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“Multidisciplinary approach to Cheetahs affected by gastrointestinal disease: study of the immune profile and of the fecal proteome”

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LIST OF ABBREVIATIONS

A.j.h. *Acinonyx jubatus hecki*

A.j.j. *Acinonyx jubatus jubatus*

A.j.r. *Acinonyx jubatus raineyi*

A.j.s. *Acinonyx jubatus soemmeringi*

A.j.v. *Acinonyx jubatus venat*icus

APCs Antigen Presenting Cells

CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora)

DCs Dendritic Cells

EAZA European Association of Zoos and Aquaria

FeCoV Feline coronavirus

FIP Feline Infectious Peritonitis

GALT Gastric Associated Lymphoid Tissue

GHLO Gastro Helicobacter-like Organisms

GI Gastrointestinal

IELs Intra-epithelial lymphocytes

IFN-γ Interferon gamma

IL Interleukin

IUCN International Union for Conservation of Nature

MHC Major Histocompatibility Complex

NO Nitric Oxide

PBMC Peripheral Blood Mononuclear Cell

PUFA Polyunsaturated Fatty Acids

TLRs Toll-like Receptors

WCMC World Conservation Monitoring Center

"The health of animals, people, plants and the environment are interconnected. One Health is an integrated approach that recognizes this fundamental relationship and ensures that specialists in multiple sectors work together to tackle health threats to animals, humans, plants and the environment"[1].

INTRODUCTION

"One Health" concept links human health, animal health and the environment with a great potential to mutually influence the health of all species. Infectious diseases associated with wildlife can worse negative public perceptions of wild animals and public support for conservation requiring conscientious effort and urgency to avoid an undesirable outcome [2].

The exponential growth of the human population led to ecologic drastic changes related to human encroachment on wildlife habitats [3].

During the 20th century, growth of human population, invasion of wildlife habitats, changes in agricultural practices, domestication of wild animals and ecotourism have damaged the conservation of wildlife [4,5]. Establishing and maintaining protected areas are key tools for biodiversity conservation. However, this approach is insufficient for wide-ranging species. Protected areas, created for conservation purposes, do not always adequately conserve biodiversity, many terrestrial protected areas within human-dominated systems are isolated from one another increasing the risk of species extinctions [6]. The relationship between isolation and extinction is the basis of the "metapopulation theory" that many spatially distinct subpopulations reconnected by movement of individuals, leading to genetic exchange and the possibility of re-establishing extirpated subpopulations [7].

Numerous emerging infectious diseases have arisen from, or have been identified in, wildlife with implications for human health. For this reason, wildlife conservation can be considered an important resource of protection[8]. Since 1960 the IUCN Red List has

made multiple lists for a compendium of animals and plants conservation. To date, more than 134000 species have been assessed for The IUCN Red List, of these 26% are mammals [9].

In this thesis, different aspects related to an animal classified as vulnerable by the IUCN Red List, the cheetah, will be focused.

The aim is to investigate the gastrointestinal physiopathology of cheetahs, with a morbidity rate of 95% in captive animals, correlating it with the immune response.

Different studies compared diseases in captive and free-ranging cheetahs showing the prevalence of stress-induced pathologies [10]. The reduction of the population has led several cheetahs to be housed in zoos, in conditions that do not allow carrying the physiological behaviors of the species [10]. For this reason, some pathologies are defined as probably stress-induced.

The present research project is based on a multidisciplinary approach to the physiopathology of "cheetahs" GI diseases. In the first part of the study, starting from a common viral disease of cats (feline infectious peritonitis - FIP) in which the immune system plays a key role in disease progression, methods of monocytes' isolation from peripheral blood were investigated, and then macrophages were studied regarding phagocytosis and respiratory burst activities. The method was firstly performed from samples of cats with feline infectious peritonitis (FIP) and cats' samples positive for Feline coronavirus but not FIP, and then performing the same evaluations in samples from healthy cheetahs and from subjects with GI disease associated to *Helicobacter* spp. In the second part of project, the study of the cytokines profile on the same samples

was performed, comparing results between animal populations (healthy *vs* diseased cheetahs). In the third line of research, fecal proteomics to investigate possible differences in healthy and diseased cheetahs was performed.

HISTORY AND CONSERVATION STATUS OF CHEETAH

The *Felidae* family includes about 37 species, representing a great morphological variability[11]. Felids have a wide distribution with one or more species native to every continent except Australia, Antarctica and some oceanic islands [11]. As reported by CITES and by the WCMC (World Conservation Monitoring Center), in the adopted classification there are three distinguished sub-families: *Acinonychinea, Felinae, Pantherinae* [12]. Since the 1900s, the cheetah has achieved a unique taxonomic status and has been classified as the only species of the genus *Acinonyx*.

Currently the known cheetah's population is of about 7,000 animals with an estimated total population of no more than 10,000 mature individuals, representing a decline of at least 30% in 18 years [13]. Today, free-ranging cheetahs are isolated populations across Africa and the Middle East. Throughout history, the reduction of cheetahs was caused by hunters, poachers, and habitat destruction causing the extinction of the cheetah in Asia [13]. Its unique adaptations for speed, such as lightweight legs, a small skull, shortened jaws and smaller canines than other felids, makes the cheetah a poor fighter [14]. Consequently, cheetahs are easily robbed of their kills and their cubs are often attacked by other predators [13].

Currently, the competition with stronger predators such as lions (*Panthera leo*), leopards (*P. pardus*) and spotted hyenas (*Crocuta crocuta*) often results in the exclusion of cheetahs from conservation areas or protected reserves. Therefore, cheetahs frequently come into conflict with humans and are often killed by farmers protecting their livestock[13]. Hence, proactive conservation methods, including

alternative livestock protection programs and community education projects play an important role in efforts to prevent the extinction of this species.

GASTROINTESTINAL DISEASES IN CHEETAH

The decline of the cheetah population has increased the focus on the conservation of this species to study chronic diseases. Between 1989 and 1992, Munson and her team conducted the first research about diseases affecting cheetahs in captivity, to calculate the incidence of different pathologies [15]. Renal diseases, gastro-enteric disease, feline infectious peritonitis, testicular degeneration, and pneumonia in cubs, have been shown to have a high prevalence (Tables 1 and 2).

CHRONIC DISEASE	PREVALENCE	REFERENCE
GASTRITIS	99%	(Munson et al., 2005)[16]
RENAL DISEASE	81%	(Munson et al., 2005)[16]
VENO-OCCLUSIVE DISEASE*	43%	(Munson et al., 2005)[16]

*** Veno-occlusive disease (VOD) of the liver**

Table 1. Prevalence of chronic diseases in cheetahs.

INFECTIOUS DISEASE	PREVALENCE	REFERENCE
FELINE CORONAVIRUS (FECOV)	29%	(Munson et al., 2004) [17]
FELINE HERPESVIRUS TYPE 1 (FHV1)	12%	(Munson et al., 2004) [17]
FELINE PANLEUKOPENIA VIRUS (FPV)	48%	(Munson et al., 2004)[17]
CANINE DISTEMPER VIRUS (CDV)	24%	(Munson et al., 2004)[17]

FELINE LEUKEMIA VIRUS (FELV)	0%	(Munson et al., 2004)[17]
FELINE IMMUNODEFICIENCY VIRUS (FIV)	0%	(Munson et al., 2004)[17]

Table 2. Prevalence of infectious diseases in cheetahs.

Among these conditions, chronic gastritis showed high prevalence in cheetahs, and in 95% of cases it was caused by spiraliform bacteria [15]. To date, it is known that gastrointestinal diseases represent a high number of recorded cases associated with significant mortality in cheetahs, especially in captive animals[18,19]. Reasons must be sought in diseases associated to chronic gastritis as for example: Barrett's esophagus [20], gastro-esophageal reflux disease (GERD), acquired hiatal hernia, and related secondary conditions such as *ab ingestis* pneumonia[15,21,22]. Inconstant clinical signs including vomiting, diarrhea, and weight loss are described in cheetahs affected by chronic gastro-enteric disorders. Gastritis in cheetah was also classified with 3 histological degrees, depending on lesions severity[19] (Figure 1):

- **gastritis grade 1**: characterized by moderate inflammation and few necrotic areas interspersed in the epithelial layer.
- **gastritis grade 2**: characterized by inflammatory infiltrate invading the lamina propria with some glandular dilatations and necrotic area.
- **gastritis grade 3**: in which the lamina propria is invaded by inflammatory infiltrate, and the glands appear dilated, with necrotic areas associated to areas of erosion and ulceration.

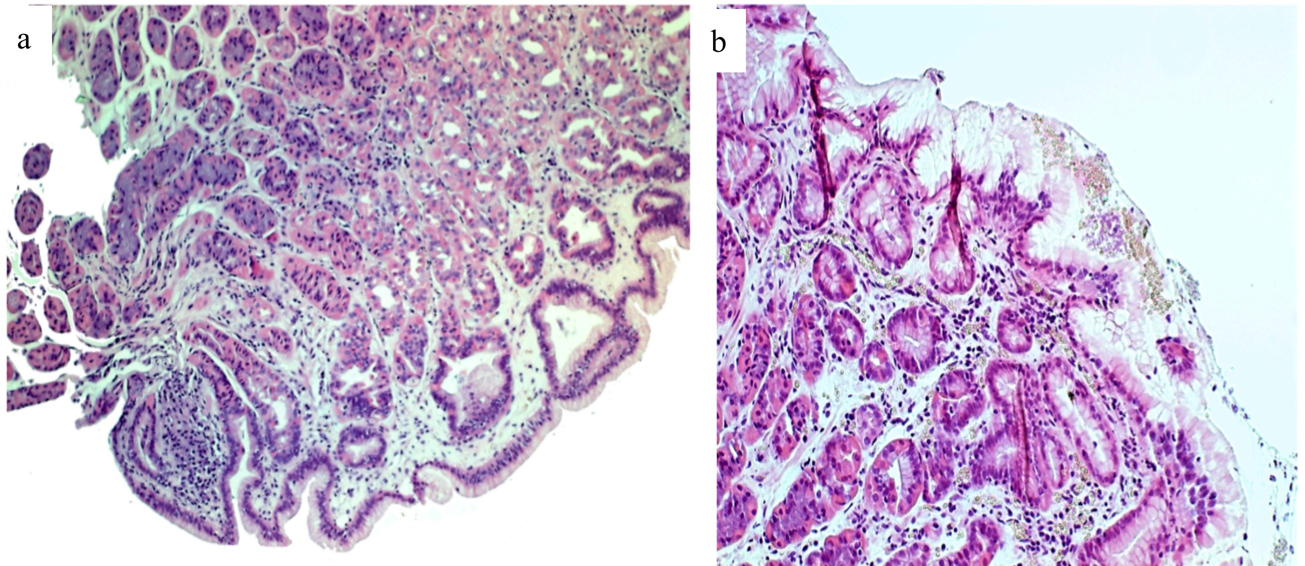

Figure 1. Histological pictures of stomach in cheetahs with gastric disorders (a-b). Stomach characterized by chronic-active interstitial gastritis with neutrophilic-lymphocytic inflammatory infiltrate and oedema of the mucosal corion (H&E. 20X).

As strict carnivores, cheetah has a simple and relatively developed stomach and a small intestine that is predominant while the large intestine is much smaller [23,24]. This anatomical conformation has been adapted to the diet of the species based on the consumption of animal tissues. These animals need to take proteins with the diet to obtain the essential amino acids and a non-specific nitrogen source that is used to synthesize other nitrogen compounds [25]. Also, felids cannot synthesize adequate amounts of arachidonic acid from linoleic acid [26], and cheetahs have limited delta-6 desaturase which catalysts the biosynthesis of polyunsaturated fatty acids (PUFAs), that converts linoleic acid in gamma-linolenic acid and hence the arachidonic acid [27]. The ratio between n-3/n-6 PUFAs is an important cofactor in the modulation of the immune system and to maintain an anti-inflammatory condition [28]. PUFAs deficiency

needs to be supplemented in the diet, however, different concentrations of arachidonic acid are present in the prey they consume; for example, rabbit (*Oryctolagus cuniculus*) meat contains lower concentrations of arachidonic acid than the meat of ungulates [29]. PUFAs concentration is elevated in hunted prey in the wild but, in the diet in captive conditions, these acids are highly unstable and rapidly deteriorate on low temperature storage of meats[30]. Depending on the type of feeding of the chicken (*Gallus gallus*), in this species the amount of n-6 PUFA is very high [28]. Free-range cheetahs hunt ungulates such as eland (*Taurotragus oryx*), kudu (*Tragelaphus strepsiceros*), springbok (*Antidorcas marsupialis*), steenbok (*Raphicerus campestris*), scrub hare (*Lepus saxatilis*), hartebeest (*Alcelaphus buselaphus*), gemsbok (*Oryx gazella*) and other small mammals, while livestock (*Bovidae family*), goats (*Capra* spp.), and sheep (*Ovis* spp.), are comprised only in a small proportion [31]. In captive cheetahs, the diets are different, being fed with meat from rabbit, chicken, beef, turkey (*Meleagris gallopavo f. domestica*), horse, lamb and goat [22]. Studies suggested that the prevalence of diarrhea associated with dietary factors was significantly higher in cheetahs fed raw meat or mixed diets (made by the alternation of raw meat, commercially prepared and carcasses), while being lower in carcass-fed cheetahs [22]. The components of proper diets are essential substrates for the proper function of the gastrointestinal apparatus and contribute to the composition of cheetah's microbiome, conditioning a healthy gut and consequently, the health of the host animal. Cheetah's GI microbiome is mainly composed by *Firmicutes*, *Fusobacteria*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* [32]. The difference in bacterial populations found in the GI microbiome

of free-range cheetahs, if compared with captive ones, highlights the presence of bacteria belonging to the classes Clostridia and Erysipelotrichi, possibly associated with the intake of perivisceral fat present in hunted prey [32]. Conversely, *Clostridium chauvoei* and *Enterococcus avium*/*E. hirae* have been detected in captive cheetah and are possibly associated with meat handling by the keepers and the presence of domestic animals [32]. Studies showed that n-3 PUFAs could reduce *Helicobacter* spp. associated gastric diseases through an inhibitory effect on bacterial growth via disruption of cell membrane leading to bacteria lysis [33] reducing iodoacetamide-induced gastritis by decreasing malondialdehyde (MDA), gastrin and nitric oxide (NO), and normalizing mucosal glutathione levels [34]. It is well established that *Helicobacter* spp. infection can enhance PGE2 synthesis and accelerate n-6 PUFAs metabolism in gastric mucosal cells. Conversely, since essential dietary fatty acids confer protection to the gastroduodenal mucosa, n-3 PUFAs represent an important gastro-protective factor against damage to the gastric mucosa induced by *Helicobacter* spp. [35]. Early studies have shown that the cause of gastritis in captive cheetahs were linked to Helicobacter-like organisms which are found in the wild population, with no signs of disease in the latter [19]. It has been hypothesized that captivity can exasperate stress conditions in cheetah including enclosure size, the presence of the people, diet, the lack of exercise and inability to perform other natural behaviors [36].

IMMUNE SYSTEM ALTERATION AND GASTROINTESTINAL DISEASE

Stressors cause an adaptive response of the organism through the interaction between the neuroendocrine and the immune systems [37]. In the evolution, vertebrates developed adaptations in response to harmful or dangerous stimuli [38]. This primary biological response, aimed at survival, is characterized by activation of the hypothalamus-pituitary-adrenal axis, followed by the release of catecholamine and cortisol from the medullary and the cortical of the adrenal gland area [38]. However, the variability in the physiological stress response in animal species is not fully understood [39]. Knowledge of disease susceptibility of endangered species is fundamental to understand the population dynamics of such species and for planning conservation strategies. The global cheetah's population has diminished drastically, yet the health status and disease susceptibility of cheetahs have been studied predominantly in captive cheetahs. It is supposed that cheetahs kept in captivity can suffer from infectious and chronic degenerative diseases, with subsequent mortality. The high prevalence of infectious diseases in captive cheetahs was suggested to be a consequence of a lack of genetic variability at the *class I* loci of the major histocompatibility complex (MHC-I) [40]. The most prevalent disease reported in the cheetah is chronic gastritis associated with increased stress levels [10]. To assess stress in cheetahs, cortisol levels were measured using different methods such as the evaluation of adrenal gland cortico-medullary ratio or fecal corticosterone concentrations [41-42]. In chronic stress condition a greater release of adreno corticotropic hormone (ACTH), stimulating the cells of the fasciculate area,

increases synthesis and secretion of cortisone, causing hypertrophy and hyperplasia of the glandular area [9,41]. In Japan, fecal corticosterone was monitored on cheetahs between two seasons (spring to winter) to identify a possible relationship between the climatic conditions and corticosterone levels. The study showed that cheetahs were susceptible to climatic variations as suggested by higher fecal corticosterone concentrations, caused by the decrease in temperature[43].

