UNIVERSITA’ DEGLI STUDI DI CAMERINO
FACOLTA’ DI
MEDICINA VETERINARIA

PhD Thesis

PATHOGENETICAL MECHANISM AND
DEVELOPMENT OF A NEW DIAGNOSTIC
KIT FOR THE PARROT
PROVENTRICULAR DILATATION
DISEASE

PhD Student
DVM, Pesaro Stefano

Tutor
DVM, PhD, Prof. Rossi Giacomo

22° Ciclo
Dedicated to my family and my grandfather Giorgio
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Abstract

PDD is a progressive disease often fatal, that occurs in several parrot species but a common susceptibility of all parrots is suspected. It also may occur in non psittacine bird like, gooses, hawks, doves tucans and flamingos. The ill birds develop gastrointestinal or central nervous system signs. These presentation can be occur like a combination of both or alone. The clinical signs are caused by histological nervous lesions, characterized by a non suppurative encephalomyelitis and/or perineural lymphoplasmycatic infiltrates around peripheral nerves. The intramural neural plexa of digestive tract were constantly involved. The diagnosis is characterized by inconsistent clinical laboratory findings. A presumptive diagnosis of PDD is often based on anamnestic information, contrast radiographs, fluoroscopy in PDD suspect birds. Until now the only specific and reliable method used for the diagnosis is the crop biopsy. The presence of characteristic histological perineural infiltrates are strongly suggestive of the disease and necessary for a definitive diagnosis. Until now the etiology and the pathogenesis are unclear, even if, many Authors suppose the potential role of unclear virus as the causative agent of PDD. The different aspects of this disease show a lot of analogies with the human Guillain Barrè syndrome, so we have focalised the our study to clarify the pathogenesis. To do this, we investigated if the PDD can be an autoimmune disease and if a possible presence of the blood antiganglioside antibodies can be the starter of this autoimmune pathological mechanism, like was observed in more than 50% of the GBS’s cases.

Keywords
Proventricular dilatation disease (PDD), antiganglioside antibodies, Guillain Barrè syndrome, parrot
1) Compilative Part

i) Proventricular Dilatation Disease (PDD)

a) Introduction

Proventricular dilatation disease (PDD) has been reported since the late 1970’s in USA (Graham, 1984; Phalen, 1986). Initially, the disease seemed to be limited to macaws. This fact, in conjunction with an unknown clearly aetiology, gave rise to the terms macaw wasting or fading syndrome, wasting macaw syndrome and gastric distention of macaws (Huges, 1984; Mannl et al., 1987). Until now even if it was diagnosed in more of 50 different species of birds, the most susceptible group appear to be the psittacines (Ritchy, 2006; Kistler et al., 2008). The development of the disease is progressive, variably contagious and often fatal. The typical clinical presentation is characterized of gastrointestinal and central nervous system signs present both or alone in the affected birds (Gregory, 1995). The symptoms reflect the pathognomonic microscopically lesion, represented by lymphoplasmacytic perineural and perivascular infiltration in the trigger organs (Berhane et al., 2001).

Several viruses have been identified in birds with PDD or in flocks where PDD was a problem, but until now different dubts persist about the etiology (Phalen, 2006).

b) Clinical aspects

PDD occurs most frequently in african grey parrots, macaws, amazon parrots, cockatoos and conures, but it is possible that all parrot species are susceptible (Phalen, 1994). It also may occur in non-psittacine birds, as a disease with similar lesions has been observed in like Canada geese, redtailed hawk, toucan, spoonbill, flamingos, hooney creeper, canary and finch (Ritchy, 2006). There is no sex predilection for PDD. The median age of onset of PDD is 3 to 4 years, but birds as young as 10 weeks and as old as 17 years have been documented with lesions consistent with PDD. Domestically raised and imported birds are equally susceptible to disease. The incubation period for this disease is not known but may be long, as birds isolated from contact with other birds for up to 2 years still have developed this disease (Phalen , 2006).

The clinical signs of PDD vary between individuals and species, but in general they exhibit two general types of disease. Some develop neurologic signs such as depression, seizures, ataxia, blindness, tremors, incoordination, slowly progressive, proprioceptive deficits, paresis and less commonly paralysis. They may reflect disease of the brain or the
spinal cord, and recent evidence suggests that they also may reflect lesions of the lower motor neurons. (Phalen, 2006; . A peripheral neuritis has also been reported in some birds involving the sciatic, brachial and vagal nerves (Berhane et al., 2001). Alternatively, birds may develop gastrointestinal problems such as crop stasis, regurgitation, inappetance, and undigested food in feces secondary to damage to the enteric nervous system. This damage leads eventually to starvation and death. Death due to circulatory collapse or food aspiration is also common. Birds may show neurologic signs or gastrointestinal signs or both. It is also suspected that some affected birds may show minor or no clinical signs (Villanueva et al. 2009).

Physical examination, however, reveals an emaciated bird. Often the ventriculus can be palpated in the coelomic cavity caudal to the edge of the sternum. Radiographically, the proventriculus is often massively dilated, filling the left side of the coelomic cavity (Fig. 1). Contrastographic x-ray study show distention of the proventriculus and ventriculus and often the proximal duodenum. Transit time of the contrast material is markedly reduced. Various permutations of this disease may occur, and dilation of the crop, ventriculus, proventriculus or duodenum may be seen alone or in combination. Typically, the stomachs develops a “J” shape causing the ventriculus to be displaced to the right and ventrally (Fig. 2). Ultrasound will demonstrate a widely distended proventriculus and ventriculus (Phalen, 2006) and fluoroscopy has been used to demonstrate reduced gastric motility which can be an indication of PDD (Taylor et al., 1997).

(Fig. 1) (Fig. 2)

Figure 1: Radiographic aspect of dilatated proventricula in severe affected parrots.
Figure 2: Proventricular aspect during necropsy in PDD affected bird.

c) Diagnosis
Many clinical aspect can help the clinician to diagnosticate the disease but there are other pathologies that can cause nearly identical signs (Antinoff, 2001). Unfortunately until now the only *in vivo* “golden standard” examination to obtain the definitive diagnosis of this disease is the demonstrating of the pathognomonic microscopically lesions in a crop biopsy, represented by a lymphoplasmacytic perineural and perivascular infiltration (Fig. 6-7). Not all birds with PDD have crop lesions, and the percentage of the infiltration in the nervous structure of the crop is 40-76% (Schivaprasad *et al.*, 1995; Gregory *et al.*, 1997; Berhane *et al.*, 2001), so failure to find lesions in a biopsy does not rule out the disease. It is suggested that biopsying the right cranial ventral aspect of the crop, while making sure that it contains a blood vessel, will increase the probability of a lesion being found (Gregory *et al.*, 1998). (photo 3).

(Fig. 3)

![Image](image-url)

Figure 3: Surgery procedure to obtain the crop biopsy sample.

d) Etiopathogenesis

Several viruses have been identified in birds with PDD or in flocks where PDD was a problem. These include Eastern Equine Encephalitis, Enterovirus, Coronavirus, Reovirus, Avian Paramyxovirus 1 and Paramyxovirus 3. (Gought and Harcourt-Brown, 1998; Gregory, 1998, Grund *et al.*, 2002). Recently, the use of high-throughput viral screens has enabled investigators to identify the presence of a new virus, avian Borna virus (ABV), from several cases of biopsy-confirmed PDD (Honkavouri *et al.*, 2008; Villanueva *et al.*, 2009) sequent studies using PCR and immunohistochemistry on tissues of affected birds have confirmed the association between ABV infection and PDD.
PCR and serological methods was used to identified the nucleoprotein p40 (De Kloet and Dorrestein, 2009). In BDV different studies show the fact that anti-p40 antibodies are the first virus-specific antibodies synthesized, around day 15 p.i., and the fact that the p40-specific T-cell activity is also detectable early after infection make this virus-specific protein a major target antigen in both the humoral and cellular immune reaction in BD (Planz, and Stitz, 1999). The seropositivity obtained for viral nucleo-protein P40 using the Western blot and the ELISA in apparently healthy parrots that shedding virus (De Kloet and Dorrestein, 2009; Liertz et al 2009) evidence the unclear role of this virus in the development of the histological lesion and the subsequent associated clinical signs. Actually, the seropositivity of “healthy” birds is difficult to explicate.

