNOSI DI PERITONITE INFETTIVA FELINA (FIP). Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Sara Meazzi. February 2019
- Margherita Peri: LEISHMANIOSI CANINA: CORRELAZIONE TRA MICROBIOTA INTESTINALE E ASSETTO IMMUNITARIO. Thesis supervisor: Alessia Giordano; Thesis co-supervisor: Sara Meazzi. October 2019

ACKNOWLEDGMENTS

Grazie a tutte le persone che mi hanno accompagnata alla fine di questo progetto, a tutti quelli che ogni giorno mi insegnano qualcosa e che hanno arricchito la mia vita professionale e personale.

Ad Alessia, tutor ed amica, e con lei tutte le persone con cui ho lavorato in questi anni, ma anche tutti i colleghi e gli studenti che questo dottorato mi ha dato modo di conoscere.

Ai miei genitori, che ancora cercano di ascoltarmi quando parlo di microbiota (che pazienza) ed a mia nonna (anche ai miei nonni che in questi anni se ne sono andati, ma so che sarebbero comunque molto orgogliosi di me: Ecco). A Hiro.

Alle amiche che non vedo mai, ma che ci sono sempre.

Al karate e a tutto il meraviglioso gruppo che è cresciuto e mi ha fatto crescere in questi vent'anni di pratica. Il karate si pratica tutta la vita.

A Matteo per ogni cosa. Sei la mia Domenica.

Una somma di piccole cose.