In the GI system, stress can induce a catecholamines mediated ischemia of the gastric mucosa, decreasing mucus secretion[44].

Furthermore, a greater quantity of corticosteroids produced under stressful conditions, induces a reduction in the synthesis of prostaglandins through the suppression of the constitutive enzymes COX1 and COX2. The activity of the COX1 enzyme is fundamental for the synthesis of prostaglandins which favor the production of mucus by the epithelium of the gastric body and antrum regions. Elevated cortisol levels therefore strongly reduce gastric mucus production.

In this stress condition, *Helicobacter* infection can interfere with the processes of physiological control of gastric acidity. Physiologically, gastrin-producing G cells and somatostatin-producing D cells regulate gastric acid secretion; they are regulated by a paracrine feedback system of mutual regulation, but also by endocrine and nervous mechanisms [45]. Gastrin, released by G cells, responsible for acid secretion, is influenced by histamine, gastrin, and vagal nerve stimulation [46]. It has been shown that the gastric colonization of some *Helicobacter* species (for example *H. pylori*, *H. canis*, etc.) is associated with an increase in the concentration of G cells in the antral mucosa

and in gastrin values [45]. These changes in gastrin and somatostatin increase acid secretion and lead to ulceration. It is therefore possible that in cheetahs, the combined action of stress and infection lead to a serious alteration of the mucous layer, exposing the mucosa to infection-induced hyperacidity [45]. The inflammatory products that are generated further altering acid secretion and leading to gastric atrophy with hypochloridria over time. Additionally, chronic stress induces changes in the neuroendocrine and immune systems, predisposing to chronic diseases as Helicobacter-associated gastritis [21]. As reported above, the ratio between n-3/n-6 PUFAs is an important co-factor in the modulation of the immune system (Figure2) and in the anti-inflammatory function [28].

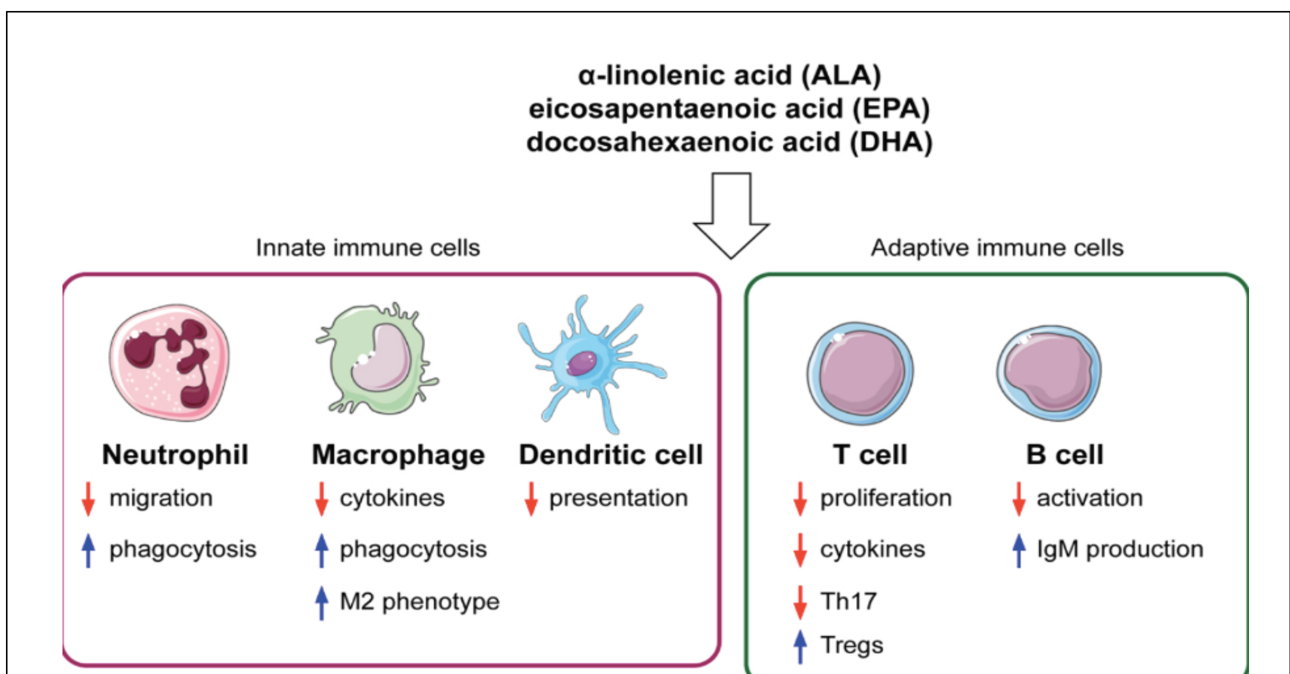

Figure 2. Effect of omega-3 fatty acids in immune system cells [47]. https://www.mdpi.com/1422-0067/20/20/5028

In cheetahs, GHLOs are not found in only sick animals. External or internal host factors can contribute to the development of the gastroenteric pathology having, an important role in the pathogenesis of chronic gastritis, related to stress, diet and individual immune response. Unlike *H. pylori*, in cheetahs, the isolated GHLOs do not seem to fulfill Koch's postulates in accordance with which:

1- the microorganism must be found in the diseased animal, and not found in healthy animals";

2- it "must be extracted and isolated from the diseased animal and subsequently grown in culture";

3- it "must cause disease when introduced to a healthy experimental animal";

4- it "must be extracted from the diseased experimental animal and demonstrated to be the same microorganism that was originally isolated from the first diseased animal"[48].

Very interestingly, however, research by O'Brien *et al.* [49] revealed that cheetah went through a historic population bottleneck leaving them genetically monomorphic at the major histocompatibility complex (MHC) [49]. It has been showed that bottlenecked populations have a highly reduced MHC variation associate with low immune adaptability and a prevalence for disease and extinction [50]. The sharp reduction of the population impairs species immune-response and the low polymorphisms in MHC I and II genes have been associated with high susceptibility to infectious diseases [51]. In MHC, two subgroups were suggested in 2000 by different metabolism of arginine after LPS injection [52]:

a) MHC-I molecules are expressed by all nucleated cells and are recognized by cytotoxic CD8+ T cells [53];
b) MHC-II which are present on antigen-presenting cells such as macrophages and lymphocytes and can present processed antigens to T-helper cells [53].

This family of genes encodes receptor molecules to recognize and bind foreign proteins driving the immune cells and the immune response. Pathogens or foreign organisms can enter cells by infection or phagocytosis into specific cells such as macrophages. Through the interaction between protein fragments of the pathogen and the MHC, the activation of the immune response starts [53] . Genetics is a key factor in the control of the immune response and, consequently, in the evolution of the disease.

SCIENTIFIC AIMS OF THE STUDY

The present research project is based on a multidisciplinary approach aiming at investigating the gastrointestinal disease in cheetahs, mainly induced by *Helicobacter* spp. infection.

In the first line of the project, I performed the study of samples from cats with feline infectious peritonitis (FIP) and in samples from cats positive for Feline coronavirus not causing FIP. The test subsequently performed in samples from healthy cheetahs and from subjects with GI disease associated to *Helicobacter* spp. Although apparently different, it was decided to refine the technique in cats with feline coronavirus infection and then to apply it on cheetahs, as in both diseases the severity of the condition is caused by the host's immune response to the pathogen. Samples have been collected from cheetahs housed in European zoos through a project authorized by Biobank EAZA.

The second line of the project aimed at studying the cytokine profile on the same samples, comparing results between animal populations.

Within the third line of research, I performed the study of the fecal proteome in both healthy and diseased cheetahs.

The different goals were achieved through different steps synthesized as follows:

- Study of macrophages activity in cats with feline infectious peritonitis and healthy Feline Coronavirus (FeCoV)-shedding cats.

- Study of macrophages activity in cheetahs with gastrointestinal diseases associated with gastric *Helicobacter*-like organisms (GHLOs) and healthy cheetahs.

- Study of the immune profile through the determination of the cytokine pattern in cats with feline infectious peritonitis and healthy FeCoV-shedding cats.
- Study of the immune profile through the determination of the cytokine pattern in cheetahs affected by gastritis associated with gastric *Helicobacter*-like organisms and healthy cheetahs.
- Evaluation of the fecal protein pattern in cheetahs, investigating possible differences between healthy and diseased subjects.

RESEARCH LINES OF THE STUDY

a. STUDY OF MACROPHAGES ACTIVITIES

a.1 INTRODUCTION

The mammalian digestive tract is a complex environment for the immune system which maintains ongoing homeostasis by highly specialized structural and cellular strategies [54]. The epithelium is constituted of a single layer of epithelial cells (IECs) that participates in innate immunity. The turnover of IECs is another important mechanism to prevent pathogen attachment to the intestinal mucosa, as well as tight junction complexes, in which claudins, occludins, zonula occludens and junction adhesion molecules create a seal between neighboring IECs and pathogens [55]. Under the epithelium, the *lamina propria* harbours dendritic cells (DCs), which are important APCs and the gut-associated lymphoid tissue (GALT). GALT includes Peyer´s patches, lamina propria-lymphocytes and intraepithelial lymphocytes (IELs) [56]. Under physiological condition, there is a dynamic regulation of all these components, however sustained inflammation or infections can lead to dysregulation and barrier breach allowing the entry of microbes [57]. In the *lamina propria* from the proximal small intestine to the distal large tract there is the largest population of resident macrophages [58]. The resident macrophages are specialized phagocytic cells characterized by bactericidal properties, and chemokines production finalized to the recruitment of effector cells from the blood [59]. Macrophages can produce IL-1β following TLR

stimulation, supporting Th17 cell development in the healthy gut [60]. Many of the macrophages found in lymphoid and non-lymphoid tissues are derived from peripheral blood monocytes that were recruited from the blood into the tissue, the differentiation occurs on exposition to cytokines favoring inflammation and local growth factors production [61]. Depending on the stimulus that actives macrophages, these cells can exist on a spectrum of phenotypes, the end of which are the pro-inflammatory or classical activated **M1 macrophages** and anti-inflammatory or alternatively activated **M2 macrophages**. M1 macrophages, also named classically activated macrophages, are identified as CD64+CD80+. This type can be polarized through interferon-γ, lipopolysaccharide, tumor necrosis factor-α, or monocyte chemoattractant protein-1 [62]. M2 macrophages are identified as CD11b+CD209+, which can be polarized through IL-4, IL-10, IL13, glucocorticoids, or vitamin D3 [62].

In general, macrophages are important cells of the immune system involved in both innate and cell-mediated immunity [61]. These cells have different functions:

- Eliminate foreign organisms and dead cellular debris by phagocytosis [61]. Internalization mechanisms are complex, and include macro-pinocytosis (the major uptake pathway for extracellular solutes, or particles), endocytosis (process for importing viruses, microorganisms, and nanometer sized particles), and phagocytosis (mechanism by which foreign particles such as dead cells, pathogens and digesting drug particles can enter cells and is characterized by the fusion of the phagosome and lysosome) [63];

- Present antigens derived from these entities to T or B lymphocytes [61];
- Produce a broad of mediators such as cytokines, growth factors, and reactive oxygen species that influence other immune cells functions [61].

Phagocytosis is a key component of the immune defense. This dynamic pathway is defined as the cellular uptake of particulates (>0.5 µm) within a plasma-membrane envelope, and different names have been applied associated with the uptake of apoptotic cells (efferocytosis) and of necrotic cells in infection and inflammatory condition (necroptosis and pyroptosis) [64]. Once internalized, the phagosome vacuole can fuse with primary lysosomes, or with the product of the endoplasmic reticulum and Golgi apparatus producing a secondary phago-lysosome [65]. The term ‘‘professional phagocytes’’ was introduced by Michel Rabinovitch, related to the highly efficient activity in leukocytes, and includes monocytes, macrophages, dendritic cells, neutrophils, and osteoclasts [59].

After phagocytosis, newly recruited monocytes and tissue macrophages use pre-existing phospholipids and arachidonates in the plasma membrane to release radicals generated by activation of a *respiratory burst* or induction of nitric oxide synthesis, and secretion induced by phagocytosis in macrophages is mainly achieved by new synthesis of RNA and a large variety of cytokines and enzymes [66].

There is ongoing interest in establishing how and to what extent the host phagocyte can mount an appropriate response [67].

Phagocytosis is commonly assessed via:

- microscopy: allows for clear discernment between engulfed particles and those associated with the cell surface [68];
- flow cytometry: relatively rapid, however intra- and extracellular particles are often indistinguishable from one another [68];
- fluorometric plate-based approach [68].

Respiratory burst is characterized by the rapid release of reactive oxygen species. As a mechanism against pathogens, the assessment of respiratory burst is used in immunological studies. Respiratory burst is often assessed via flow cytometry but alternative methods are reported. Plate-based colorimetric assays provide an alternative approach for estimating respiratory burst *in vitro* [69]. In this assay, respiratory burst may be induced via one mitogen (e.g., phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS)) [70]. Nitroblue tetrazolium (NBT), is added to isolated cell suspensions, and it diffuses into the cells where it is reduced by reactive oxygen species, particularly superoxide, to produce formazan crystals, which upon dissolution in potassium hydroxide (KOH) and dimethyl sulfoxide (DMSO) produce a blue color. This color change can be measured with a standard plate reader. The absorbance of the solution is directly related to the amount of superoxide produced [70].

Different methods have been employed to isolate monocytes from peripheral blood. Commonly used methods are plastic adhesion and magnetic bead-based isolation kits [71].

Within the first research line of the present project, it was evaluated the phagocytosis and respiratory activity of monocyte-derived macrophages in **FIPV-infected cats** and **cheetahs with *Helicobacter* spp. infection**. Knowledge of the immune mechanisms involved in animal protection plays a pivotal role in understanding the pathogenesis and clinical progression of these diseases.

Feline coronavirus (FeCoV) is the major pathogens of *Felidae* family with worldwide distribution [72]. In cats FeCoV replicates in the intestines and can spread by oral-fecal transmission but not understood changes can give rise to mutants that are referred to feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) [72]. FIP is mainly a disease of domestic cats but has also been recognized in the African lion, Mountain Lion, Leopard, Jaguar, and Cheetah [73-74]. In FIP there are two clinical forms which can occur separately or coexist [75,76]. The effusive FIP is characterized by pyogranuloma and the infection is considered as a distinct form of vasculitis, induced by Type III hyper-sensivity reaction. This form is particularly prevalent in the abdomen covering the serosal surfaces of organs, fibrin and protein-rich fluid are also deposited within and around the lesions and necrosis is often evident [77] (Figure 3).