As in most other infectious diseases, a population of seropositive healthy birds can be expected to exist that may include infected animals before to develop disease or recovered animals that have acquired an immune status to the disease (Villanueva et al. 2009). Until now even if different study show a high correlation between this infection and the disease the epidemiology, the infectivity of this virus, and the pathogenesis of virus-induced lesions still unclear.
3) **Guillain Barrè Syndrome (GBS)**

a) **Introduction**

Guillain-Barre syndrome (GBS) is an acute demyelinating disease of peripheral nerves, an immune-mediated disorder which usually follows viral illness or immunization (Bouyahia et al., 2010). The immune system attacks spinal nerve roots, peripheral nerves, and cranial nerves, resulting in focal inflammation, with variable damage to myelin sheaths and axon fibers. Described originally by French physicians working in the Sixth Army camp during the First World War (Guillain *et al.*, 1916), it has remained relatively rare, but so striking in its presentation that few doctors will not remember the clinical features. GBS is the most common acute neuropathy of adults, and occurs worldwide at an incidence of 1 to 2 cases per 100,000 annually (Guillain-Barré Syndrome Study Group, 1985; Rees *et al.* 1998; El-Sabrout *et al.*, 2001; Schwerer, 2002). It is slightly more common in men, by a ratio of 1.3 to 1. GBS affects all ages, with peaks among young adults and the elderly (Alter, 1990). The classic clinical picture is that of an acute ascending paralysis, with depressed/lost deep tendon reflexes, facial weakness, and minimal sensory loss (Asbury, 1981). The involvement of both sympathetic and parasympathetic fibers in GBS is usually observed as extra neurological manifestations such as cardiovascular, gastrointestinal, respiratory, and other systems (Kitamura *et al.*, 1998; Illyas *et al.*, 2004; Olbricht *et al.*, 2004; Bouyahia *et al.*, 2010). GBS can develop very rapidly, over hours to days, but always reaches its worst stage within 4 weeks. Although the vast majority of patients recover, 4-10% are left severely disabled. Worse prognosis is associated with more severe disease, older age, and very abnormal electrodiagnostic studies. The mortality rate of 3% to 8% reflects autonomic and respiratory problems, and indicates that all but the mildest cases should be monitored in an intensive care setting. For patients on a respirator, the mortality rate is even higher (15% to 30%). GBS is typically monophasic, but 3% of cases experience relapses (Kleopa, Brown personal communication). Laboratory hallmarks of GBS involve cerebrospinal changes (elevated protein, normal cell count) which reflect damage to the blood-cerebrospinal fluid barrier, and a series of electrodiagnostic peripheral nerve abnormalities (slowed or blocked nerve conduction, prolonged latency, delayed or absent F and H responses, abnormal temporal dispersal rates). GBS is associated with preceding infection in some 70% of cases. The most commonly identified triggering agents are Campylobacter jejuni (in 13% to 39%), followed by Cytomegalovirus (5% to 22%), Epstein Barr virus (1% to 13%), and Mycoplasma pneumoniae (5%) (Hadden *et al.*, 2001; Schwerer, 2002).
Other associations are with vaccination, pregnancy, malignancy tumors (especially Hodgkin’s and non-Hodgkin’s lymphomas) trauma and surgery. In the last few years, research studies have shed new light on a number of disease aspects that have enhanced understanding of GBS. The most common type of GBS is acute inflammatory demyelinating polyradiculoneuropathy (AIDP), the term is often used synonymously with GBS, it’s caused by an auto-immune response directed against Schwann cell membranes. Other axonal subtypes include: acute motor axonal neuropathy (AMAN) or Chinese Paralytic Syndrome, attacks motor nodes of Ranvier, it is likely due to an auto-immune response directed against the axoplasm of peripheral nerves (Asbury & McKhann, 1997).

Acute motor and sensory axonal neuropathy (AMSAN) similar to AMAN but also affects sensory nerves with severe axonal damage. Variants of GBS include: Miller Fisher syndrome (MFS) rare variant of GBS that manifests as a descending paralysis, proceeding in the reverse order of the more common form of GBS, it usually affects the eye muscles first and presents with the triad of ophthalmoplegia, ataxia, and areflexia. Acute pandysautonomia most rare variant of GBS is the most rare variant of GBS, sometimes accompanied by encephalopathy. The last one is the Bickerstaff’s brainstem encephalitis (BBE) a further form of GBS characterized by acute onset of ophthalmoplegia, ataxia, disturbance of consciousness, hyperreflexia or Babinski’s signs (Bickerstaff, 1957; Al-Din et al., 1982). In the 60% to 70% of cases of the GBS the disease are associated with the presence of IgG autoantibodies against gangliosides, these are glycosphigolipides, components of the nerves highly expressed in central and peripheral nervous system (Kusunokis et al., 2000; Ariga et al., 2001; Yuki, et al., 2001). Molecular mimicry of the infectious agent and neural ganglioside antigens are thought to result in increasing of antgangliosides blood antibodies and in the cross-reactive humoral and cytotoxic immune responses, leading to neural damage in these patients (Schmidt et al., 2006). Treatment of GBS involves plasma exchange (generally at least four) or intravenous immunoglobulin, supportive care, and careful monitoring. (Guillain-Barré Syndrome Study Group, 1985; Huge et al., 2005, 2007).

b) Clinical presentation
Although Guillain-Barré syndrome previously had been viewed as a unitary disorder with variations, it currently is viewed as a group of syndromes with several distinctive subtypes. Each of the subtypes have different, more or less distinct, clinical presentation,
electrophysiologic and pathological findings. (Tojaborg, 1998). It’s possible to divided the different forms in demyelinatings and axonals GBS (Huges et al., 2005).

Acute inflammatory demyelinating polyneuropathy (AIDP) or “classic” GBS
Acute inflammatory demyelinating polyneuropathy (AIDP) is an autoimmune process that is characterized by progressive weakness, mild sensory changes and autonomic dysfunction. It is a rare disorder, afflicting about 1 person in 100,000. The usual presentation of acute inflammatory polyradiculoneuropathy (AIDP) is subacute muscular weakness with, areflexia, ataxia and asthenia. The signs arising from the feet before and ascending and involving gradually in all 4 limbs in more than 60-90% cases. Weakness plateaux at 2 weeks after onset in 50% of patients and by 4 weeks in over 90%. It is usually symmetric, although mild asymmetry is not uncommon early in the disease course. (Lichtenfeld, 1971; Cerisola-Cardoso et al., 2007). The cranial nerve can be also compromise in 50% of patients have some facial weakness, although only 5% have varying degrees of ophthalmoplegia. Autonomic dysfunction occurs not uncommonly and is expressed in several ways include: transient hypertension or, less often, hypotension, sinus tachycardia (cardio-sympathetic hyperactivity), bradycardia, urinary retention achalasia and costipation who usually improves in parallel with motor and sensory function, excessive or reduced sudomotor function and preserved skin vasomotor function (Asahina et al., 2002; Cuciureanu and Prodan, 2004; Muller et al., 2009). Rarely, patients show sympathetic and parasympathetic overactivity simultaneously. (Lichtenfeld, 1971; Goulon et al., 1975; Tuck et al., 1978). Oropharyngeal or respiratory weakness is a presenting symptom in 40% of patients. Improvement in strength usually begins 1-4 weeks after the plateau. About one third of patients require mechanical ventilation because of respiratory failure and can cause the death in 5% of the patients (Cuciureanu and Prodan, 2004). The pain is severe in about 15% of patients, mild lower back and/or hip pain is very common and occasionally precedes the onset of weakness (Van Doorn et al., 2008; Muller et al., 2009).

Chronic Inflammatory Demyelinating Polyneuropathy (CIDP)
Also known as chronic GBS, Chronic Idiopathic Polyneuritis, or Chronic Relapsing (Dysimmune) Polyneuropathy. Patients with CIDP have a more slowly progressive weakness (over months or years) and a protracted course either monophasic or relapsing, and relapses are much more common with CIDP. While a history of viral infection is often
obtained with Guillain-Barré syndrome, this is rather uncommon in CIDP. The clinical signs are the same of AIDP (classical form of GBS) but the occurrence of respiratory failure is very uncommon (8-15%) with CIDP (Amato et al., 1995). Both conditions are associated with areflexia, typical CSF findings of increased protein, abnormal nerve conduction studies (patchy conduction slowing with Guillain-Barré syndrome and diffuse slowing with CIDP) (Coller et al., 2005). While prednisone therapy on its own has no proven role in Guillain-Barré syndrome, CIDP patients are sensitive to prednisone therapy (Austin, 1958).

**Recurrent Guillain-Barré syndrome (RGBS)**
Recurrent Guillain-Barré syndrome is a very rare entity, it's belong with CIDP to the chronic demyelinating forms. The clinical presentation are similar to AIDP, the relapses are much more frequent and numerous (more than 32) but shorter compared with CIDP. There seems to be a tendency to accumulate neurological deficits with increasing frequency of attacks of GB syndrome (Das et al., 2004).

**Acute Motor Axonal Neuropathy (AMAN)**
Although genetic predisposition has not been fully established, the AMAN type of the disease occurs more commonly in Japan and China than in North America or Europe (Yu et al., 2006). Many cases have been reported in rural areas of China. A particularly severe form, attacks the motor nerves primarily (nodes of Ranvier), causing rapid progressive weakness, flaccid symmetric paralysis with areflexia, loss of deep tendon reflexes often with respiratory failure (Visser et al., 1995). Other typical predominant motor manifestation, are recognized, i.e. a cranial nerve variant with ophthalmoplegia, ataxia and areflexia (Trojaborg, 1998). Electromyographic studies and nerve conduction studies show normal motor conduction velocity and latency with decreased amplitude of compound muscle action potentials. F-waves and sensory nerve action potentials are often normal in this illness (Biller, 2002). The patients with AMAN did not have sensory loss during a follow-up period of 6 months (Visser et al., 1995). AMAN is not generally associated with marked autonomic dysfunction except for the sudomotor hypofunction seen in patients with severe neurological deficits (Asahina et al., 2002).

**Acute Motor Sensory Axonal Neuropathy (AMSAN)**
Acute motor sensory axonal neuropathy (AMSAN) is similar to AMAN but also affects sensory nerves with severe axonal damage. Like AMAN, it is likely due to an auto-immune
response directed against the axoplasm of peripheral nerves. Recovery is slow and often incomplete (Griffin et al., 1995).

**Miller Fisher syndrome (MFS)**
Also known as Miller Fish syndrome, Miller’s syndrome and Acute Disseminated Encephalo-myeloradiculopathy. A very rare form of GBS that affects about 1-5% of GBS patients in Europe and America, 19% Asia (Mori et al., 2001). Miller Fisher syndrome occurs in more men than women by a ratio of approximately 2:1 (Berlit et al., 1992). Unlike GBS, MFS causes descending paralysis, i.e. paralysis that begins in the upper body and gradually spreads downward. A spinal tap reveals the presence of elevated protein levels. The clinical symptoms relate to dysfunction of the third, fourth, and sixth cranial nerves, similar to ocular myasthenia (Winer, 2001). The patient experiences the classical triad of ataxia, ophthalmoplegia (bilateral hypometric saccades, bilateral saccadic pursuits) and areflexia: loss of tendon reflexes and coordination, difficulty walking and standing, vision problems (Mori et al., 2001). Also tingling, numbness, dizziness, nausea. The patient experiences blurred or double vision. Damage to cranial nerves weakens the eye-muscles, causing the double-vision. It also weakens the facial muscles, causing facial sagging and sometimes making speech unintelligible.

**Acute pandysautonomia**
Similarity between acute pandysautonomia and the autonomic failure of severe Guillain-Barre syndrome suggests that acute pandysautonomia may be an uncommon variant of Guillain-Barre syndrome. Acute pandysautonomia was first described by Young et al. in 1969. Since this time, 27 cases have been reported. The disorder presents with clinical features reminiscent of the autonomic failure of severe Guillain-Barre syndrome, including severe orthostasis, impairment of gastrointestinal motility and bladder function, impotence, impairment of pupillary reactivity and accommodation, and dryness of the eyes, nasopharynx, and skin and photophobia (Suarez et al., 1994). Recovery occurs slowly and is often incomplete. Commonly, patients are left with disabling residual symptoms (Mericle and Triggs, 1997). It is associated with a high mortality rate, due to cardiovascular involvement, and associated dysrhythmias.

c) Ethiology
Although Guillain, Barré, and Strohl did not comment on the association of this illness with infection, extensive clinical observations supported by epidemiological studies suggest that about 75% of patients have a history of preceding symptoms of infection 1-3 weeks prior to show nervous signs. The onset of neuropathic symptoms being preceded by respiratory infection in 30–45% of cases and by gastrointestinal infection in 15–20% (Winer, 1988; Yu et al., 2006). Serological studies reveal evidence of antecedent bacterial and viral infection in about 30% to 50% of cases (Hughes, 1990; Mishu et al., 1993; Jacobs et al., 1998) (Fig. 4). These data are supported by accounts of outbreaks of Guillain Barré syndrome and an association between clinical cases of food poisoning and Guillain Barré syndrome within communities. Case controlled studies confirm a significant association with C. jejuni, Cytomegalovirus, and probably Epstein-Barr virus (Winer, 1988; Rees et al., 1995; Kuwabara, 2004). Numerous anecdotal reports of associations with other infections exist in the literature Herpes Simplex Virus and others viral agents were described (Dowling et al., 1981; Winer, 1988;). Same thing happened in the bacterial antigens where Escherichia coli, Helicobacter pylori, Campylobacter fetus, Haemophilus influenzae, and Chlamydia pneumoniae were also correlated with GBS (Haidl et al., 1992; Chiba et al., 1998; Mori et al., 2000; Kono et al., 2007) (Tab. 1). Even if the chemical structures of the infectious agents contributes to the GBS genetic susceptibility of patients to infectious agents may also contribute in clinical outcomes of the patients (Yu et al., 2006). Some immunizations also appear to be recognised triggers of the disease, including swine flu and rabies (Toro et al., 1977; Langmuir et al., 1984). Cases of GBS were also observed during the pregnancy an after a surgery procedures even if are rarely compared the other noxae’s correlation Tab 2.

Figure 4: Correlation between GBS and viral and bacterial antigens.
Table. 1: Different noxae ascribed to Guillain Barrè Syndrome modified from Novelli and Dini, 2007.

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*Campylobacteriosis and Guillain Barrè Syndrome*
*Campylobacter jejuni* is a species of curved, rod-shaped, non-spore forming, Gram-negative microaerophilic, bacteria commonly found in animal feces. It is one of the most common causes of human gastroenteritis in the world. Food poisoning caused by *Campylobacter* species can be severely debilitating but is rarely life-threatening. *C. jejuni* is commonly associated with poultry and naturally colonises the digestive tract of many bird species. Contaminated drinking water and unpasteurized milk provide an efficient means for distribution. Contaminated food is a major source of isolated infections, with incorrectly prepared meat and poultry normally the source of the bacteria. Infection with *C. jejuni* usually results in enteritis, which is characterised by abdominal pain, diarrhea, fever, and malaise. The symptoms usually persist for between 24 hours and a week, but may be longer. Diarrhea can vary in severity from loose stools to bloody stools. (Ryan, 2004). *Campylobacter jejuni* enteritis is the most frequently identified infection preceding the Guillain-Barré syndrome (GBS) and neural damage is thought to be induced through molecular mimicry between *C. jejuni* lipo-oligosaccharide (LOS) and human gangliosides (Dzieciatkowska *et al.*, 2007) (Fig. 5). The reported frequency of previous *C. jejuni* infections in GBS patients varies substantially (13 to 66%). The association of *C. jejuni* with GBS seems to vary in different geographic regions. In northern China, the association reaches 66%, whereas in Europe, it may be as low as 15% (Schmidt *et al.*, 2006), even if in a recent study in Germany Schmidt *et al.* 2006 using a highly specific enzyme-linked immunosorbsent assay based on two recombinant outer antigens encoded by *C. jejuni* genes Cj0017 (P39) and Cj0113 (P18), found serological evidence of a preceding *C. jejuni* infection in 80.6% cases of GBS patients. The development of GBS is likely to be a consequence of special properties of the infecting organism, since some strains such as Penner 0:19 and 0:41 are particularly associated with GBS but not with enteritis. Other isolated strains are: 0:1, 0:2, 0:5, 0:10, 0:16, 0:23, 0:37, 0:44, and 0:64 (Huges *et al.*, 1999).
Cytomegalovirus and Guillain Barrè Syndrome