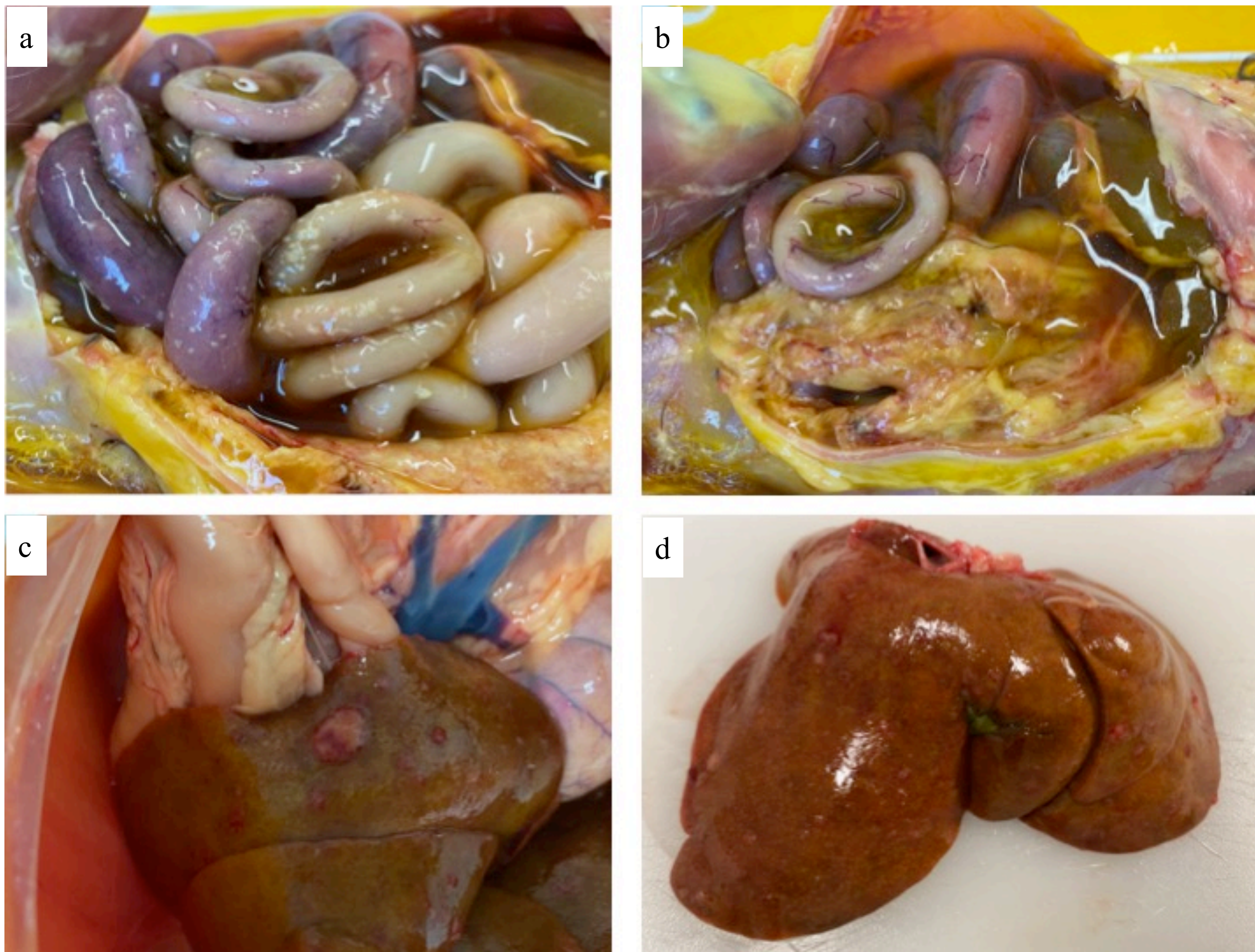

Figure 3. Gross lesions at necropsy in a cat with FIP. All lesions were confirmed by subsequent histological examination and immunohistology demonstration of feline coronavirus (FeCoV) antigen within the lesions. Abdominal cavity shows fibrous and fibrinous adhesions between the organs and the body wall: a) presence of extensive fibrinous plaque on intestinal serosal surfaces; b) yellow effusion visible in the abdominal cavity; c-d) macro and micro-granulomatous granulomas lesions on the surface of the liver.

Pyogranulomatous lesions are histologically made up of central aggregates of macrophages adjacent to venules and surrounded by a rich inflammatory infiltration composed by neutrophils and macrophages with a scattering of plasma cells and T-lymphocytes [78]. Macrophages within pyogranulomas lesions contain viral antigen [77]. Though the pyogranulomatous process is usually surface oriented, focal lesions of

vasculitis and a mixed inflammatory cells infiltrate may be seen deep in underlying muscle or organ parenchyma (Figure 4).

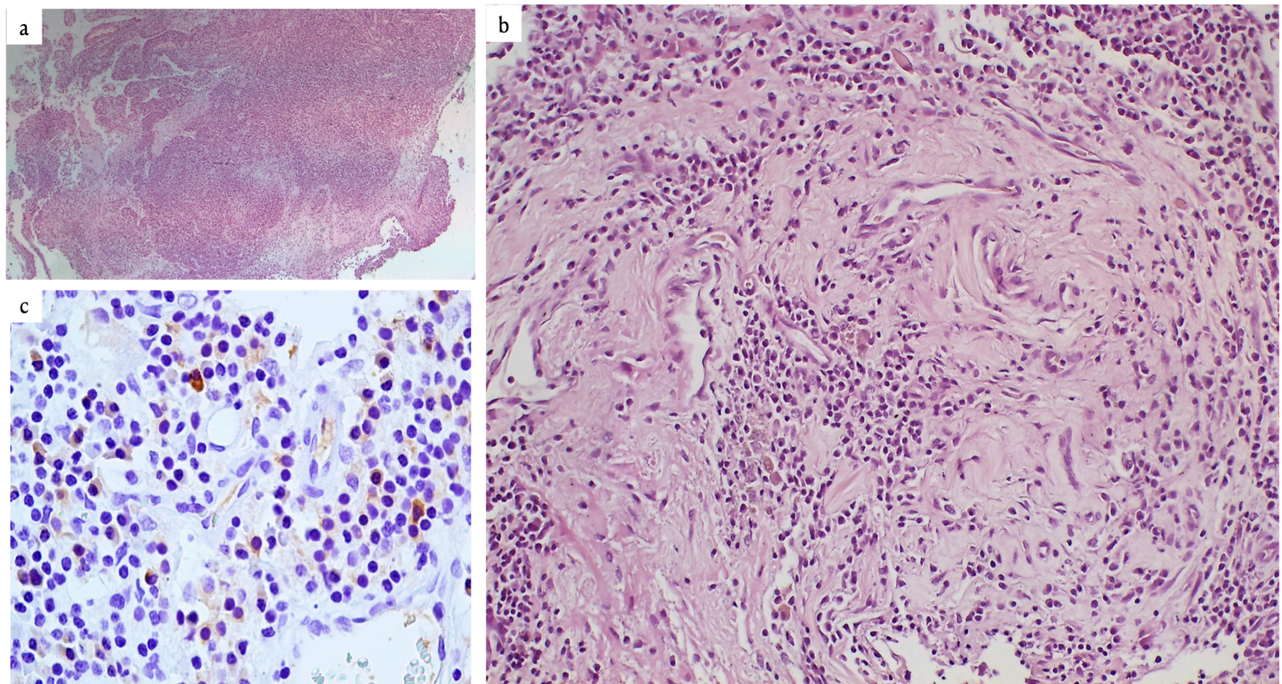

Figure 4. Histological findings in a cat with FIP: a) Perivascular infiltrates in the white matter and diffuse infiltrates in the leptomeninges. (b) The infiltrate is dominated by macrophages and lymphocytes. (c) The perivascular infiltrate as well as the leptomeningeal infiltrate FIPV3 positive cells.

The pathogenesis of FIP is still not fully understood, studies suggested that responses of macrophages to the virus followed by depletion of CD4+ and CD8+ T-lymphocytes are crucial for understanding the virus-host interactions [79,80]. A predominant theory has been that the mutation of FeCoV virus leads to pathogenic FIPV and consequently tropism for monocytes/macrophages which transport the virus systemically, causing the disease [81,82]. FIPV-infected macrophages cause the typical immunopathological damage. As a result of apoptosis, severe T-cell depletion in association with FIPV-

positive macrophages causing release of pro-inflammatory cytokines and cytokine dysregulation has been observed in lymphoid organs [83]. However, to date no convincing studies have been published that demonstrate incontrovertibly that this viral mutation occurs, transforming FeCoV into FIPV.

In cheetahs, gastritis associated with *Helicobacter* organisms has been an important clinical disease reported predominantly in the captive population [15]. Chronic gastritis may predispose to other chronic pathologies up to the death of the animal, and the severity of the clinical presentation is different between captive and free-ranging animals [15,18]. Two *Helicobacter* species have been identified in cheetahs with gastritis: *Helicobacter acinonychis* (or *H. acinonyx*), associated with lymphoplasmacytic gastric infiltration, lymphoid follicles with hyperplastic gastric associated lymphoid tissue (GALT), evidence of gastric reflux, erosion, and necrosis; and *H. heilmannii*, that seems to be less frequently associated to gastric disease [18]. The correlation between captivity and severity of clinical symptoms in captive cheetahs, if compared to those in free ranging, would seem related to internal and external factors. In captivity, the social composition of groups, the enclosure, the visitors, the vocalization and the lack of predatory activity or exercise are associated with stress conditions that predisposes animals to gastritis. In addition, the differences in the diet between captive and free-range cheetahs, may lead to nutritional deficiencies and alteration of the cheetah's microbiome.

a.2 MATERIALS AND METHODS

To assess biological variation and repeatability of the described methods, each assay was conducted in separate trials, each consisting of one sample obtained from different subjects for each trial.

Ethical review and approval were not required for this study because this case report referred to a spontaneous medical condition of client-owned cats; samples used were those collected for medical purpose. Written informed consent was obtained from the owners before enrolling their animals in the study.

For cheetahs' samples, written informed consent was obtained from the zoos for the participation of their animals in this study. Blood samples used were those resulting from routine programmed screening/monitoring evaluations. The present research project was approved by the EAZA Biobank and the European Cheetah Protection Program Coordinators. With this agreement, all European zoos belonging to EAZA were included for sending cheetah samples.

For the study of macrophage activity in cats the study population consisted in:

- Study group or (FIP +): 26 cats diagnosed with feline infectious peritonitis of which 14 with the clinical wet form and 12 with the dry form. The diagnosis for the wet form was made by PCR examination on a sample of abdominal effusion, which was positive for FIPV. The diagnosis for the clinical dry form was made by PCR assay on abdominal lymph node biopsy or CSF. All subjects tested negative for other infectious diseases (FIV, FeLV, and *Toxoplasma* spp.).

- Control group or (FIP-): 32 healthy cats, positive for feline coronavirus (FeCoV) in fecal samples. All subjects were tested for infectious diseases (FIV, FELV), resulting negative. Complete blood test and serum biochemical examination were performed and resulted within the reference values for all parameters.

Pathology	Whole blood	Serum	Feces	Necropsy
FIP (wet form)	14	14	14	10
FIP (dry form)	12	12	12	5
FeCoV infection	32	32	32	0

Table 3. Samples from cats with FIP (wet and dry form) and *feline coronavirus* infection including whole blood, serum, feces, necropsy tissue samples.

For the study of macrophage activity in cheetahs the study population consisted in:

- Study group or (GD): 7 cheetahs with a clinical history of recurrent gastrointestinal disorders and positive for *Helicobacter* spp.; all subjects were tested with complete blood tests, and FIV-FeLV tests.
- Control group or (Healthy): 4 healthy cheetahs; all subjects were tested for infectious diseases (FIV, FeLV) and tested negative. Complete blood test and serum biochemical examination were performed and resulted within the reference values.

Cheetahs	Whole blood	Serum	Faeces	GI biopsies
GI disorder	7	7	19 (7+12)	3
Healthy	4	4	7 (4+3)	1

Table 4. Cheetah samples examined during my PhD. Samples were sent from European zoos but not all biological matrices were received for each animal. The cheetahs included in my study were those that were shipped whole blood and serum and feces.

i) Macrophage's phagocytosis activity in cats

Whole blood was obtained from residual venous whole blood collected for medical purpose from 26 cats with feline infectious peritonitis and 32 healthy FeCoV-shedding cats. Monocytes were isolated in parallel by either CD14positive selection with QuadroMAcs separator as reported by the manufacturing instructions (Miltenyi Biotec). PBMCs for CD14positive selection were diluted to 1×10^8 cells/ml Leibovitz's L-15 cell media supplemented with 10% FCS, 100 U/100 µg/ml penicillin/streptomycin (ThermoFisher Scientific). 200 µl of monocyte suspension with and without PMA was distributed in duplicate in polarized slides. Slides were placed in a 30°C humidified incubator to recover overnight. After 24h the solution in excess on each slide was removed. 200 µl of solution with *Saccharomyces cerevisiae* was added. Slides were placed in a 30 °C humidified incubator for 90 minutes to allow phagocytosis. Three washes with PBS were carried out on each slide, always with

extreme caution until a background opacity was obtained, indicating the presence of the cells. The slides dried and staining was carried out with May-Grunwald Giemsa stain. Phagocytosis was measured by counting, microscopically, the number of macrophages that had ingested one or more yeast cells (*Saccharomyces cerevisiae*) per HPFs. The percentage of phagocytosis, on the other hand, was obtained by counting on a total of 100 macrophages, those who had engulfed the yeast cells in their cytoplasm.

The phagocytosis activity methods using fluorescent liposomes were also investigated. After incubating for 24h at 37°C, the media was carefully aspirated and then 200µL liposomes resuspended and added to the cells culture. These cells were also incubated at 37°C for 4 hours to measure internalization of the fluorescent liposomes. After 3×PBS washes, slides were then analyzed with C2 Plus confocal laser scanning microscope (Nikon Instruments, Firenze, Italy). Optimized emission detection bandwidths were configured by Zeiss Zen control software. Images were processed using NIS Element Imaging Software (Nikon Instrumentes, Firenze, Italy).

ii) Macrophages respiratory burst in cats

Whole blood was obtained as previously reported. Monocytes were isolated in parallel by either CD14pos selection with QuadroMAcs separator as reported by the manufacturing instructions (Miltenyi Biotec). PBMCs for CD14positive selection were diluted to 1×10^8 cells /ml Leibovitz's L-15 cell media supplemented with 10% FCS, 100 U/100 µg/ml penicillin/streptomycin (ThermoFisher Scientific). Cell viability was

evaluated by MTT assay and resulted greater than 98%. Isolated cells were cultured in 96-well plates at 37°C, 5% CO_2 for 24h allowing the cells to recover overnight. The following reaction mixes were prepared: nitroblue tetrazolium (NBT) at stock solution concentration 2.5 mg/mL, and phorbol 12-myristate 13-acetate (PMA) at stock solution concentration 100 μg/mL. Using a multichannel pipette, 50μL of media was removed from the top of each well. Cells should have settled to the bottom and therefore not be removed from the plate, as reported by Hampton *et al.* [84]. After vigorously vortexing, 5μL of NBT and 0,5 μL of PMA in triplicate were added to each sample and then returning immediately the plate to the incubator for 1h. Following incubation, 100μL 70% MeOH to each well are added, then immediately removed all solution from the wells and washed each well with an additional 100μL 70% MeOH two more times. After the second wash, remove any remaining solution and allow the plate to air dry at room temperature. After the plate was completely dry (30min), 120μL of 2M KOH was added to each well followed by 140 μL of DMSO and mixed 15 times to dissolve formazan. The absorbance of the solution on a standard plate reader at 620 nm is measured.

iii) Macrophage's phagocytosis activity in cheetahs

Whole blood was obtained from residual venous whole blood collected for screening/monitoring purpose from 7 cheetahs with gastrointestinal disorders and 4 healthy cheetahs. Monocytes were isolated in parallel by either CD14+ selection with QuadroMAcs separator as reported by the manufacturing instructions (Miltenyi

Biotec). PBMCs for CD14+ selection were diluted to 1×108 cells/ml Leibovitz's L-15 cell media supplemented with 10% FCS, 100 U/100 μg/ml penicillin/streptomycin (ThermoFisher Scientific). Two hundred μl of monocyte suspension with and without PMA was distributed in duplicate in polarized slide. Slides were placed in a 30°C humidified incubator to recover overnight. After 24h the solution in excess on each slide was removed. 200 μl of solution with *Saccharomyces cerevisiae* was added. Slides were placed in a 30°C humidified incubator for 90 minutes to allow phagocytosis. Three washes with PBS were then carried out on each slide, always with extreme caution until a background opacity was obtained, indicating the presence of the cells. The slides dried and staining was carried out with May-Grunwald Giemsa stain. Phagocytosis was measured by counting, microscopically, the number of ingested yeast (*Saccharomyces cerevisiae*) within the macrophages (as previously reported).

iv) Macrophages respiratory burst in cheetahs

Whole blood was obtained as previously reported. Monocytes were isolated in parallel by either CD14+ selection with QuadroMAcs separator as reported by the manufacturing instructions (Miltenyi Biotec). PBMCs for CD14+selection were diluted to 1×108 cells/ml Leibovitz's L-15 cell media supplemented with 10% FCS, 100 U/100 μg/ml penicillin/streptomycin (ThermoFisher Scientific). Cell viability was evaluated by MTT assay and resulted greater than 98%. Isolated cells were cultured at a density of 1×106/mL in 96-well plates (Nunc, Roskilde, Denmark), at 37°C, 5%

CO_2 for 24 h allowing the cells to recover overnight. The following reaction mixes were prepared: nitroblue Tetrazolium (NBT) at stock solution concentration 2.5 mg/mL, and phorbol 12-myristate 13-acetate (PMA) at stock solution concentration 100 μg/mL. Using a single-channel or multichannel pipette, 50 μL of media was removed from the top of each well. Cells should have settled to the bottom and therefore not be removed from the plate, as reported by Hampton *et al.* [84]. After vigorously vortexing, 5μL of NBT and 0.5μL of PMA in triplicate was added to each sample, and then the plate immediately returned to the incubator for 1h. Following incubation, 100μL 70% MeOH were added to each well; immediately removing all solution from the wells and washing each well with an additional 100μL 70% MeOH two more times. After the second wash, any remaining solution was removed, and the plate air-dried at room temperature. After the plate was completely dry (30min), 120μL of 2M KOH was added to each well followed by 140μL of DMSO and mixed 15 times to dissolve formazan.