Cytomegalovirus, CMV, is a member of the family of human herpesviridae, and is known as human herpesvirus 5 (HHV-5). CMV is a Betaherpesvirinae, a subfamily of herpesviridae (Todd and Will, 2006). CMV has a double stranded linear DNA genome. It is more the 240 kilobase pairs long making it the largest member of the herpesvirus family. It is capable of encoding more than 200 potential protein products, but the function of most of these proteins is presently unknown. The most representative is the glicoprotein B (gB), known to be the highest immunogen viral protein (Todd and Will, 2006). Most healthy people who are infected by HCMV after birth have no symptoms (Ryan and Ray, 2004). Some of them develop an infectious mononucleosis/glandular fever-like syndrome, with prolonged fever, and a mild hepatitis. HCMV is one of the TORCH infections that lead to congenital abnormalities. Cytomegalovirus (CMV) infections are also the most frequent viral infections preceding Guillain-Barre syndrome associated with severe sensory loss, cranial nerve involvement, and respiratory insufficiency. High titres of CMV’s IgM antibody has been implicated in 10%-15% of the patients with GBS (Winer et al., 1988; Visser et al., 1996). Like in Campylobacter jejuni and others viral and bacterials antigens the molecular mimicry between biological antigens and neural host tissues could be postulated through the synthesis of autoantibodies against myelin and gangliosides (Grygorczuk et al., 2005, Hernandez et al., 2005). The appearance of GBS signs in cytomegalovirus infection is correlate with the significantly decrease of viremia and with increasing high-avidity antibodies (Steininger et al., 2007). In particular CMV express ganglioside-like epitopes that recognize specifically anti-GM2 antibodies (Khalili et al., 1999; Ang et al., 2000).
Epstein–Barr Virus and Guillain Barrè Syndrome

The Epstein-Barr virus (EBV), also called human herpesvirus 4 (HHV-4), is a cancer causing virus of the herpes family, which includes herpes simplex virus 1 and 2, and is one of the most common viruses in humans. Epstein-Barr virus occurs worldwide, it is known to cause infectious mononucleosis, is implicated in the causation of Burkitt's lymphoma and Nasopharyngeal carcinoma, and is suspected to have a role in the pathogenesis of chronic fatigue syndrome. Epstein-Barr virus (EBV) infection are also associated with a wide spectrum of clinical syndromes involving the CNS and peripheral nervous system (PNS) included Guillain Barrè syndrome (Connelly et al., 1994; Portagies et al., 2000; An et al., 2008). There are less and contradictory dates in comparison with the others study that analysed the correlation between GBS and above mentioned bacterial and viral antigens. Different authors described the correlation from 2% to 29% in the GBS patients (Grose et al., 1975).

Mycoplasmosis in Guillain Barrè Syndrome

*Mycoplasma pneumoniae* is a very small (0,3-0,6 m) bacterium in the class Mollicutes, this species lacks a peptidoglycan cell wall. It causes the disease bacterial pneumonia, primary atypical pneumonia (PAP), infections of both the upper and lower respiratory tracts. Even if this antigen is the 4th cause of GBS in the patients, the publications about it are rare and they associate the *Mycoplasma pneumoniae* with Miller Fischer syndrome (MFS), AMAN and AIDP forms (Hsueh et al., 2004; Susuki et al., 2004). In a study Goldschimdt et al. 1980 using a counterimmunoelectrophoresis (CIEP) to determine precipitating antibodies of *Mycoplasma pneumoniae* retrospectively in sera from 100 patients with Guillain-Barré syndrome (GBS) they found specific antibodies in 5 patients with GBS, in 1 with acute cerebellar ataxia, and in 1 with acute disseminated encephalomyelitis. The GBS forms subsequently to *Mycoplasma pneumoniae* infection were often correlate, even at 12%, with serum anti-Galactocerebroside (Gal-C) IgM antibody, caused by mycoplasma molecular mimicry (Ang et al., 2002). In contrast Susuki et al. 2004 describe that the presence of Anti-GalC antibodies may be an epiphenomenon in particular in AIDP form and that in certain cases, anti-GM1 antibodies may cause acute motor axonal neuropathy.

Miscellaneous of viruses implicated in Guillain Barrè syndrome

The different forms of GBS are also described in correlation with others viral antigens. Neuropathy occur in approximately 10% to 15% of human immunodeficiency virus
infection and acquired immunodeficiency syndrome (Verma, 2001), the HIV can play a rule in the appearance of different forms of GBS, normally it was described in AIDP and in CIDP form, one case was also reported in the acute motor axonal neuropathy (Wagner and Bromberg, 2007). The mechanisms proposed for GBS in HIV-1-infected patients include a direct action of HIV-1 on the nerves by neurotropic strains, or of autoimmune mechanisms, with the formation of antibodies against myelin or gangliodides secondary to the abnormal immunoregulation determined by HIV infection (Dalakas et al., 1988). In a case of Fisher/Guillain-Barré syndrome form the serum was positive for anti-GQ1b IgG antibody and show a markedly decreased CD4 cell counts. This indicates that Fisher/Guillain-Barré syndrome may occur even in severely immunosuppressed patients and that anti-ganglioside antibody production is possibly mediated by a T cell-independent process (Hiraga et al., 2007). This fact are confirm by Deraga et al. 2006 by the description of an case where no perivascular and perineural lymphocyte infiltration are detected. This appear in contradiction with the hypothesis that the in GBS induced by HIV, in particular in the first moment of the pathogenesis, T cells may also influence the recruitment of other cells of the immunologic response such as macrophages that react against Schwann cells and fibroblasts, resulting in the process of demyelination (de Castro et al., 2006). These findings suggest that the changes mediated by antibodies are the most important process, in agreement with the pathogenetic mechanism proposed for the onset of GBS, which occurs after infections, as is the case for Campylobacter jejuni infection (Wilson, 2005). Influenza Virus A and B, Parainfluenza Virus 1, Adenovirus and Herpes Virus are also described like a possible causes in the 1% of the cases of the GBS. In particular in the cases of the Influenza Virus 1 the viral protein NS2 can mimicry the sequence region of the human myelin protein P2, inducing a mitoses and the subsequent attack by specific T lymphocytes against myelin of peripheral nerves (Jacobs et al, 1998).

Miscellaneous of bacteria implicated in Guillain Barrè syndrome

Haemophilus influenzae is a Gram-negative human pathogen whose outer membrane contains LPS. Guillain–Barré syndrome patients with H. influenzae infection were characterized clinically as having had a preceding respiratory tract infection, less frequent cranial and sensory nerve involvement, and good recovery (Apicella, 1994). In 67% of the cases was diagnosticated the AMAN pattern. Strain of H. influenzae has a GM1-like structure and elicits Guillain–Barré syndrome (Mori et al., 1999) is supported by the strong association between the anti-H. influenzae positivity and the anti-GM1 positivity. In different
studies the patients showed the positivity for IgG and IgM anti-ganglioside antibodies against ganglioside GM1, GM1b, GD1a, GalNac-GD1a, GD1b or GQ1b (Mori et al., 2000). The secretion of anti GM1 antibody were also observed in three cases of Chlamydophila pneumoniae infections (Haidl et al., 1992; Coste et al., 2002). Kono et al. in 2007 describe the first case of woman who developed rapidly progressive GBS, AMAN form with high titer of IgG antibodies to GM1 and GalNAc-GD1a, 18 days after sustaining urosepsis due to Escherichia coli. Helicobacter pylori is a common cause of gastroduodenal disorders. Antigenic mimicry between H. pylori and the host is speculated to be a possible mechanism for the mucosal damage. Cheba et al. 1998 have reported the presence of several IgG antibodies against crude H. pylori antigens in the cerebrospinal fluid (CSF) of patients with GBS. The demyelinating polyradiculoneuropathy of GBS occurred also in some cases after disappearance of erythema migrans subsequently Borrelia burgdorferi infection (Wiszniewska et al., 2005). Watanabe et al., 2005 reported that an LPS of Brucella melitensis had a GM1 ganglioside-like structure and that B. melitensis is a new etiological agent for GBS. Immunization of mice with B. melitensis induced mice to show flaccid limb weakness and ataxia-like symptoms that may be due to molecular mimicry between B. melitensis LPS containing GM1 ganglioside-like structures and peripheral nerve gangliosides.

Parassitosis implicated in Guillain Barrè syndrome
There are only four reports in the world that correlate (Toxoplasma gondii), endocellular parasite, in GBS patients (Dano et al. 1985; Bossi et al., 1998). One cases o RGBS was described in 1993 by Bahatia and Misra caused following acute filariasis (Wuchereria bancrofti)

Pathology implicated in Guillain Barrè syndrome
Effects of lymphoma on the peripheral nervous system have been reviewed by (Hughes et al., 1994; Vallat et al., 1995). There have been at least 10 reports of GBS in association with Hodgkin's disease, but association with non-Hodgkin's-lymphoma is rare. Chronic inflammatory demyelinating polyneuropathy (CIDP) in lymphoproliferative disease has been distinguished from GBS by its subacute or chronic course but both might be regarded as a part of a spectrum (Re et al., 2000). Systemic lupus erythematosus (SLE), in the peripheral neuropathies are relatively uncommon and rarely present as the initial
symptom can be consider. Like described in the others cases, anti-GM1 blood antibody is elevated in the serum of such rheumatic disorders, as SLE (Zeballos et al., 1994).