The absorbance of the solution on a standard plate reader at 620 nm is measured.

a.3 STATISTICAL ANALYSIS

All data are presented as the means ± SEM. A two-way Analysis of Variance (ANOVA) with Sidak's multiple comparisons test was used to analyze differences between phagocytic and non-phagocytic cells percentage between FIP+ and healthy FeCoV-shedding cats (FIP-) to investigate differences in PMA-stimulated respiratory burst activity between the same two categories of cats. Moreover, a Mann-Whitney test

was used to compare differences within FIP+ and FIP- groups both for phagocytic percentage and respiratory burst activity. All statistical analyses were performed with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered significant.

a.4 RESULTS

i) Macrophage’s phagocytosis activity in cats

For each animal in both groups, 3 microscopic fields were examined (Figure 6). The mean of the number of phagocytic cells undergoing yeast phagocytosis was evaluated and reported in tables 5 and 6.

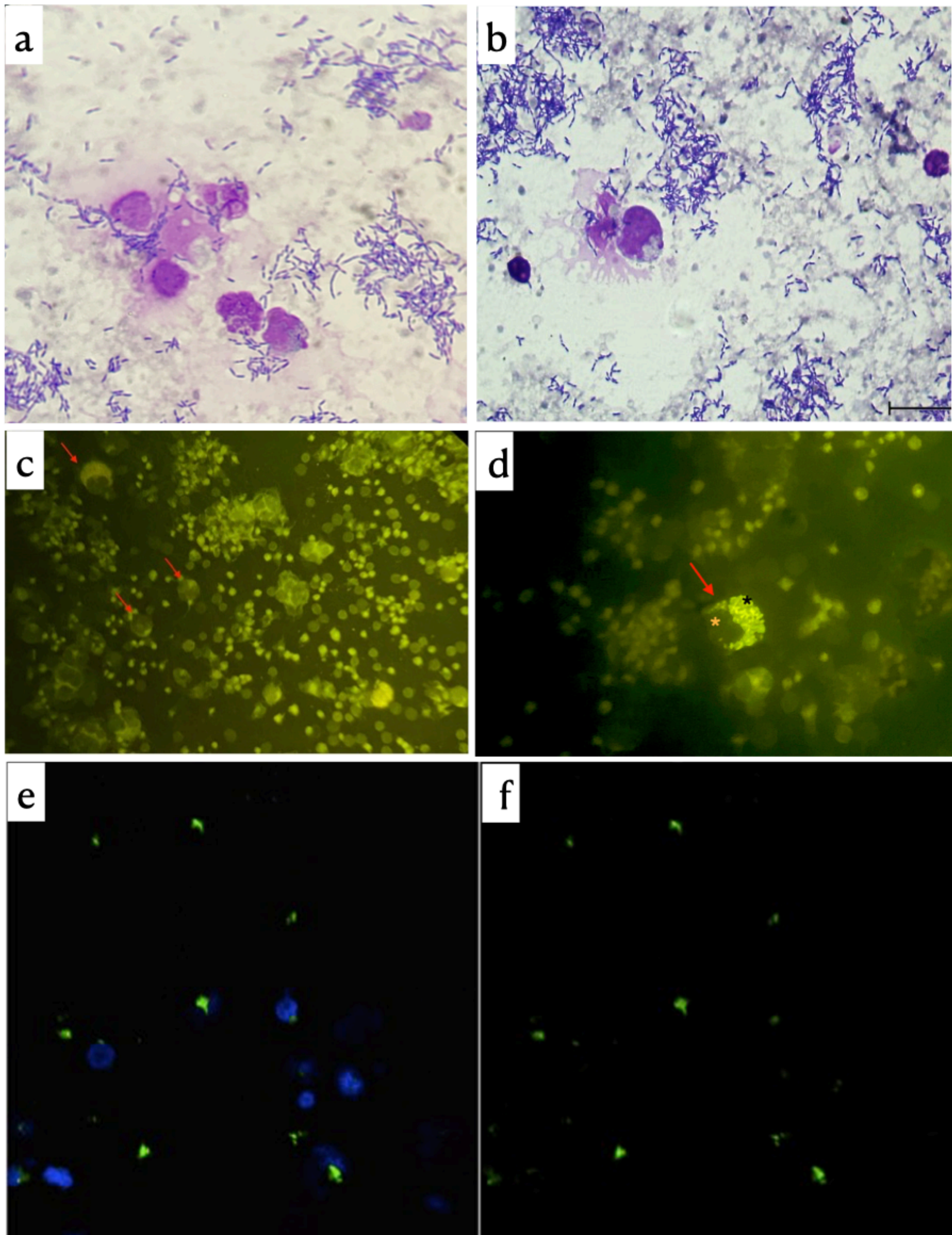

Figure 6. Cat's samples (FIP+). a-b) Light microscopy: Culture of macrophages with stimulated cells in the presence of PMA. Note the presence of vacuoles of uniform dimension in the macrophages. c-d) Fluorescence microscopy: Co-culture of macrophages (red arrows) with intra-cytoplasmatic liposomes (black asterisk). Note the intra-cytoplasmatic material by the macrophages (d) (yellow asterisk). e-f) Note

the nuclear (DAPI stained) material by the macrophages and the intra-cytoplasmatic liposomes (f) by confocal microscope. Scale bars 12µm.

Cat (FIP+)	Phagocytic cells (%)	Non-Phagocytic cells (%)
1	13	87
2	6	94
3	10	90
4	15	85
5	20	80
6	18	82
7	23	77
8	13	87
9	5	95
10	10	90
11	8	92
12	20	80
13	15	85
14	15	85
15	25	65
16	15	78
17	12	88
18	21	79
19	19	81
20	15	85
21	25	75
22	25	75
23	12	88
24	22	78
25	30	70
26	33	67
Mean	**17,1**	**82,9**

Table 5. Mean of phagocytic cells evaluated in cat FIP+.

Cat (FIP-)	Phagocytic cells (%)	Non-Phagocytic cells (%)
1	25	75
2	25	75
3	60	40
4	48	52
5	44	56
6	39	61
7	46	54
8	86	14
9	31	69
10	17	83
11	39	61
12	23	77
13	80	20
14	54	46
15	74	26
16	56	44
17	48	52
18	43	57
19	51	49
20	29	71
21	52	48
22	38	62
23	89	11
24	21	79
25	60	40
26	57	43
27	77	23
28	40	60
29	73	27
30	46	54
31	82	18
32	88	12

Mean	51,3	48,7

Table 6. Mean of phagocytic cells evaluated in cat FIP-.

The mean value of phagocytic cells in the FIP + group was low compared to the mean of phagocytic cells in the FIP- group.

ANOVA with Sidak's multiple comparisons test was used to analyze differences between phagocytic and non-phagocytic cells percentage between FIP+ and FIP- (Fig7). P-Values $p < 0.05$ is significant. Our results reached statistical significance with $^{****}p < 0.0001$.

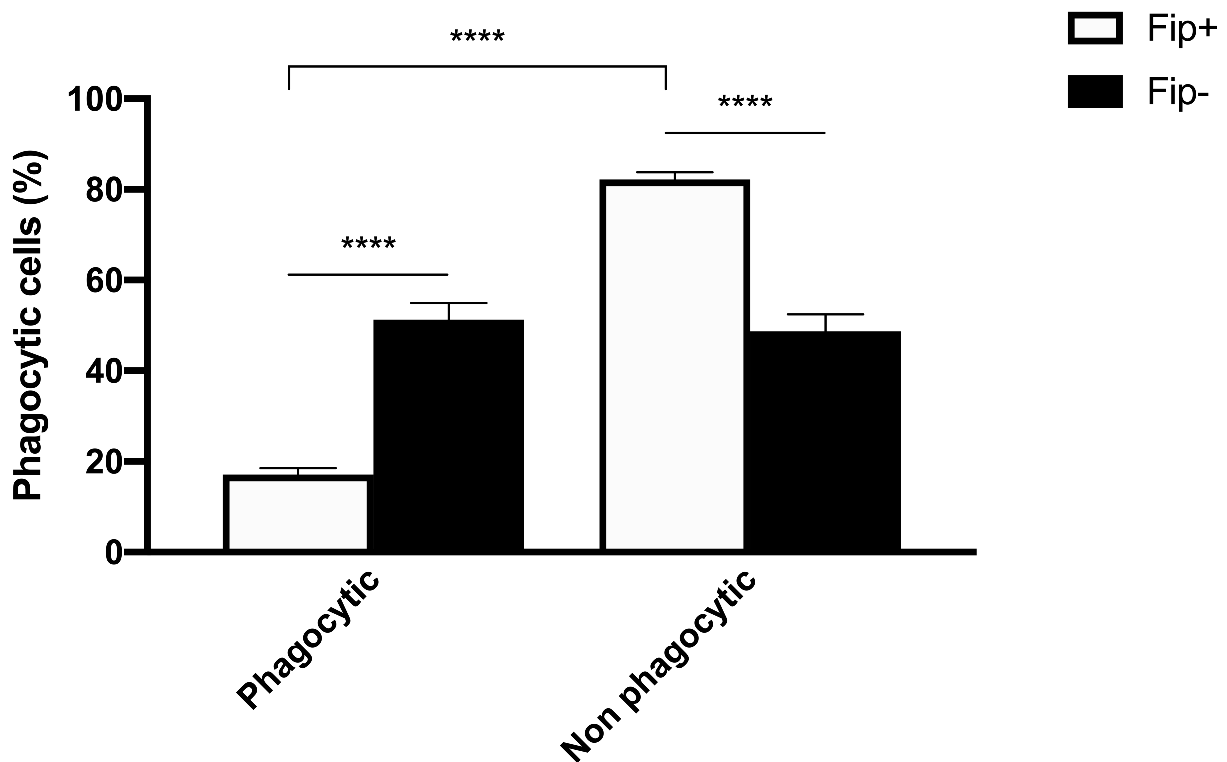

Figure 7. Schematic representation of phagocytic and non-phagocytic cells percentage in FIP+ and FIP– groups. $^{****}p < 0.0001$.

ii) Macrophage's respiratory activity in cats

The absorbance of cultured cells on a standard plate reader at 620nm was measured in FIP+ and FIP- without PMA and stimulated with PMA respectively (Tables 7 and 8).

Cat (FIP+)	PMA-	PMA+
1	0,517	0,761
2	0,671	0,744
3	1,213	1,397
4	0,493	0,573
5	0,578	0,69
6	0,507	0,646
7	1,26	1,37
8	1,47	1,56
9	1,11	1,44
10	1,16	1,32
11	0,96	1,2
12	0,87	1,15
13	1,15	1,41
14	1,16	1,47
15	0,835	0,859
16	1,156	1,341
17	1,086	1,219
18	1,145	1,219
19	1,09	1,124
20	0,771	0,992
21	0,953	1,112
22	1,160	1,32
23	0,798	1,04
24	1,060	1,192
25	1,030	1,23
26	1,035	1,39

Mean	0,1	1,14

Table 7. Absorbance measured in cats FIP+ without PMA and stimulated with PMA.

Cat (FIP-)	PMA-	PMA+
1	1,232	0,811
2	1,115	1,039
3	1,057	1,004
4	1,275	1,131
5	1,964	1,591
6	1,272	1,482
7	1,804	1,573
8	1,564	1,493
9	1,067	1,189
10	1,311	1,493
11	1,041	1,005
12	0,820	1,444
13	1,206	1,5
14	1,293	1,388
15	1,249	1,456
16	1,350	1,48
17	1,974	1,919
18	1,500	1,68
19	1,585	1,807
20	1,330	1,54
21	1,238	1,357
22	1,355	1,506
23	1,260	1,47
24	1,300	1,48
25	1,11	1,44
26	1,32	1,64
27	0,966	1,405
28	0,871	1,151
29	1,154	1,415
30	1,169	1,477
31	1,526	1,757
32	1,31	1,455
Mean	1,3	1,4

Table 8. Absorbance measured in cats FIP- without PMA and stimulated with PMA.

The (*Mean ± Standard deviation)* of absorbance calculated without PMA stimulation in the FIP+ group (0,1 ± 0,25) was low compared to the absorbance calculated in the FIP-group (1,3 ± 0,26). The (*Mean ± Standard deviation)* of absorbance calculetd wit PMA stimulation in the FIP + group was low (1,14 ± 0,27) compared to the absorbance calculated in the FIP- group (1,4 ± 0,23).

ANOVA with Sidak's multiple comparisons test was used to analyze differences in PMA-stimulated respiratory burst activity between FIP+ and FIP- (Figure 8).

Our results reached statistical significance ($^{**}p < 0.01$, $^{****}p < 0.0001$).

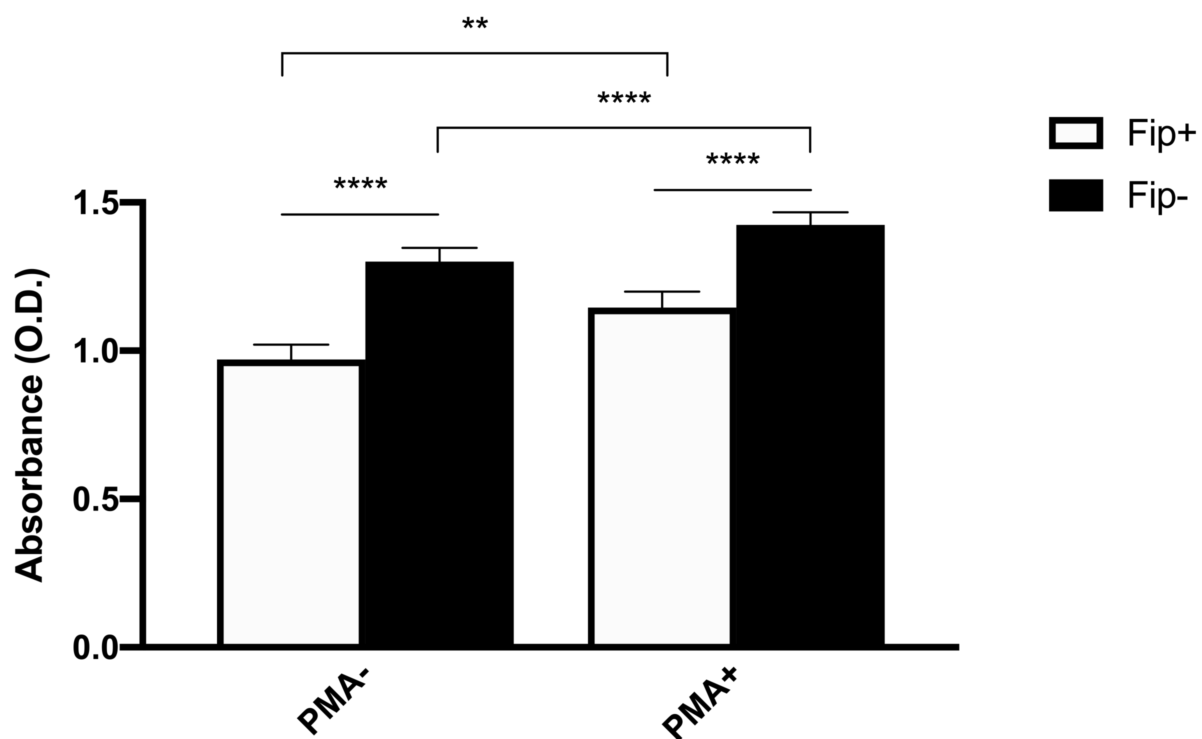

Figure 8. Schematic representation of absorbance in non-stimulated (PMA-) and stimulated (PMA+) macrophages in FIP+ and FIP– groups. $^{**}p < 0.01$, $^{****}p < 0.0001$.

iii) Macrophage's phagocytosis activity in cheetahs

For each animal in both groups, 3 microscopic fields were examined (Figure 9) The mean of the number of phagocytic cells undergoing yeast phagocytosis was evaluated and reported in tables 9 and 10.