**Vaccines**

In the United State during 1976-77 was the first observation of an increase of GBS cases in the population after the administration of swine influenza vaccine. The signs appears inside the 5 weeks after the vaccination in 1 case every administrated 100000 doses (Schonberger et al., 1979). Studies of influenza vaccines used in subsequent years, however, have found small or no increased risk of GBS, 5-18 cases every 10 millions of administrated doses. Older formulations of rabies vaccine cultured in mammalian brain tissues have been found to have an increased risk of GBS, but newer formulations of rabies vaccine, derived from chick embryo cells, do not appear to be associated with GBS at a greater than expected rate. In an earlier review, the Institute of Medicine concluded that the evidence favoured a causal association between oral polio vaccine and tetanus toxoid-containing vaccines and GBS. However, recent evidence from large epidemiological studies and mass immunization campaigns in different countries found no correlation between oral polio vaccine or tetanus toxoid-containing vaccines and GBS. Spontaneous reports to the US Vaccine Adverse Events Reporting System shortly after the introduction of quadrivalent conjugated meningococcal vaccine (MCV4) raised concerns of a possible association with GBS (Haber et al., 2009). Others correlations was describe between A-B hepatitis and the GBS, the development of nervous system signs occurs 10 weeks after immunization with recombinant hepatitis B vaccine (Khamaisi et al., 2004) . Comparisons with expected rates of GBS, however, were inconclusive for an increased risk, and lack of controlled epidemiological studies makes it difficult to draw conclusions about a causal association (Tab 1).

**Medical and surgical problems**

Various types of trauma, including intracranial and peripheral lesions and in general surgical procedures, orthopaedic operations, and spinal anaesthesia can be the causes of the onset of GBS (Duncan and Kennedy, 1997, de Freiatas et al., 2006). Inexplicable diffuse peripheral neuropathy in the postoperative period is a diagnostic conundrum in which Guillain-Barré syndrome must be considered. Guillain-Barré syndrome is an unusual postoperative complication that usually has onset in the lower extremities (Bamberg and Thys, 2005). AIDP was also reported during all the three trimesters of pregnancy and in
the post-partum period. It is known to worsen during the post-partum period due to a rapid increase in delayed type hypersensitivity during this period. Relapse during successive pregnancies has been reported (Goyal et al., 2004). Though the incidence of AIDP in pregnancy (every 100000) is similar to that in the normal population: 0.89 for pregnant women, 1.37 for women during the first 90 days, and 2.93 during the first 30 days after delivery, in particularly during the first 2 weeks postpartum, only 50 cases of AIDP during pregnancy have been reported. (Cheng et al., 1998; Goyal et al., 2004)

d) Pathogenesis
Guillain Barré syndrome remains one of the most fascinating yet challenging conditions despite considerable advances in its understanding and treatment over the past 10 years. The studies of Asbury and colleagues 1969, suggested that the earliest hall mark of Guillain Barré syndrome was the presence of perifascicular lymphocytic cuffs of small vessels in the endoneurium and perineurium. This appears to be associated with demyelination, which is typically macrophage associated (Prineas, 1981). The evidence from histological examination of peripheral nerve biopsy and postmortem samples suggests that both cell mediated and humoral mechanisms are involved in the pathogenesis (Huge, 2001). Pathological studies of patients with the AMAN and in AMSAN forms, where the prevalence trigger is the axons, the alterations are caused by the multiple action of antibodies, complement and mostly by the macrophages that through the periaxonal space of the internode damaged the axolemma without altered the myelin. In contrast in AIDP and CIDP the studies reveal that even if the signs of primary injury to myelin sheath and Schwann cells of sensory and motor nerves are targeted by the auto-antibodies (humoral response), the most significant damages are caused by the presence of T lymphocytes and monocytes (cells response). In these lasts two case when the alteration are heavy and elderly the axolemma can be altered by the same mechanism of AMAN and AMSAN (Winer, 2001, Willson and Yuki, 2002). Much of the research into GBS over the last decade has focused on the forms mediated by autoantibodies against the different components of the myelin, in particular antiganglioside antibodies (Ariga et al., 2001, 2005; Wilson, 2005) (Tab.2)

Table 2: Components of nervous myelin (Novelli and Dini, 2007).

<table>
<thead>
<tr>
<th>Substances</th>
<th>Component</th>
<th>Percentage of components</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROTEINS (20-25%)</td>
<td>P0 (protein 0)</td>
<td>50%</td>
</tr>
<tr>
<td></td>
<td>MBP (myelin basic protein)</td>
<td>18%</td>
</tr>
</tbody>
</table>
The discovery of antiganglioside and others antibodies in the serum of patients with Guillain Barré syndrome has sparked an enormous proliferation of publications. The frequency of such antibodies varies from as low as 29% up to nearly 70% (Gregorson et al. 1993, Kusunoki, 1999) although the average figure is probably around 30%. Another possible antibody that might be relevant is some of these patients is antibody directed towards the myelin protein PMP 22, MBP, P0, P2 particularly in AIDP and in CIDP (Gabriel et al., 2000; Quarles, 2002; Csurhes et al., 2005), galactocerebrosides (GalC) (Ang et al. 2002), sulfatide (Obara et al. 2003). The different forms of the GBS presented various affinity with the gangliosides and the others antigens (Tab 3).

Table 3: Association between different gangliosides and protein component of myelin with Guillaine Barrè syndrome forms and antibodies secretion.
These antibodies providing a possible auto immune mechanism for the disease. This "mistaken immune attack" may arise because the surface of C. jejuni and others antigens contains polysaccharides (Lipopolisaccharide LPS, Lipooligopolisacharide LOS) that resemble glycoconjugates of the human nerve tissues (Fig. 5) (Tab.4). This resemblance has been termed "molecular mimicry," which is defined as the dual recognition, by a single B- or T-cell receptor, of a microbe's structure and an antigen of the host (gangliosides, cerebroside, myelin protein), and is the mechanism by which infections trigger cross-reactive antibodies or T cells that can lead to autoimmune diseases (Yuki, 1997; Ang et al., 2004).

Table. 4: Association between different gangliosides and protein component of myelin with different viral and bacterial antigensin Guillaine Barrè.

<table>
<thead>
<tr>
<th>Antigens</th>
<th>Gangliosides Structure</th>
<th>Non Ganglioside structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Campylobacter jejuni</td>
<td>GM1, GM1b, GM3, GD1a, GD1b, GalNac-GD1a, GD2, GD3, GT1a, GQ1b, asialo GM1 like (GA1),</td>
<td>SGBG (L2/HNK-1),</td>
</tr>
<tr>
<td>Citomegalovirus</td>
<td>GM2</td>
<td></td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>GM1, GT1a, GQ1b</td>
<td></td>
</tr>
<tr>
<td>Mycoplasma pneumoniae</td>
<td>GM1</td>
<td></td>
</tr>
<tr>
<td>Brucella melitensis</td>
<td>GM1</td>
<td></td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>asialo GM1 like (GA1)</td>
<td></td>
</tr>
<tr>
<td>Helycobacter pylori</td>
<td>P0, P2, PMP22, MBP</td>
<td></td>
</tr>
</tbody>
</table>
However, T cells are also likely to play a role in GBS. T cell help is an important prerequisite for B cell maturation and antibody production. T cells are found in the peripheral nerves of some subjects with GBS (Pollard et al., 1986, 1987; Honavard et al. 1991). Increased numbers of circulating activated T cells in patients with GBS altered proportions of T cell subsets, (Dahle et al., 1994) raised circulating levels of IL-2 and soluble IL-2 receptor, (Bamsil et al., 1991; Hartung et al., 1991) and oligoclonal expansion of T cells bearing particular T cell receptor V and V genes (Koda et al., 2003) all support a role for T cells in GBS and CIDP. Furthermore, experimental autoimmune neuritis (EAN), an animal model of GBS, which can be induced by inoculation of susceptible animals with myelin proteins, or peptides from P2,30 P0,31–34 or PMP-22 can also be passively transferred by activated T cells specific for peripheral myelin antigens. The spectrum of cytokines produced by T cells has a strong influence on the outcome of immune responses. Human CD4+ helper T cells can be divided into two categories. The first one, Th1 cells, produce the cytokines IL-2, IFN-γ, tumour necrosis factor TNF-α, and TNF-α. Th1 responses promote cell mediated inflammatory immune responses by the proliferation of B-T activated lymphocytes, the secretion of antibodies by plasmacells, the proliferation and the improvement of citotoxic activity of NK and citotoxic T lymphocytes, all important factors for development of many organ specific autoimmune diseases. The second one, Th2 type T cells, produce IL-4, IL-5, IL-10, and IL-13, and are thought to suppress inflammation and help eosinophils and B cell antibody production, and may play beneficial roles in human organ specific inflammatory diseases. Th1 responses have been shown to be important in EAN-GBS induction, whereas the recovery phase is associated with a switch to Th2 (Csurhes et al., 2005).
iii) Autoimmune disease in birds