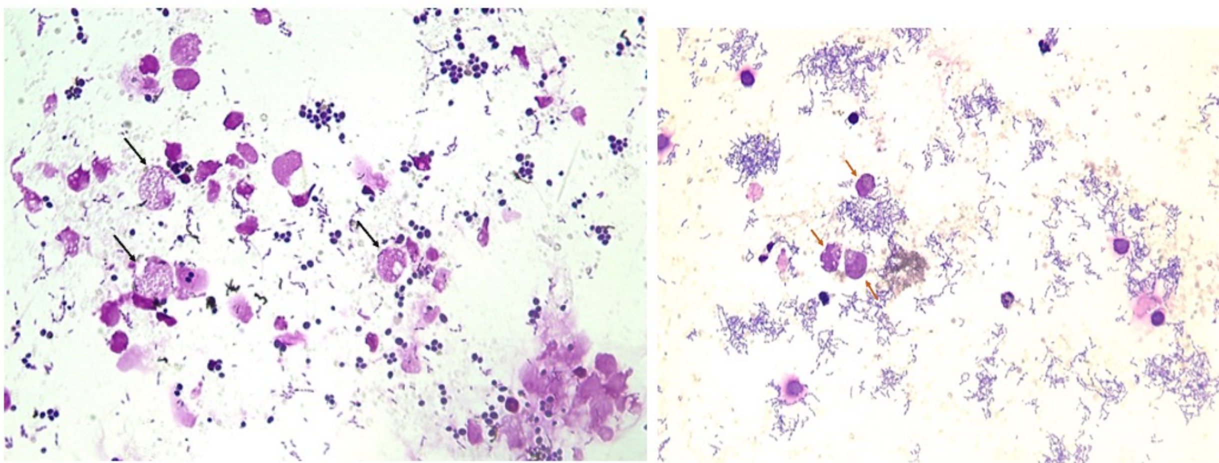

Figure 9 Cheetah's samples (Healthy). Light microscopy: Culture of macrophages with stimulated cells in the presence of PMA. Note the presence of vacuoles of uniform dimension in the macrophages (black arrows) and non-phagocytic macrophages (orange arrows).

Cheetah with GI disorder	Phagocytic cells (%)	Non-phagocytic cells (%)
1	46	54
2	86	14
3	31	69
4	17	83
5	39	61

6	23	77
7	29	71
Mean	38,7	61,3

Table 9. Mean of phagocytic cells evaluated in cheetahs with GI disorders.

Healthy cheetahs	Phagocytic cells (%)	Non-phagocytic cells (%)
1	48	52
2	44	56
3	39	61
4	60	40
Mean	47,8	52,2

Table 10. Mean of phagocytic cells evaluated in healthy cheetahs.

The mean value of phagocytic cells in the Cheetah with GI disorder group was low compared to the mean of phagocytic cells in the healthy group.

A two-way Analysis of Variance (ANOVA) with Sidak's multiple comparisons test was used to analyze differences between phagocytic and non-phagocytic cells percentage between healthy cheetahs and cheetahs with gastrointestinal disorders (Figure 10). Our results did not reach statistical significance.

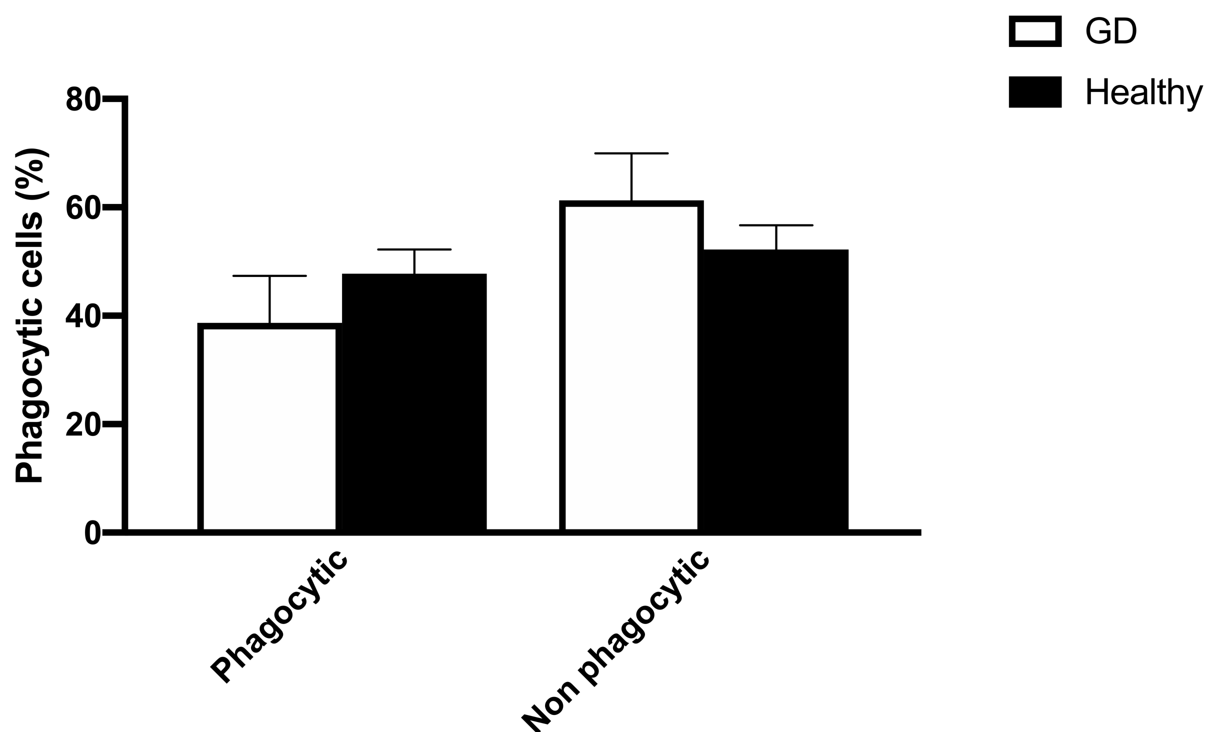

Figure 10. Schematic representation of phagocytic and non-phagocytic cells percentage in cheetahs with gastrointestinal disorders (GD) and healthy cheetahs (Healthy).

iv) Macrophage's respiratory activity in cheetahs

The absorbance of cultured cells on a standard plate reader at 620nm is measured in cheetahs with gastrointestinal disorders and healthy cheetahs, without PMA and stimulated with PMA respectively (Tables 11 and 12).

Cheetah with GI disorder	PMA-	PMA+
1	1,591	1,964
2	1,272	1,482
3	1,573	1,804
4	1,493	1,564
5	1,067	1,189
6	1,311	1,493
7	1,005	1,041
Mean	**1,3**	**1,5**

Table 11. Absorbance measured in cheetahs with gastrointestinal disorders without PMA and stimulated with PMA.

Healthy Cheetahs	**PMA-**	**PMA+**
1	0,811	1,232
2	1,039	1,115
3	1,004	1,057
4	1,131	1,275
Mean	**0,9**	**1,6**

Table 12. Absorbance measured in healthy cheetahs without PMA and stimulated with PMA.

The (*Mean ± Standard deviation)* of absorbance calculated without PMA stimulation in GD group (1,3 ± 0,21) was higher compared to the absorbance calculated in the Healthy group (0,9 ± 0,11). The (*Mean ± Standard deviation)* absorbance calculated

with PMA stimulation in GD group (1,5 ± 0,29) was higher compared to the absorbance calculated in the Healthy group (1,16 ± 0,08).

ANOVA with Sidak's multiple comparisons test was used to analyze differences in PMA-stimulated respiratory burst activity between cheetahs with gastrointestinal disorders (GD) and healthy cheetahs (Figure 11). Our results reached statistical significance ($^{*****}p < 0.0001$).

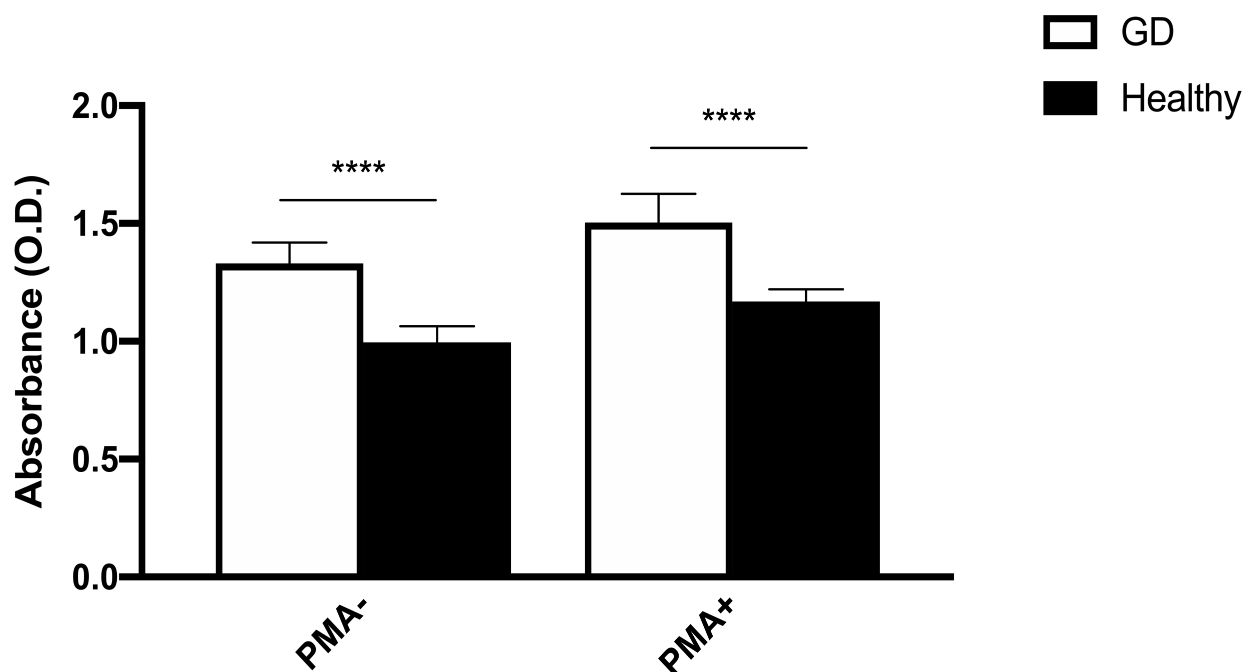

Figure 11. Schematic representation of absorbance in non-stimulated (PMA-) and stimulated (PMA+) macrophages in cheetahs with gastrointestinal disorders (GD) and healthy cheetahs (Healthy). $^{****}p < 0.0001$.

a.5 DISCUSSION

Macrophages are considered professional phagocytes that are important for pathogen clearance. In this first line of the PhD project, it is presented a method for studying *ex*

vivo macrophages functional capacity. This technique uses fluorescent-tagged or yeast particles and whole blood, preserving, at least in part, the physiological *in vivo* conditions. This method offers several advantages due to its speed and simplicity, by this assay, cellular phagocytic capacity can be quantified.

The experiments reported demonstrate that in cats with FIP monocyte-derived macrophages have significantly lower phagocytic activity than healthy cats, and similarly, in cheetahs with GI disorders associated with *Helicobacter* spp. infection monocyte-derived macrophages have lower phagocytic activity than healthy cheetahs. In physiologic condition, after inflammatory response M1-*like* macrophages switch to the M2-*like* phenotype to initiate the resolution of inflammation. M1 macrophages constitute the first line of defense against intracellular pathogens and promote or amplify Th1 polarization of CD4+ lymphocytes by IL-12 production [85]. This phenotypic switch is characterized by decreased production of inflammatory mediators and increased release of anti-inflammatory cytokines such as IL-10 [85]. M1 macrophages differentiate under the influence of IFN-γ and/or LPS and are usually characterized by increased microbicidal activity and the secretion of high levels of pro-inflammatory cytokines (TNFα, IL-6, production of reactive oxygen intermediates (ROI) and nitric oxide synthase-2 (NOS-2/iNOS)-dependent reactive nitrogen intermediates [86]. M2 macrophages have been initially identified under the influence of IL-4 and IL-13 produced during a strongly Th2-polarized response. M2-like cells have been described in pathological conditions such as infections by intracellular bacteria or virus, allergy, diabetes, or cancer [59]. M1 macrophages are characterized by the selective

expression of markers such as arginase 1 (Arg1), CD36 (fatty acid translocase), and CD206, and the production of low levels of IL-12 and iNOS. M2-associated phenotypic markers have been recognized: M2a (induced by exposure to IL-4 or IL-13); M2b (by stimulation with TLR, or the IL-1 receptor antagonist), both subtypes drive Th2 responses; and M2c type (generated by stimulation with IL-10) play a predominant role in the suppression of immune responses and tissue remodeling [87].

The interaction between pathogens and the host is characterized by strategies. M2 macrophages appear to be a "favorable situation" for long-term persistence of intracellular pathogens.

In this study, it was also optimized the use of a colorimetric plate-based respiratory burst. Oxidative burst activity was assessed by stimulating phagocytes with and without phorbol 12-myristate 13-acetate (PMA) in all groups and measuring the absorbance. Present results show that in cats with FIP, monocyte-derived macrophages have significantly lower burst activity than healthy cats, as well as in cheetahs with GI disorders associated with *Helicobacter* spp. infection monocyte-derived macrophages have higher burst activity than healthy cheetahs.

Present results demonstrate that these assays can be used to study immune function and to detect perturbation of cellular function in animals with immunological impairment (cats with and without FIP; cheetahs with gastrointestinal disorders related to *Helicobacter*-like organisms' infection and healthy cheetahs).

a.6 CONCLUSION

Monocytes are considered an intermediate stage between bone marrow precursors and tissue macrophages. Circulating monocytes have important effector functions in homeostasis and repair functions and during infections by having inflammatory effects[88]. Several populations of tissue resident macrophage originate from yolk sack embryonic precursors but some tissues (such as dermis and the intestine) host adult monocyte-derived macrophages [88]. During infectious process, blood inflammatory monocytes migrate to inflamed tissues and differentiate into monocyte-derived macrophage populations.

Present results seem to suggest that in cats with FIP the macrophage is deficient in the recognition and elimination of the virus, with a reduction of phagocytic activity and respiratory burst. This result was not found in healthy positive FeCoV shedding cats where there is an increase in phagocytosis and burst activity.

In cheetah, present results show that in subjects with GI disorders the macrophage is deficient in phagocytic activity if compared to healthy cheetahs. However, the respiratory burst activity was found higher in cheetahs with GI disorders if compared with healthy animals. These results show that, in absolute terms, the phagocytic capacity of macrophages belonging to cheetahs with GE pathology is lower, reducing the host's defense capacity, but that the macrophages that can carry out phagocytosis, produce a higher respiratory burst, from the moment which they differ in an inflammatory background.

b. STUDY OF CYTOKINES PROFILE

b.1 INTRODUCTION

Cytokines are mediators and modulators within localized environments regulating immunological responses, hematopoietic development, and cell-to-cell communication. Cytokines are important for migration, maturation, and proliferation of immune cells [89]. Differences in cytokines expression are associated with disease activity in immuno-mediated and inflammatory disorders [90]. The abnormalities of cytokines may reflect the imbalance among different immune cell subsets, such as Th1/Th2 immune response [90]. Cytokines can be classified into several categories including tumor necrosis factors (TNFs), interferons (IFNs), interleukins (ILs), monokines, lymphokines, colony stimulating factors (CSFs), and transforming growth factors (TGFs). Based on their cellular source, cytokines can be classified into: type 1 cytokines, produced by cluster of differentiation 4 (CD4)+ Th1 cells, including IL-2, IL-12, IFN-γ, and TNF-β; and type 2 cytokines, produced by CD4+ Th2 cells, including IL-4, IL-5, IL-6, IL-10, and IL-13 [91]. Cytokines may also be classified as pro-inflammatory or anti-inflammatory [91]:

- Pro-inflammatory: IL-1β, IL-6, IL-8, IL-12, TNF-α, and interferons, that facilitate inflammatory reactions and tend to stimulate immunocompetent cells.
- Anti- inflammatory: IL-4, IL-6, IL-10, IL-11, IL-13, IL-1 receptor antagonist (IL-1RA), and TGF-β. These molecules inhibit inflammation and suppress immune cells.

A single cytokine may be secreted by different cells and have both pro-inflammatory and anti-inflammatory activities (such as IL-6) [92].

The characterization of cytokine profiles in different pathological condition, should make it possible to estimate a "peripheral immune state" in a specific subject.

Feline infectious peritonitis is a progressive *Arthurs-type* immune-mediated disease that is triggered in cats are infected with enteric coronavirus [81]. As described above, the disease is characterized by multiple organ failure, generalized effusion, or neurological disease [81]. The development of FIP lesions is triggered by activated virus infected monocytes that induce the granulomatous phlebitis that is the first and hallmark lesion occurring in all organs[93]. The overactivation of the immune system appears characterized by an excessive release of pro-inflammatory cytokines (or also called *"cytokine storms"*) which results in multi-organ dysfunction and fatal outcome of the disease [94,95]. The cytokine pattern of healthy cheetahs and cheetahs with gastrointestinal disease has never been studied.

b.2 MATERIAL AND METHODS

For ethical aspects please see above what reported for the first research line.

Frozen serum samples collected from 7 cheetahs with GI disorders associated with *Helicobacter* spp. infection (GD) and 4 healthy cheetahs (Healthy) are used.

- study group or GD: 7 cheetahs with a clinical history of recurrent gastro-energy disorders and positive for *Helicobacter* spp. All subjects were tested with complete blood tests, and FIV-FELV tests.
- control group or Healthy: 4 healthy cheetahs. All subjects were tested for infectious diseases (FIV, FELV) and tested negative. Complete blood test and serum biochemical examination were performed and resulted in the norm of all values.

Collected Serum samples were cryopreserved at −80°C and remained frozen until analysis.

A commercial feline-specific multiplex assay was utilized allowing for simultaneous measurement of 10 Feline cytokines suitable for serum, plasma, cell culture media, other body fluids, cell and tissue lysates: Fas, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p40 and RANTES. Serum samples were assayed according to manufacturer's recommendations.

Serially diluted standards were prepared. 100 μL of Sample Diluent was added into each well and incubate at room temperature for 30 minutes to block slides. 100 μL standard cytokines was added in 8 wells and 100 μl of samples (GD and Healthy group) was added to the rest of well. The slide was incubated at room temperature for 1-2 hours. Samples was decanted from each well and washed 5 times.

with 150 μL of 1X Wash Buffer I. After decanted the 1X Wash Buffer I from each well, wells were washed 2 times with 150 μL of 1X Wash Buffer II. The buffer was completely removed. 80 μL of the detection antibody cocktail was added to each well.

and incubate at room at 2-8°C for overnight.

After 24 h, the samples were decanted from each well, and wash 5 times with 150 µL of 1X Wash Buffer I and then 2 times with 150 µL of 1X Wash Buffer II. The wash buffer was completely removed.

The Cy3 equivalent dye-conjugated streptavidin was prepared as shown in the Reagent Preparation section and 80 µL of Cy3 equivalent dye-conjugated streptavidin was added to each well. The slide was covered with aluminum foil and incubated at room temperature for 1 hour. The samples were decanted from each well, and wash 5 times with 150 µL of 1X Wash Buffer I. The device was disassembled and place in the slide Washer/Dryer with 1X Wash Buffer I (about 30 mL) to cover the whole slide. The glass array slide was dried by centrifuge at 1,000 rpm for 3 minutes. The signals was visualized through use of fluorescence microscopy with a Cy3 wavelength.

2.6 CONCLUSION and FUTURE PERSPECTIVES

The incidence of chronic disease in cheetahs is associated with chronic systemic inflammation. Cytokines regulate cell differentiation, proliferation and communication and difference in their expression is associated with disease activity in immuno-mediated and inflammatory disorders [90]. The ability to misure cytokines pattern in serum sample is usefull to assess levels of systemic inflammation during chronic disease. Few studies reported to study the cytokine profile in cheetah[96]. Cytokine concentrations in cheetah serum were based on the use of domestic cat standards. but studies on cytokine profiles in cats with FIP provided controversial results[97]. In this

part of my research, I performed the study of the immune profile through the determination of the cytokine pattern in healthy cheetahs and cheetahs with gastroenteric disorder associated with *Helicobacter spp. infection*. A commercial feline-specific multiplex assay was utilized allowing to measure 10 Feline cytokines suitable for serum: Fas, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-10, IL-12p40 and RANTES (tab. 13).

Cytokine	*Main Sources*	*Classification*	*Major function*
Fas	T cells and natural killer cells	Pro-inflammatory	-
IFNγ	Natural killer cells, innate lymphoid cells (ILCs), T helper 1 (TH1) cells and CD8+ cytotoxic T lymphocytes (CTLs)	Pro-inflammatory	Anti-viral, macrophage activation, increases neutrophil and monocyte function, MHC-I and -II expression on cells
IL-1β	Monocytes and macrophages, B cells, DCs	Pro-inflammatory	Pyrogenic, pro-inflammatory, proliferation and differentiation
IL-2	Th1-cells	Adaptive immunity	Proliferation of B cells, activated T cells, NK cell function
IL-4	Th cells	Adaptive immunity	Proliferation of B and cytotoxic T cells, enhances MHC class II expression, stimulates IgG and IgE production
IL-5	Th2 Cells and mast cells	Adaptive immunity	B-cell proliferation and maturation, stimulates IgA and IgM production

IL-8	Macrophages	Pro-inflammatory	Chemotaxis for neutrophils and T cells
IL-10	T cells, B cells, macrophages	Anti-inflammatory	Inhibits cytokine production and mononuclear cell function
IL-12p40	T cells, macrophages, monocytes	Anti-inflammatory	Activates NK cells, phagocyte cell activation, endotoxic shock, tumor cytotoxicity.
RANTES	Hematopoietic and non-hematopoietic cell	Pro-inflammatory	Chemotaxis for monocytes, NK cells, memory T cells, eosinophils and DCs

Table 13. Summary of selected cytokines and their functions.

However, the final interpretation of the method has not been developed. These molecules can provide key insight into disease progression or regression, and can be considered at as biomarkers of a disease process providing insight into the possible effect of treatment, the disease state or future disease development[92].

Future research could be carried out on the study of the citchin profile of cheetahs with different stages of gastroenteric disease associated with Helicobcater spp infection. The correlation between histological, haematobiochemical and cytokine profile tests could suggest the porgnostic course of the disease and be used for preventive therapeutic purposes.

c. STUDY OF THE FECAL PROTEOME

c.1 INTRODUCTION

Since the mapping and sequencing of the human genome, different new technologies are improved to obtain a huge number of molecular measurements in biological samples [98]. The scientific fields associated with the study of biological molecules in a high-throughput way are called "omics." Omics study includes transcriptomics, proteomics, metabolomics, genomics, lipidomics, and epigenomics [98].

The term "proteome" describes the set of proteins encoded by the genome [99]. Proteome study is called "proteomics", including all protein isoforms and modifications [100]. Potential biological samples for proteome analysis can be *in vitro* cultured cell lines or *in vivo* sources including animal tissues, organs, feces, and body fluids [101]. Fecal samples offer accessible alternative biological samples to investigate a range of diseases of the gastrointestinal tract [101]. Proteins and peptides present in stools have potential as biomarkers in different bowel-related diseases. Mass spectrometry (MS) is the method of choice for analysis of complex protein samples [102]. One of the most reported proteomics methods consists of different stages. The proteins to be analyzed are isolated from biological samples (blood, tissue, feces, urine) by biochemical fractionation [102], after this a step one-dimensional gel electrophoresis and second dimension electrophoresis are performed [102]. Proteins are degraded enzymatically to peptides by trypsin, leading to peptides with C-terminally protonated amino acids. The peptides are separated and after evaporation multiply protonated peptides enter the

mass spectrometer and a mass spectrum of the peptides eluting is performed [102]. Among proteomics technologies, conventional, advanced, and quantitative techniques are recognized [100] (Tab.14).

Proteomics technology	Subtypes
Conventional	• Chromatography-based techniques • Enzyme-linked immunosorbent assay • Western blotting
Advanced	• Protein microarray • Gel-based approaches
Quantitative	• ITAC • SILAC • iTRAq
High-throughput techniques	• Mass spectrometry • NMR spectroscopy
Bioinformatics analysis	

Table 14. Schematic representation of different proteomics techniques[100].

The two-dimensional polyacrylamide gel electrophoreses (2D-PAGE) is an efficient method for separation of proteins based on their mass and charge [100,103]. By 2D-PAGE, proteins, that are firstly separated by charge in the first dimension, are separated because of differences between their mass [101]. The selection of appropriate experimental model and preparation of sample are the most important step in proteomic

study that can affects the results [104]. The major impediments associated with this analysis, are the wider range of protein abundance, such as severe copies of a protein in a singular cell [105].

Within this third line of the present PhD research project, the study of proteome in fecal samples of cheetahs with GI disorders associated with *Helicobacter* spp. infection, and then comparing it with the fecal proteome from healthy cheetahs, was performed. The present study protocol applied to cheetahs' samples, was previously performed in fecal samples from healthy dogs and cats and from dogs with lymphangiectasia [106,107].

c.2 MATERIAL AND METHODS

As above (two previous research line), the study population consisted of:

- Study group or GD: made by 7 cheetahs with GI disorders associated with *Helicobacter* spp. infection. *Helicobacter* spp. presence was detected in fecal samples by RT-PCR analysis. Other pathologies were excluded by complete blood exams and clinical history. All subjects were tested for infectious diseases (FIV, FeLV) and tested negative
- Control group or Healthy: made by 4 healthy cheetahs. All subjects were tested for infectious diseases (FIV, FeLV) and tested negative. Complete blood test and serum biochemical examination were performed and resulted in the norm of all values.

All animals included in the study did not undergo dietary changes or medical treatments. Parasitic and other gastrointestinal diseases were excluded.

Fecal samples naturally eliminated from each cheetah were collected and stool samples from the GD and Healthy groups were frozen and stored at -20°C within 8 h of sampling. 2 grams of feces for each animal was mixed in a pool and resuspended in 42 mL of phosphate-buffered saline (PBS) diluted 1:100, containing a protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA), and extracted as previously described [106]. After centrifugation at 10000xg for 20 minutes, samples were filtered using 0.45μm filters and subsequently 0.20μm filters. To the final filtrate, 90 % ammonium sulphate in the solution was added. The solution was divided equally in 1ml Eppendorf and centrifuged at max speed for 30 minutes. The supernatant was discarded. The precipitate was resuspended in 100 ml of PBS buffer and total protein content was determined according to the Bradford method [108]. Before 2DE, one milligram of total protein was treated with the 2D Clean-Up Kit (GE-Healthcare Life Sciences, Uppsala, Sweden). The first dimension was performed using the IPGphor isoelectric focusing cell apparatus (GE-Healthcare) a pH range of 3-10 (Immobiline DryStrip, IPG-strip, length 18 cm was used (Figure 12), and 13% SDS-PAGE (Protean II apparatus, Bio-Rad, Hercules, CA, USA) for the second dimension, as described in previous studies [109]. The analyses were performed in triplicate.

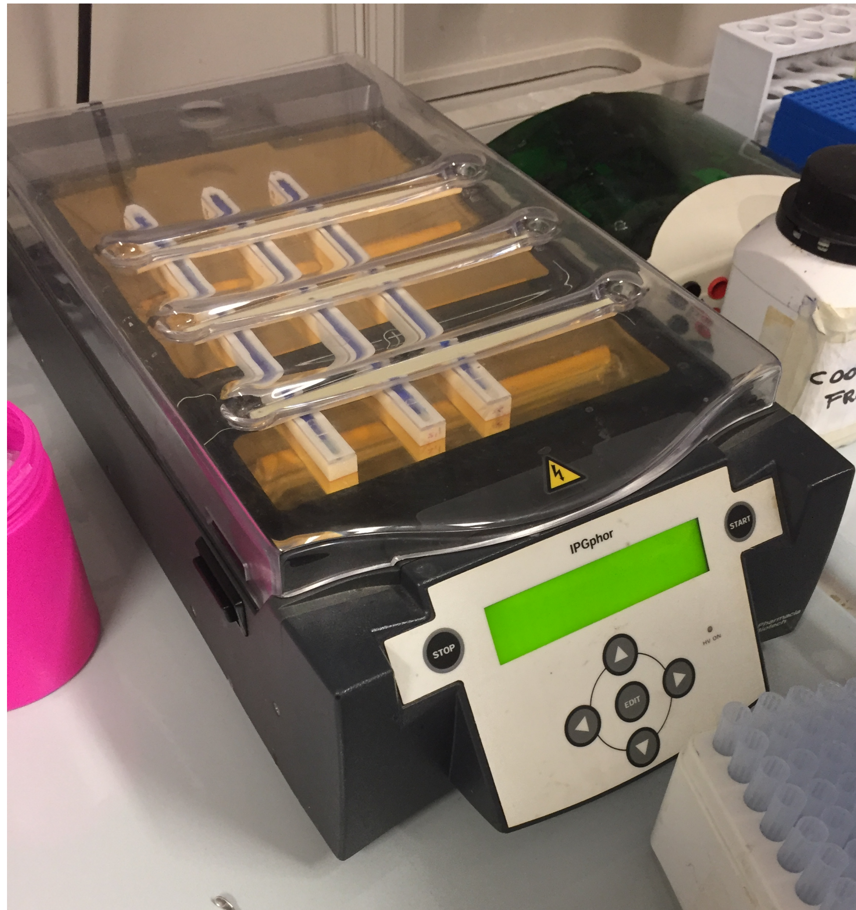

Figure 12. First dimension with pH range of 3–10 (Immobiline DryStrip, IPG-strip, length 18 cm; IPGphor isoelectric focusing cell, GE-Healthcare) used for cheetahs' samples.

The stained gels were scanned at 600dpi and subjected to image analysis using the PDQuest software (Version 7.1.1; Bio-Rad Laboratories), to calculate the isoelectric point (pI), the molecular mass (Mr), and the normalized quantity of each protein spot (Fig.13). The selected spots were manually excised, and the protein was extracted from the gel as previously described, then subjected to LC-MS/MS analysis for protein identification [110].