a) Introduction

The immune system has developed many effective ways to protect an individual from environmental insults and disease. Adaptive (specific) immunity will focus these defensive efforts very specifically on a given antigen in order to remove and/or destroy that antigen. However, when this specific response is directed against self-antigen, the result is autoimmune disease. Autoimmune disease is defined as a disease caused by a breakdown of self-tolerance such that the adaptive immune system responds to a self (autologous)-antigen and causes cell and tissue damage. Autoimmune diseases can be categorized into two basic types: organ-specific and systemic diseases. In organ-specific or localized autoimmune diseases, immune recognition is cell-/ tissue-specific as is the resulting immunopathology. Examples of organ-specific autoimmune diseases include Hashimoto’s thyroiditis, type-1 diabetes, vitiligo and many others. In systemic autoimmune disease, such as systemic lupus erythematosus (SLE), multiple organs are affected. Self-tolerance is an essential property of the immune system designed to ensure that individuals do not respond to their own antigens. Tolerance to self-antigens is normally maintained by selective processes that prevent the maturation of self-antigen-specific lymphocytes and that inactivate or delete self-reactive lymphocytes that do mature and enter the periphery. Loss of self-tolerance may result from abnormal selection or regulation of self-reactive lymphocytes and by abnormalities in the way that self-antigens are processed and presented to the immune system. Self-tolerance is primarily inherent in the T cell compartment. This is due in part to the rigorous selection processes T cells encounter during maturation in the thymus, the important regulatory function of T cells in adaptive immune responses and the restriction of T cells to recognition of antigenic-peptides in association with self major histocompatibility complex (MHC).

Failures of self-tolerance within the T cell compartment can result in autoimmune diseases in which the autoimmune lesion is caused by cell-mediated and/or humoral immune responses. Autoimmune diseases typically are multifactorial in nature, requiring several components such as genetic susceptibility, immunological influences and environmental factors for expression. Unfortunately, the relative contribution of these factors to the development of autoimmune disease is not clear cut and cannot be easily dissected. In organ-specific autoimmune diseases, genetic susceptibility is frequently associated with an inherent target cell defect that predisposes it to immunorecognition and may include
aberrant immunological activity at various levels (e.g. dendritic cells/macrophages, B cells and T cells). The autoimmune destruction of cells has been found to be associated with a lack of regulatory function within the immune system, heightened immune activity and altered responsiveness of immune components to factors from other physiological systems. The role of environmental factors in the development of autoimmune disease is also multifaceted and may include infections by microbes as well as exposure to chemicals. The consequences of having adaptive immune mechanisms specifically focused on a self-antigen are clearly destructive. It is puzzling, however, that autoimmune attacks against components such as the myelin sheath in multiple sclerosis, pigment cells in vitiligo and thyroid cells in autoimmune thyroiditis, do not necessarily destroy all of these target cells as would be implied by the specificity of the recognition. Rather, cell/tissue destruction tends to be progressive in nature, suggesting an important interplay between the target tissue and the antigens it displays, immune recognition and immunoregulation. Understanding these interrelationships will be important in the prevention and treatment of autoimmune disease.

b) Autoimmune diseases of poultry

Autoimmune diseases have rarely been reported in birds. This may be a result of our inability to recognize autoimmune disease rather than a resistance to autoimmune problems (Harrison, 1984). The specie most studied for the autoimmune disease is the poultry. These pathologies are studied by avian and humane medicine researchers, over the years, the chicken has made significant contributions to the understanding of the components and mechanisms involved in autoimmune diseases, primarily because some lines of chickens spontaneously, and predictably, develop autoimmune disease. For example, the Smyth line (SL) chicken is the only animal model for the pigmentation disorder vitiligo, that manifests all the clinical and biological symptoms of the human disease. The obese strain (OS) chicken is one of the most-valued and best models for spontaneously occurring Hashimoto thyroiditis and the University of California at Davis (UCD) 200/206 chicken lines are the only model for spontaneously occurring scleroderma that presents the combination of symptoms observed in humans. Autoimmune disease does not appear to be of widespread concern in poultry production. Although, poultry breeding programmes have selected against inherited diseases, susceptibility to autoimmune diseases may be selected for in the absence of disease expression. Considering the frequently observed disorders associated with high intensity poultry
production, such as leg, endocrine and nervous system problems, as well as metabolic, integumental, pulmonary, alimentary and reproductive tract problems, one may ask whether or not there is an underlying autoimmune component to these disorders. Given the multifactorial nature of autoimmune disease, the danger lies in the combination of factors encountered by a susceptible population that will lead to the expression of the disease. Hence, poultry breeding programmes will need to include attention to the role of the immune system in health and disease. The study of autoimmune disease in susceptible lines of chickens has been a driving force for the development of assay systems, probes and tools to study fundamental aspects of immune function, immunopathology and immunophysiology in poultry. Moreover, the SL, OS and UCD-200/206 lines are excellent models to study the cause–effect relationship between a genetically controlled disease, immune function and environmental factors, and have been an incentive for the mapping of susceptibility genes and studies on the mechanistic links between susceptibility genes and failure of self-tolerance. Even if the most common and studied disease are represented by vitiligo, thyroiditis, scleroderma, reports on other occurrences of, and investigations into, autoimmune disease in poultry can be found in the literature. Included are experimental viral arthritis, experimental allergic encephalomyelitis and ovarian autoimmune disease (Blaszczyk et al., 1978; Pertile et al., 1996; Barua and Yoshimura, 2001).
**Experimental Studies**

**i) Introduction**

How is possible to understand reading the introduction parts that explain the proventricular dilatation disease, even if it’s studied for more than 30 years, many different aspects of it appear still unclear. In particular this is really true for what concern the epidemiology, aetiology, and the pathogenesis of this disease.

This is caused by many difficult aspects that the researcher meet during their studies: the few numbers of PDD affected birds that is possible to analysed in the same time also in very big breeding centres, the difficult to reproduce experimentally the disease, the rapidity of the development of the pathology in the grater part of the cases and the difficult to diagnose it before the birds died.

Because the high mortality rate of affected birds, until the beginning the researcher focalized their studies to discover the exactly aetiological agent to reduce the risk of the dissemination of the PDD. Different biological antigens were investigated, more than six different viruses were suppose associated with the disease in the last twenty years, Avian Bornavirus the last one. Unfortunately none have reproduce experimentally the disease using an isolated virus from affected bird satisfying the Koch’s postulates, so until now the really aetiological antigen remain unclear.

Analysing this aspect and the clinical presentation of this disease we found various analogies with the human Guillain Barrè syndrome, so we have focalised the our study to clarify the pathogenesis. To do this, we investigated if the PDD can be an autoimmune disease and if a possible presence of the blood antiganglioside antibodies can be the starter of this autoimmune pathological mechanism, like was observed in more than 50% of the GBS’s cases. This aspect also to understand more the pathogenesis of this disease, can be apply to help the breeders and the veterinaries to diagnose this still unclear.

**ii) Materials and Methods.**

During the last three years (2007-2010) we have collected 200 crop biopsy tissues samples of affected and healthy birds, different organs samples of 10 PDD’s suppose death parrots, 1140 plasma samples, obtained from 14 different psittacine bird generas of italian, spanish, portuguese and brasillian breeding centers. Furthermore for the great part
of the animal we collected the datas rappresented by: age, sex, signs of disease. All these samples was analysed in the University of Camerino, Faculty of Veterinary Medicine.

The different crops and organs samples was utilised for different analyses: to the diagnosis and of the possible PDD’s affected birds (golden standard method) and to observed the most significant alteration of crops and organs that occur during the disease. The plasma samples was analysed to put in evidence the possible presence and the correlation between the antiganglioside antibodies and the pathological PDD’s status of the parrots diagnosed ante mortem by the evaluation of the eventually perigangliar infiltration of the nervous crop structures.

The first steps of our study, using HE staining for the morphological examination, luxol fast blue (LFB) for the myelin status, Dane & Hermann’s tetrachromic stain (DHTS) for characterization of the mucins and for different epithelial layers of the crop and the IHC for the evaluation of the mitotic activity, was focalized to described the morphological microscopically aspects of the lesions observed in the crop tissues of affected birds comparing with healthy subject. Totally we analysed 60 crop biopsy obtained from unaffected and from symptomatic and asymptomatic affected parrots.

Subsequently, using the IHC, we have immunophenotypized the lymphoplasmacytic perigangliar infiltrate of the PDD positive crops, to understand if it presented the typical aspects of the infiltration observed during an immunomediated process.

At the end we used the serological methods, E.L.I.S.A., to check in the 1140 blood sample the eventually presence of antiganglioside antibodies direct to: the specific GM1, GT1b gangliosides, two of the most representative glicosfingolipides of the nervous system, and to all different gangliosides of the bird myelin. To do this we used like antigens the commercial bovine GM1 e GT1b (SIGMA), while for the last analysis we extract the antigens (gangliosides pool) directly from CNS and PNS of the parrots in our laboratory. Furthermore we compare in 140 cases the histological aspect of the crop biopsy (which represent the in vivo “golden standard” diagnostic method), with the serological data. In 102 of these, we also obtained the clinical presentation of the patient during the period of 1 year and the radiological aspect in L/L and V/D position. This last comparative study that analized the different serological, histological, symptomatological and radiological presentations obtained from the same subjects, it was very important to evidence a possible alternative use of the blood antigangliosides evaluation for the diagnostic and non invasive method for this disease.
In February 2008 to further investigate in vivo the role of anti-ganglioside autoimmunity in the pathogenesis of the disease, we challenged 6 cockatiels (Nymphicus hollandicus) intraperitoneally and orally with a 1 mg of purified gangliosides diluted in 1 ml of 0.9% NaCl solution with adjuvant added, and two control cockatiels by same route only with NaCl solution.

**Morphological staining**
Tissue samples were collected from each birds and fixed in 10% neutral buffered formalin for a minimum of 12h, embedded in paraffin, sectioned in 3µm thick sections, and stained.

**Haematoxylin Eosin Staining**
Bring sections to distilled water, stain nuclei with the alum haematoxylin (Carazzi) for 10 minutes, rinse in running tap water for three time, the last one for 15 minutes. Stain the slide with eosin for 1 minutes and wash rapidly, 2 seconds, three time. Dehydrate with decrease concentration of alcohol baths, put the section in two xilolo’s baths, and mount by resin (Permound, Histoline,Milan, Italy).

**Luxol fast blue (LFB)**
Deparaffinize and hydrate to 95% alcohol, the section and put it in Luxol fast blue solution overnight at 60°C. Rinse in 95% alcohol and wash by distilled water. Put the slide in lithium carbonate solution for 5 seconds and then in 70% alcohol, two changes, 10 seconds each. Wash in distilled water. Rinse slide in 70% alcohol end color with eosin for 1 minute. Rinse in distilled water and stain by cresyl violet for 1 minute, wash again by distilled water and dehydrate with decrease concentration of alcohol baths, put the section in two xilolo’s baths, and mount by resin (Permound, Histoline,Milan, Italy).

**Dane & Hermann’s tetrachromic stain (DHTS)**
Deparaffinize and hydrate to 95% alcohol, the section and put it in Meyer’s emallume for 10 minutes, wash with distilled water. Color the section by a 1% floxina B water solution for 3 minutes, wash for 1 minute with water as soon the discoloration of section and rinse by distilled water. Color by a solution 1:1 of 1% alcian blu and 1% acetic acid. Wash for 1 minute with water and rinse by distilled water. Colour for 13 minutes the section by a 0,5% orange G solution in 2% phosphotungstic acid solution in distilled water. Immerse 10 times
in 95% ethanol. Immerse 35 times in 100% ethanol. Clarify by renew xylene many times and mount by resin (Permount, Histoline, Milan, Italy).

**Immunohistochemical stain**

To evaluate the mitotic activity of cells constituting the epithelial basal layer of the crop’s mucosa, and to characterized the phenotype of cell-mediated infiltrates, an immunohistochemical test was employed. In particular to assess the mitotic index, we used a rabbit polyclonal antibody (pAb) specific to PCNA antigen (Novocastra, London), diluted 1:100 in TBS+1% BSA.

Periganglia lymphoplasmacytic infiltrate was immunohistochemically phenotyped by a panel of antibodies constituted by a monoclonal rat-anti CD3 antibody (mAb) (Serotec, USA) diluted 1:200 in TBS+1% BSA, mouse-anti CD8 mAb (Southern Biotechnology Associates Eching, Germany) diluted 1:200 in the same buffer, and mouse-anti lysozyme mAb (Novocastra, London) diluted 1:500. For histochemical and immunohistochemical tests, sections were placed on pretreated slides (Bio-Optica, Milan, Italy) to promote adhesion and dried overnight at 37°C. After being dewaxed, sections were placed in EDTA buffer, pH 9.0, and processed in a microwave oven at 650 W for two cycles of 10 min each to enhance their antigenicity. Slides were then allowed to cool at room temperature for at least 20 min before being further processed for immunostaining employing standard procedures. Tissue sections were incubated overnight in a moist chamber at 4°C with different primary antibodies, diluted in Tris-buffered solution (TBS) containing 0.1% crystalline bovine serum albumin (BSA). Binding of the previously mentioned antibodies was detected with ABC-peroxydase (Vector Laboratories Inc., Burlingame, CA) techniques using 1:200 diluted biotin conjugated goat anti-rabbit immunoglobulin G (Vector Laboratories Inc., Burlingame, CA) and a 1:200 diluted biotinylated goat anti-mouse immunoglobulin (AO433; DAKO, Glostrup, Denmark), applied for 45 min at room temperature as secondary antibodies. The enzymatic reaction was developed with 3,1-diaminobenzidine (DAB) (Sigma, St. Louis, MO) as substrate for ABC-peroxydase technique, by using Meyer hematoxylin as nuclear counter stain. Vector Vip and Vector Blue (Vector Laboratories Inc., Burlingame, CA) were used as second and third chromogen in IHC triple stains tests. Specific primary antibodies substituted with TBS or non-immune sera were used as negative controls in immunohistochemical techniques. Histological examination of crop’s samples included assessment of thickness of epithelial layer, the rate of PCNA expression by cells of the basal layer of mucosal epithelium and
the phenotype characterization of the mononuclear cells that constituting the periganglia infiltrate. For immunohistochemical evaluation, ten 40x randomly selected fields were analysed for each sample and the number of positive cells/field was recorded. The value of expression of PCNA per sample was expressed as the mean value obtained from all ten values.

**Ganglioside extraction**

Gangliosides were extracted from brain, spinal cord, sciatic nerves during the necroscopical examination of PDD non affected parrots. Tissue was cut with a freezing microtome into approximately 40 coronal sections throughout the sample. The tissue slices for each area were collected and homogenized for 10 min at 4°C in acetone and incubated at 20°C for 15 min. After centrifugation, the supernatant was discarded and 4 ml of cold acetone was added. This procedure was repeated twice. The pellet was re-extracted (three times) with 3.3 ml of chloroform-methanol-distilled water (1 : 2 : 0.3 v/v/v) and incubated for 15 min at 37°C in a water bath. The ratio of chloroform-methanol-distilled water was adjusted to 1 : 1 : 1 v/v/v. This solution was shaken overnight and gangliosides were then separated from other lipids by partitioning into the aqueous methanol (upper) phase. The lower phase was removed with a syringe and discarded. The membrane between both phases was saved together with the remaining upper phase and dried under air in a 37°C water bath and then used for antiganglioside antibodies blood analysis (Fig. 14).