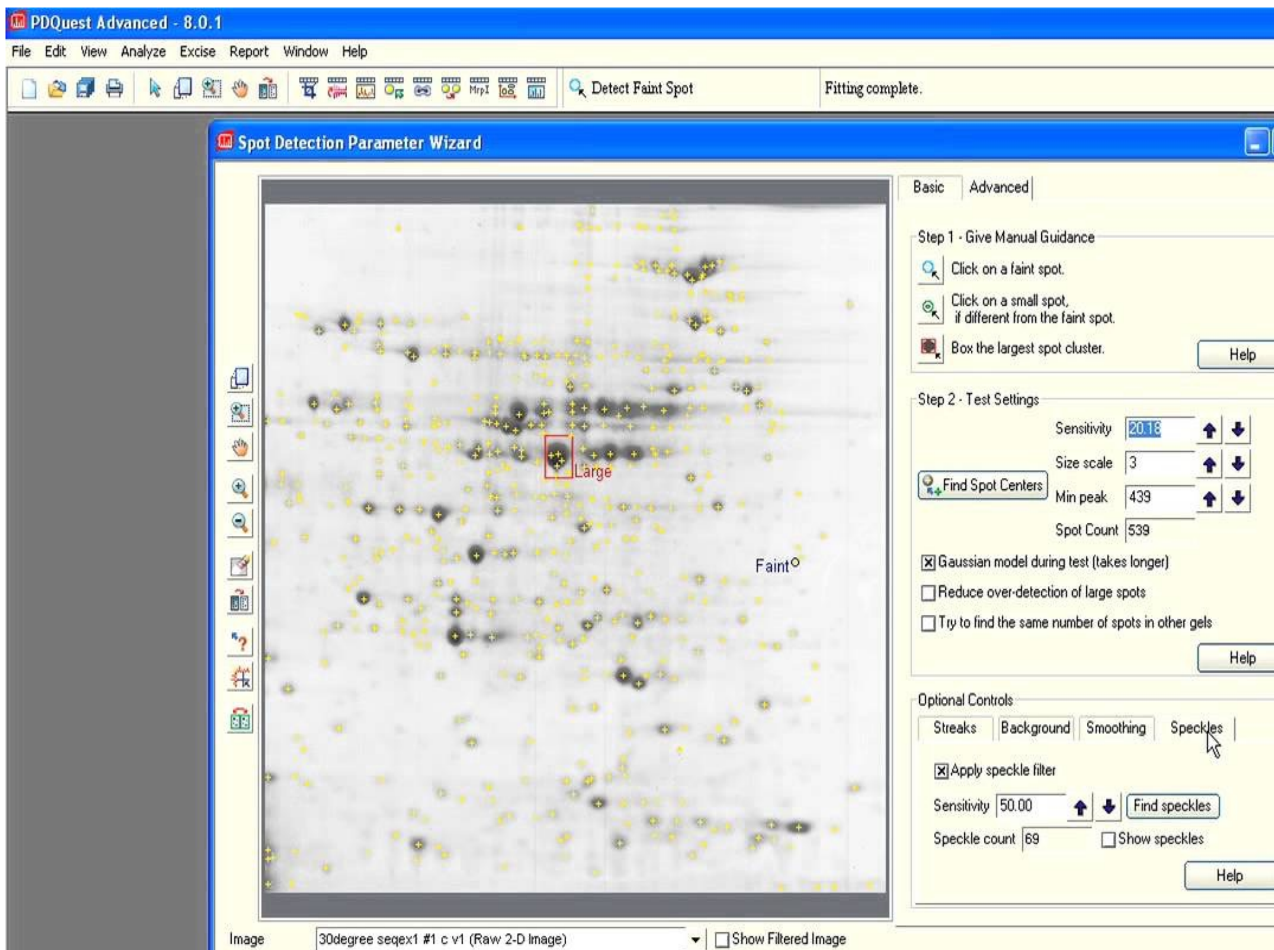

Figure 13. PDQuest software (Version 7.1.1; Bio-Rad Laboratories), to calculate the isoelectric point, the molecular mass, and the normalized quantity of each protein spot.

The MS spectra were extracted and analyzed by MASCOT software (www.matrixscience.com; http://hs2.proteome.ca/prowl/knexus.html [accessed on 10 March 2021]).

c.3 STATISTICAL ANALYSIS

2DE experiments were performed in triplicate. Quantitative data of protein level in fecal samples are presented. The differences of protein spots were compared between groups.

c.4 RESULTS

The protein expression profiles of fecal samples of healthy cheetahs and cheetahs with GI disorders associate with *Helicobacter* spp. infection were examined by 2DE in the pH range 3-10.

In healthy cheetahs, PDQuest analysis revealed the presence of 13 spots differentially expressed in the fecal samples. In samples of cheetahs with GI disorders, 6 different spots were identified (Figure 14 and 15).

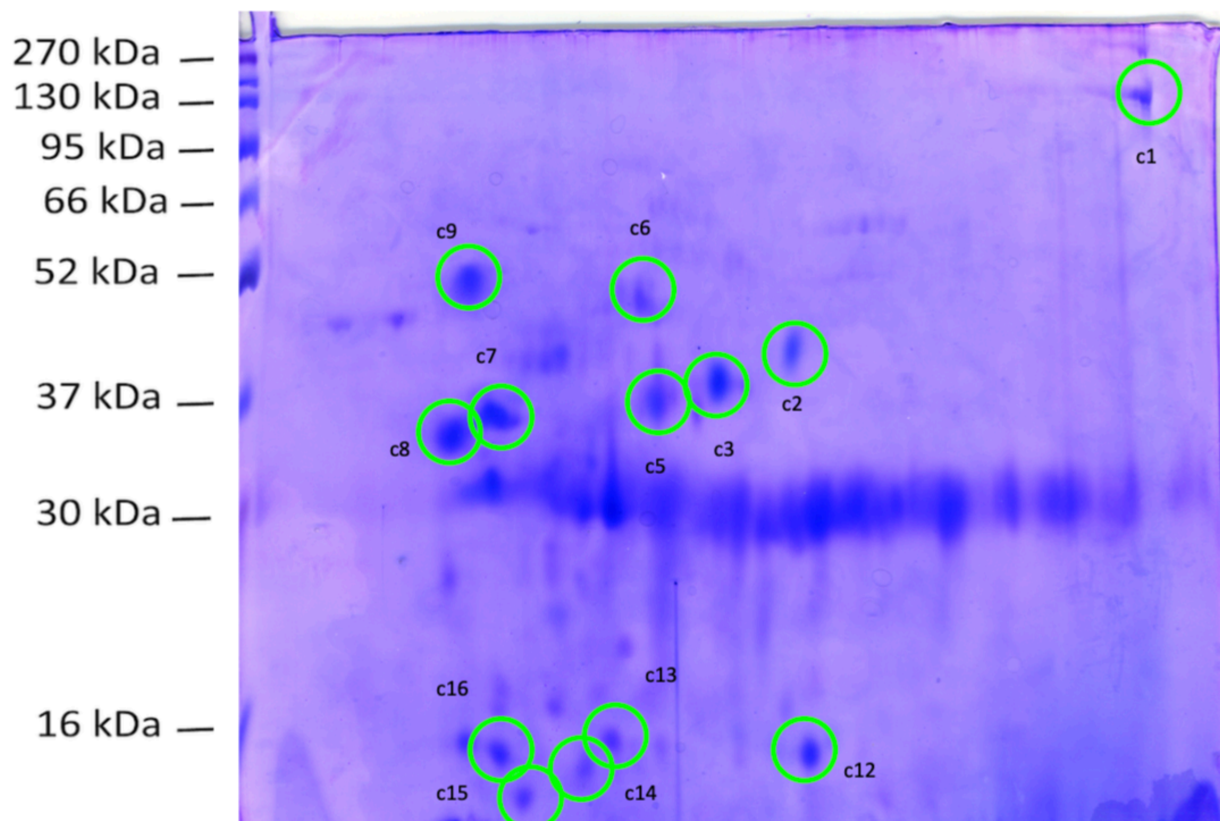

Figure 14. 2D electrophoresis gel shows a representation of the protein spots in healthy cheetahs. The spots that were identified by the mass spectrometry are evidenced in red. The proteins were separated on an immobilized pH 3–10 linear gradient strip and subsequently subjected to a 13% SDS-PAGE. The standards were Bio-Rad low molecular weight (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

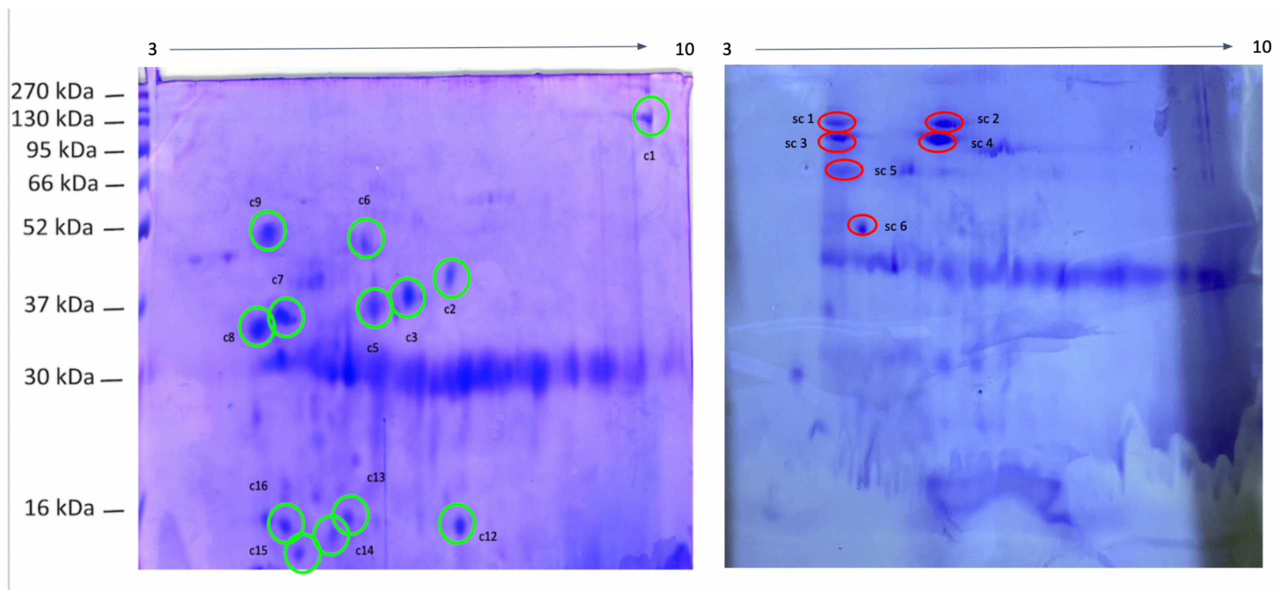

Figure 15. 2D-electrophoresis gel of the protein spots in cheetahs with GI disorders associated with *Helicobacter spp*. infection (right) and healthy cheetahs (left). The spots that were identified by the mass spectrometry are evidenced in red. The proteins were separated on an immobilized pH 3–10 linear gradient strip and subsequently subjected to a 13% SDS-PAGE. The standards were Bio-Rad low molecular weight (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin 45.0 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

In table 14 and 15 it is shown the normalized quantity of each spot, as well as the experimental Ip values and the molecular weights of the spot proteins, compared with the theoretical values found by the MASCOT or SONAR software. Before to perform the spots identification, the spots of interest were excised from 2-D gels and digested with trypsin. In healthy cheetahs, the most representative spots present in triplicate have been selected and identified as follow: C1 (*Collagen alpha-1*), C2 (*Transthyretin*), C3 (*Transthyretin*), C5 (*Transthyretin*), C6 (*IgGFc-binding protein*), C8 (*IgGFc-binding protein*), C9 (*Titin*), C12 (*Dystonin*), C13 (*Isopentenyl-diphosphate Delta-isomerase 1*), C14 (*Sodium/potassium-transporting ATPase subunit alpha-1*), C15 (*Protein disulfide-isomerase A6*), C16 (*Dystonin*) (Tab. 14). (Tab. 15).

Spot ID C [a]	**Mr (kDa)/pI** [b]	**Normalized quantity ($x10^3$)** [b]	**Protein name** [c]	**Mr (kDa)/pI** [c]	**Score** [c]	**Sequence** [c]
C1	135.5/9.3	175±147	*Collagen alpha-1 (II) chain*	141.8/6.6	40	*GIAGPQGPR*
C2	44.2/6.9	128±50	*Transthyretin*	15.7/5.9	109	*GSAPAANVGVK*
C3	40.7/6.4	307±2	*Transthyretin*	15.7/5.9	144	*GSAPAANVGVK*
C5	39.1/5.9	324±48	*Transthyretin*	15.2/5.9	59	*KAADDTWEPFASG*
C6	48.1/5.7	149±27	*IgG Fc-binding protein*	571.6/5.1	128	*VLVENEHRG*
C7	36.9/4.7 39.8/4.7	401±25 3298±518	n.d.	n.d.	n.d.	n.d.
C8	35.7/4.4	460±15	*IgG Fc-binding protein*	571.6/5.1	50	*LDSLVAQQLQSK*
C9	51.8/4.5 50.2/4.4	288±130 7634	*Titin*	3813.0/6.0	85	*APTPSPVR*
C12	18.6/7.1	207±40	*Dystonin*	833.7/5.2	73	*AVTTALK*
C13	18.7/5.5	163±36	*Isopentenyl-diphosphate Delta-isomerase 1*	26.4/5.6	55	*AANGEIK*

C14	17.8/5.3	144±43	*Sodium/potassium-transporting ATPase subunit alpha-1*	112.6/5.6	81	*CRGAGIKV*
C15	16.4/4.8	125±10	*Protein disulfide-isomerase A6*	48.1/4.9	51	*KAATALKD*
C16	18.1/4.7	192±6	*Dystonin*	833.7/5.2	83	*AVTTALK*

Table 15. Identification of fecal proteins from healthy cheetahs by LC-MS/MS followed by MASCOT and SONAR software analysis.
[a] Assigned spot ID as indicated in figure 1 (1A: healthy cheetahs; 1B: diseased cheetahs);

[b] Experimental values calculated from the 2DE maps by the PDQuest software;

[c] Data obtained from MASCOT results (SwissProt databases).

In cheetahs with GI disorders, the spots present in triplicate in this study group have been selected and identified as follow: SC1 (*Albumin*), SC2 (n.d.), SC3 (n.d.), SC4 (*Albumin*), SC5 (*n.d.*), SC6 (Titin) (Tab. 16).

Spot ID SC [a]	Mr (kDa)/pI [b]	Normalized quantity ($x10^3$) [b]	Protein name [c]	Mr (kDa)/pI [c]	Score [c]	Sequence [c]
SC1	61.7/4.6	6477±3214	*Albumin*	68.6/5.5	190	*KAPVSTPTLVEV*
SC2	62.6/5,5	6290±864	n.d.	n.d.	n.d.	n.d.
SC3	57.3/4.7	5880±1240	n.d.	n.d.	n.d.	n.d.
SC 4	58.9/5.7	7922±922	*Albumin*	68.6/5.5	421	*KAPVSTPTLVEV*
SC7	36.9/4.7 39.8/4.7	401±25 3298±518	n.d.	n.d.	n.d.	n.d.
SC6	51.8/4.5 50.2/4.4	288±130 7634	*Titin*	3813.0/6.0	85	*APTPSPVR*

Table 16. Identification of fecal proteins from cheetahs with GI disorders associated with *Helicobacter* spp. infection by LC-MS/MS followed by MASCOT and SONAR software analysis.

[a] Assigned spot ID as indicated in figure 1 (1A: healthy cheetahs; 1B: diseased cheetahs);

[b] Experimental values calculated from the 2DE maps by the PDQuest software;

[c] Data obtained from MASCOT results (SwissProt databases).

c.5 DISCUSSION

Recently, useful advances are made in the field of proteomics. This method is considered rapid and sensitive providing greater proteome coverage. Different fields related to biological sciences have been benefited with increasing use of proteomics techniques. These methods can be used in clinical research suggesting potential new biomarkers for specific pathologies possibly helpful as diagnostic and prognostic tools. Proteomics can be a useful research method in veterinary medicine, in different biological samples (such as blood, urine, body fluids or stool). The aim of this part of my research is the proteomic analysis of fecal samples from a population of healthy cheetahs and cheetahs with GI disorders associated with *Helicobacter* spp. infection.

In healthy cheetahs included in our study, 13 different spots were identified. 12 spots (not the C7) were identified with LC-MS/MS followed by MASCOT analysis (for a total of 8 proteins, considering duplicates). Analyzing proteins from healthy cheetahs, the most interesting spot were the spot C1, identified as Collagen alpha-1protein, which is a fibril-forming collagen found in most connective tissues and with a different

functions within the organism [111]. Spots C2-C3-C5 were identified as transthyretin, a protein secreted from the liver and choroid plexus into the blood and transporter for thyroid hormones and a retinol binding [112]. In animals, few studies reported a lower concentration of transthyretin in diarrheic calves and in *Mycobacterium avium* paratuberculosis seropositive cows [113]. Recently, the presence of transthyretin precursor in the feces was reported in clinically healthy cats [106], and was found to be more expressed in dogs suffering from chronic enteropathy than in in healthy controls [114]. Spots C6 and C8 were identified as IgGFc-binding protein was identified in healthy cheetahs as. This protein fragment, is abundant in the intestinal mucus as MUC2 produced and secreted by goblet cells [115]. Also the presence of IgGFc-binding protein was reported in proteomic study of clinically healthy cats [106]. Spot C9 was identified as Titin, which inserts the NH_2 terminus, in the Z-disk and reaches all the way to the center of the sarcomere [116]. This protein is expressed in different isoforms, its presence could be attributed to the raw animal meat on which cheetahs are fed. Spots C12 and C16 were identified with Dystonin, which is a member of the plakin family and a cytoskeletal linker protein with both actin- and tubulin-binding domains [117]. Different isoforms of Dystonin are found in neural, muscle and epithelial tissues [117]. The role of loss of this protein in nervous system is known leading to a fatal peripheral neuropathies in humans and mice [118]. The spot C13 in fecal proteomic was identified as Isopentenyl-diphosphate Delta-isomerase 1, an enzyme that participates in the cholesterol biosynthesis pathway [119]. The presence of Isopentenyl-diphosphate Delta-isomerase 1 was reported in proteomic study of proteomic analysis in healthy dogs and

in dogs with food responsive diarrhea [120]. Spot C14 was identified in healthy cheetahs as sodium/potassium-transporting ATPase subunit alpha-1 which belongs to the family of P-type cation transport ATPases, and Na+/K+-ATPases subfamily. This protein is responsible to maintain the electrochemical gradients of Na and K ions across the plasma membrane essential for osmotic regulation [121]. In general, the Na+-K+-ATPase comprises two subunits (α and β). The catalytic α-subunit encompasses the sites of nucleotide and cation binding, and multiple isoforms of the α- (α1, α2, α3 and α4) are expressed in tissues [122]. Spot C15 in fecal proteome of healthy cheetahs, was finally identified as Protein disulfide-isomerase A6, one of more than 20 protein disulfide isomerases in the eukaryotic endoplasmic reticulum [123]. This protein is an active oxidoreductase with similar enzymatic properties to protein disulfide isomerases [124].

In fecal samples of cheetahs with GI disorders, 3 spots (out of 6 – not SC6, SC2 and SC3) were identified (for a total of 3 proteins, considering duplicates) were selected and analyzed with LC-MS/MS. The spot SC5 was identified as *Titin*, it was present also in helathy group, while SC1 and SC4 were identified as corresponding to different albumin isoforms belong to *Canis lupus familiaris.* The presence of the different albumin isoforms that characterize the group of sick cheetahs could be attributed to chronic gastrointestinal disease present in the study group.

In this study, the protein fragments identified by mass spectrometry belong to species different from *Acinonyx jubatus*, because the absence in database of peptide fragment spectra from *Acynonix jubatus* species. In particular, regarding to species attribution,

proteins were attributed to other species such as *Mus musculus, Bos taurus, Ovis aries, Homo sapiens, Macaca fascicularis*, and *Canis lupus familiaris*.

The cheetah belongs to the genus *Acinonyx o*f which it is the only extant species. This species has unique phenotypic characters that distinguish it from other big felids[125]. The present results show for the first time the fecal proteomic pattern of healthy cheetahs and cheetahs with gastrointestinal disease affected by *Helicobacter* spp. infection. Comparing the present results with those already present in literature in healthy dogs and cats [106], it is interesting to notice that cheetahs' fecal proteome looks more similar to the canine than it is with the feline one; but further studies are needed in all such species before a comparison could provide strong evidences.

c.6 CONCLUSION

In the previous several years, useful advances have been made in the field of proteomics. The technologies are rapid, sensitive and provide greater proteome coverage. Furthermore, combination of these technologies has achieved success in purification, analysis, characterization, quantification, sequence and structural analysis and bioinformatics analysis of large number of proteins in all types of eukaryotic and prokaryotic organisms. All fields related to biological sciences have been benefited with increasing use of proteomics techniques. However, further work is still required to improve the reproducibility and performance of well-known proteomics tools. This is the first study of fecal proteomic study and healthy cheetahs and cheetahs with GI disorder associated with *Helicobacter* spp. infection. The cheetahs included in the

study are housed in European zoos, implying subjective and group variability. The present results need to be confirmed by further studies in order to interpret more in depth and to eventually identify possible tools or biomarker in cheetahs suffering from chronic GI disease associated with *Helicobacter* spp. but may represent the starting point for these kind of studies.

GENERAL DISCUSSION

Wildlife conservation involves the managing of wildlife populations and their environment leading to not despoil or extinguish species or their habitats. Wildlife conservation can be considered a social value in a normative belief system, and associated actions are collectively the practice of wildlife conservation [2].

My PhD research project is based on a multidisciplinary approach aiming at investigating the gastrointestinal disease in cheetahs, mainly induced by *Helicobacter* spp.

In the first line of the project, it is presented a method for studying *ex vivo* macrophages functional capacity. This technique uses fluorescent-tagged or yeast particles and whole blood, preserving, at least in part, the physiological *in vivo* conditions. This method offers several advantages due to its speed and simplicity, by this assay, cellular phagocytic capacity can be quantified. Through the study of samples from cats with feline infectious peritonitis (FIP), and then comparing these results with the study done in samples from cats positive for Feline coronavirus not causing FIP, it has been perfected the test subsequently performed in samples from healthy cheetahs and from subjects with GI disease associated to *Helicobacter* spp. Although apparently different, it was decided to refine the technique in cats with feline coronavirus infection and then to apply it on cheetah, as in both diseases the severity of the condition is caused by the host's immune response to the pathogen. Present results suggest that in cats with FIP the macrophage is deficient in the recognition and elimination of the virus, with a reduction of phagocytic activity and respiratory burst. This result was not found in

healthy positive FeCoV shedding cats where there is an increase in phagocytosis and burst activity. In cheetah, present results showed that in subjects with GI disorders the macrophage is deficient in phagocytic activity if compared to healthy cheetahs. However, the respiratory burst activity was found higher in cheetahs with GI disorders if compared with healthy animals. These results show that the phagocytic capacity of macrophages in cheetahs with GE pathology is lower, reducing the host's defense capacity, but that the macrophages that can carry out phagocytosis, producing a higher respiratory burst.

The second line of the project aimed at studying the cytokines profile on the same samples, comparing results between cheetah's populations. In human, studies support the hypothesis that host genotype and cytokine pattern could have a key role in *H. pylori* susceptibility[126]. However, the final interpretation of the method has not been developed. Future research could be carried out on the study of the citchin profile of cheetahs with different stages of gastroenteric disease associated with Helicobcater spp infection. The correlation between histological, haematobiochemical and cytokine profile tests could suggest the porgnostic course of the disease and be used for preventive therapeutic purposes.

In the third line of research, I performed the study of the fecal proteome in both healthy and diseased cheetahs. This method is considered rapid and sensitive providing greater proteome coverage. Different fields related to biological sciences have been benefited with increasing use of proteomics techniques. These methods can be used in clinical research suggesting potential new tools or biomarkers for specific pathologies, with

both diagnostic and prognostic perspective. Present results showed that 13 different spots were identified in fecal samples of healthy cheetahs. In fecal samples of cheetahs with GI disorders, only partially overlapping results were found, and, interestingly, different spots corresponding to different albumin isoforms were identified, probably due to chronic gastrointestinal disease present in the study group. It has been very difficult to compare our results with previous ones as very few proteomic studies are present in the veterinary scientific literature and therefore the meaning of some findings remains unclear; the study, however, provide a first step towards the study fo such topic. In this study, the protein fragments identified in both groups belong to species other than *Acynonyx jubatus*. This seems to be due to the absence of data from the *Acynonix jubatus* species. The present results show for the first time the fecal proteomic pattern of healthy cheetahs and cheetahs with gastrointestinal disease affected by *Helicobacter* spp. infection.

The cheetah belongs to the genus *Acinonyx o*f which it is the only extant species. This species has unique phenotypic characters that distinguish it from other big felids [125]. The survival of this species is linked to many aspects including the high incidence of chronic diseases [16]. Among chronic disorders, chronic gastrointestinal diseases have been shown to have a high incidence of disease in captivity [15]. Studies about gastrointestinal disease in captive and wild cheetahs and archived biopsies between 1993 and 2021 of indistinct *A. jubatus* subspecies are presented in Table (Tab.17), correlating the presence of *Helicobacter* spp. with diet, and the immune response with gastroenteric disorders.

Reference	*Aims*	*No. Cheetah*
Eaton et al., 1991 [127]	Gastritis Associated with Gastric Spiral Bacilli in Cheetahs	25
Eaton et al., 1993 [128]	Isolation of Helicobacter Acinonyx from cheetah with gastritis	4
Munson, 1993 [15]	Diseases of Captive Cheetahs (*Acinonyx jubatus jubatus*) in South Africa	69
Lobetti et al., 1999 [21]	Prevalence of Helicobacteriosis and gastritis in Cheetahs	28
Dailidiene et al., 2004 [129]	Helicobacter pylori-like gastric pathogen of cheetahs and other big cats	6
Munson et al., 2005 [16]	Extrinsic factors affect pattern of disease in free-ranging and captive cheetah	227
Terio et al., 2005 [18]	*Helicobacter* species in captive cheetahs with gastritis	33
Lane et al., 2012 [28]	Effect of diet on the incidence of and mortality owing to gastritis and renal disease in captive cheetahs	72
Terio et al., 2012 [19]	Characterization of the gastric immune response in cheetahs with H*elicobacter*	21
Rossi et al., 2014 [20]	Severe gastritis with double Helicobacter spp. infection associated with Barrett's esophagus in a cheetah	1
Whitehouse-Tedd et al., 2015[22]	Dietary factors associated with fecal consistency and other indicators of gastrointestinal health in the cheetah	184

Table 17. Studies about gastro-enteric pathologies in cheetahs.

Although captive cheetahs have been shown to have a higher severity of gastritis than free-ranging animals, however, due to the difficulty of monitoring the free-ranging population it is not known to what extent the prevalence to chronic diseases affects the lifespan of the free-ranging population. Species survival has been linked to the immunocompetence that is influenced by genetic factors such as the Major histocompatibility Complex (MHC) [40]. Studies showed that cheetahs have low genetic variability in the loci of the MHC, although recent studies have shown that this is more evident for MHC- II than MHC-I thus predisposing to a decreased function of the phagocytic system [53]. In captivity, the composition of social groups, size of the enclosure, the number of visitors, the vocalization and the lack of predatory activity or exercise are associated with stress conditions that predisposes animals to gastritis. In addition, the differences in the diet between captive and free-range cheetahs, may lead to nutritional deficiencies and alteration of the cheetah's microbiome. Knowledge of how extrinsic and intrinsic factors affect cheetah may aid in successful management.

The present research project is based on a multidisciplinary approach to study the physiopathology of cheetahs' GI diseases.

CONCLUSIONS and FUTURE PERSPECTIVES

Wildlife conservation can be considered an important resource of protection. Specific knowledge of the individual factors contributing to species extinction and endangerment is critical to the development of efficient conservation strategies. Since 1960 the IUCN Red List has made multiple lists for a compendium of animals and plants conservation. More than 134,000 species have been assessed for The IUCN Red List, among these the cheetah the cheetah plays a prominent role as it is the only specimen of the genus *Acinonyx*. Currently the known adult cheetah population is of about 7,000 animals with an estimated total population of no more than 10,000 mature individuals, representing a decline of at least 30% in 18 years [13]. In this thesis I investigated a disease with a high mortality and morbidity rate in cheetahs. However, little is known about the pathogenesis of the disease and the role that the immune system plays. Through approval of my project by the Biobank of the European Association of Zoos and Aquaria (EAZA), I have examined samples from European zoos of healthy cheetahs and cheetahs with gastrointestinal disease. Future studies should include a larger population and further investigate the role of MHC and macrophage cells in determining pathogenesis with conservation pourpose and improve our ability to reduce the extinction; fecal proteomics needs to be completely developed to understand the real potential

SUPPLEMENTAL ACTIVITIES

During my 3-year PhD I improved my skills in veterinary pathology and animal gastroenterology.

I have carried out techniques of histology, immunohistochemistry, autopsy techniques and *ex vivo* cellular studies.

I attended the activities related to small animal gastroenterology at the University Veterinary Teaching Hospital.

I developed proteomics techniques on fecal samples from healthy cats and dogs, dogs with lymphangiectasia, dogs with acute diarrhea, healthy cheetahs and cheetahs with gastrointestinal disease.

During my PhD I participated in national and international congress, as a speaker presenting researches carried out by my research group.

I spent 3 months abroad at the Complutense University of Madrid where I deepened and developed my knowledge in small animal clinical gastroenterology. I attended the Forensic Pathology Department of the Complutense University of Madrid where I deepened and developed histological and immunohistochemical techniques.

SCIENTIFIC ARTICLES

Chronic Stress-Related Gastroenteric Pathology in Cheetah: Relation between Intrinsic and Extrinsic Factors
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CONGRESS

European College of Veterinary Clinical Pathology – European Society of Veterinary Clinical Pathology – International Congress 2021
"Serum concentration of cholinesterase in cats with feline infectious peritonitis: what is the role?"
Gavazza, Alessandra.; Rossi, Giacomo.; Cerquetella, Matteo; Rosorani, Ilaria;
Mangiaterra, Sara.

European College of Veterinary Clinical Pathology – European Society of Veterinary Clinical Pathology – International Congress 2020
"Renal biomarkers and serum protein electrophoresis in captive Northern-East African cheetahs (Acinonyx jubatus soemmeringii): A comparison with the Southern cheetah (Acinonyx jubatus jubatus)"
Mangiaterra, Sara; Petit, Thierry; Cerquetella, Matteo; Galosi, Livio; Rossi, Giacomo; Gavazza, Alessandra.

INTERNATIONAL YABOUMBA CONGRESS 2020
Administration of the probiotic Slab51 in captive and rescued cheetahs (acinonyx jubatus) with gastrointestinal disorders: a clinical evaluation
Mangiaterra, Sara; Marker, Laurie; Schmidt-Kuentzel, Anne; Piccinini, Renato; Guadagnini, Davide; Turba Maria, Elena; Berardi, Sara; Galosi, Livio; Preziuso, Silvia; Cerquetella, Matteo; Rossi, Giacomo

INTERNATIONAL YABOUMBA CONGRESS 2020
Characteristic Presentation of Avian Tuberculosis in Different Species of Birds
Rossi, Giacomo; Mangiaterra, Sara; Zanoni, Mariagrazia; Pesaro, Stefano; Mari, Subeide; Maschio, Manuel; Cattarossi, Diego; Pelicella, Fabio; Preziuso, Silvia; Attili, Annarita; Galosi, Livio

SISVET CONGRESS 2019

A Case of Bronchioloalveolar Carcinoma - Associated Systemic Toxoplasmosis in a Mountain Lion (Puma Concolor)
Galosi, Livio; Mangiaterra, Sara; Gambi, Raffaello; Piccinini, Renato; Veronesi, Fabrizia; Filippo, Bertero; Mari, Subeide; Rossi, Giacomo

SISVET CONGRESS 2018
First detection of Helicobacter canis and related gastric pathology in cheetahs (Acinonyx jubatus)
Mangiaterra, Sara; Galosi, Livio; Scarpona, Silvia; Berardi, Sara; Marini, Maria Cristina; Cerquetella, Matteo; Preziuso, Silvia; Rossi, Giacomo

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