**Serological evaluation of antiganglioside antibodies (PATENTED PROCEDURE).**

1 μg of antigens, bovine Gm1 ganglioside C_{73}H_{131}N_{3}O_{31} (SIGMA) or bovine GT1b ganglioside Gt1b bovino (SIGMA), C_{95}H_{195}N_{5}O_{49} (SIGMA), or extracted pool of parrot gangliosides, was adsorbe overnight (12 h) on ELISA plate (Nunc) at 4°C in 100 ml of PBS pH 7.2 (phosphate buffer saline). Rinse 3 times with 200 μl of PBST 0.01% (phosphate buffer saline + tween 20). Fixing of the antigens on the plate by 200 μl of BSA 1% (bovine serum albumine+PBS) then wash again 3 times with 200 of PBST 0.01% (phosphate buffer saline + tween 20). Bindind and applied for 3 h at 37°C 100μl of rabbit anti-bovine-GM1 and rabbit anti-bovine-GT1b diluted 1:5000 in PBS and an hyperimmune parrot serum diluted 1:50 in PBS for extracted pool of gangliosides in positive control wells, 100μl of healthy bird plasma diluted 1:50 in PBS in negative control wells and 100μl diluted 1:50 in PBS of parrot’s plasma in examination. At the end of incubation period rinse 3
times with 200 μl of PBST 0.01% (phosphate buffer saline + tween 20). Subsequent apply for 90 minutes (continuously shaking): 100μl of secondary peroxidate antibodies consisting by polyclonal goat anti rabbit IgG diluted in 1:2500 in PBS in the GM1 and GT1b control positive wells, while in the positive control well containing extracted gangliosides pool, in all three negative controls wells and into the well of the serum sample in examination, 100μl of secondary peroxidate antibody consisting by monoclonal rabbit anti chicken IgG diluted in 1:2500 in PBS. At the end of the incubation time, rinse 3 times with 200 μl of PBST 0.01% (phosphate buffer saline + tween 20) and apply 100 μl of ABST (SIGMA) for 30 minutes, subsequently stop the peroxidase activity with 100 μl of SDS 1%. After 3 minutes read by a spectrophotometer working at 405 nm. The cut-off value for each antigen resulted from the mean of the positive and negative control O.D. values, after subtraction of the O.D. value of a ganglioside-coated well incubated only with secondary antibody (without primary antibody incubation). In each test, positive serum sample (potentially indicative of positive PDD parrot) consisted in O.D. value higher than the mean O.D. one resulting from the mean subtracted of the white O.D. value.

iii) Results

During the histological evaluation of the 60 crop samples the periganglia infiltrate of lymphomononocytic nature was observed in 17. Of those 17, 8 (66.6%) came from the group of the 12 symptomatic birds and 9 (18.7%) from the clinically healthy birds (Photo. 1). Severity of chronic inflammatory infiltrate, was graded from 0 to 3, according to the prevalence and amount of the difference inflammatory cells, score 0 , score 1 , score 2 , score 3; the score values are determined as reported by Martini et al. 2005. In the 17 affected and symptomatic birds the mean obtained was evaluated in 2.8 in contrast to the value of 0.9 obtained from healthy parrots. In the 9 asymptomatic PDD positive animals all perigangliar infiltrates consisting in a high percentage of CD3+ lymphocytes, with a variable percentage of plasma cells and macrophages (Fig. 8). Only occasional CD8+ cells (citotoxic T lymphocytes, CTL) and macrophages were observed mixed with scattered CD3+ T cells. On the contrary in the 8 symptomatic positive birds a large percentage of these CD8+ lymphocytes and macrophages were associated with moderate to severe (score 2 to 3) periganglia mononuclear cell infiltration (Fig. 8). In these 8 samples, LFB stain also revealed a moderate to severe degree of demyelination (Fig. 9). Differences were also observed in basal crop epithelium PCNA positive cells-count when pathological (Fig. 12) and healthy (Fig. 13) crop specimens were compared. In particular
the mean value of PCNA positive cells counted in affected crop samples resulted 75.8. The value obtained in samples belonging to asymptomatic – unaffected parrots was of 186.6 PCNA positive cells more than the double value of the cells population count in the healthy. Notably, in the cells of basal layer, PCNA cells count varied independently to the degree of the pathology and the severity of the lymphoid infiltrate in the two groups. Similarly, specimens stained with DHTS showed a different thickness in pre-keratinized epithelial layer of the crop between groups, with an evident thinness of this stratum in affected birds (Fig. 10-11).

About the 900 plasma samples obtained from asymptomatic birds we found 13.5% of birds positive for all the three antigens. To identified the more susceptible genera for an increasing of antiganglioside antibodies values we create 5 groups subdivide on the basis of the morphology, behaviour and geographic origin of the parrots: “Group Amazon”: Amazona sp., Pionus sp. Pionites sp.; “Group Macaw”: Ara sp. Cyanopsitta sp. Anodorhynchus sp.; Aratinga sp., Cyanolisus sp., “Group Grey”: Psitthacus sp.; Poicephalus sp., Choracopsis sp. “Group Cockatoo” Cacatua sp., Nynphicus sp., Eolophus sp., Calyptorinchus sp., “Group Lory”: Lori sp., Lorius sp., Eclectus sp.. The results show the positivity rate quantificated in 23% for “Group Amazzon”; 11% for “Group Macaw”, 10% for “Group Greys”, 15.73% for “Group Cockatoo” and the 2% for “Group Lory”.

On the basis of the age we subdivided the animals in two groups, under and up of the 1 year of age, and we found a low positivity only 1% in the subject under the year of age and the gender ratio for susceptibility appear same between male and female 1.3:1 of all ages. In the comparative study of the 140 cases, we examined the correlation between the histological and the serological aspect of the same birds.

None of the 120 animals with crop histologically negative showed the positivity for all three antibodies and the 85% presented a negativity for all three antigens, the 98% of the 30 seras obtained from symptomatic and histologically positive birds showed high antibody titres at least one of three antigens. The most important data is that 70% of sera obtained from these histological positive birds showed high antibody titres for all of three antigens in particular for parrot’s gangliosides pool. About these 140 animals histologically and serologically tested, during the year 2008-2009 we had the opportunity to follow for a period of 1 year the clinical status of 102 animal in an italian and spanish breeding centers. Of these parrots 89 presented negativity for both examination, 6 (1 Psittacula finschii, 1 Amazona rhodocorita, 1 Cacatua moluccensis, 1 Cacatua galerita, 2 Amazona viridiginalis) presented a positivity for both aspects, 1 (Ara glaucogularis) was positive
histologically and positive for 2 (GT1b, pool of gangliosides) of the three antigens, 7 (Psittacula krameri, Amazona ocrocephala, Pionus fuscus, Eclectus roratus, Amazona farinosa, Cacatua alba) appeared histologically positive and negative subsequently the serological examination and only 1 subject (Cacatua moluccensis) presented a not clear signs of histological positivity with a very light perigangliar infiltration and show positive value for GM1, dubt for GT1b and negative for ganglioside pool. The 88 of the 89 different parrots totally negative, didn't show any kind of symptomatical and radiological alterations. During the all period of examination, only one (Psittacula krameri) showed a scab and ulcerative lesions round the beack, reduction of body condition, with apatia and enlargement of proventriculum observed by a contrastographic study. The remission of the clinical alterations occurred after a treatment of antibiotic (enrofloxacine) and NSAD (meloxicam). The histological examination of crop biopsy evidenced a granulomatous ingluvitis. The 6 totally positive presented in all the cases the typical symptomatic signs correlate with the x-ray proventricular enlargement. After a period of one year from the first examination, 5 of it died and the in vitam PDD diagnosis was confirmed during the necropsy and by the subsequent histological evaluation. Only one of it (Amazona viridiginalis) still alive after one year, even if present undigested food in the feces, very bad feather condition and the enlargement of the proventriculium, furthermore a second serological and histological examination continue to indicate a positive status for PDD. The one cases (Ara glaucogularis) that demonstrated a positive evaluation for the histology and for GT1b and gangliosides pool, died with the characteristic lesion and clinical signs of PDD, the causes of the death was confirm by post mortem laboratory examination; while the cases of Cacatua moluccensis with unclear situation after three subsequent histological evaluations continue to demonstrate a border line mycroscopical pattern even if the values of antibodies decrease and became negative, the animal never shows during all the period of the study any clinic-pathological signs correlated with PDD. Different development of the disease was observed in the parrots histologically positive and serologically negative. The Psittacula krameri and the Amazona ocrocephala died respectively after 2 and 30 days subsequently of the comparison of the clinical signs of the disease, and the in vitam diagnosis was confirmed by the histology on the different sample of the trigger organs, even if the amazon show very light lymphoplasmacitic infiltration of adrenal glands and crop. The other 4 birds showed a remission of clinical signs characterized by enlargement of proventriculium and gastrointestinal alterations present both or alone. To confirm the PDD negativization of the patients, three subsequent session
of crop biopsy with related histological examination and serological evaluation was made. All results appeared negative and confirm the apparent recovery of the four subjects.

The 6 subjects of cockatiels (Nymphicus holladicus) inoculated by the extracted pool of gangliosides, two weeks after the booster (1 month p.i.), 100% of I.P. inoculated and 33% of orally challenged parrots developed typical PDD signs. Currently, four inoculated and symptomatic cockatiels show typical ganglioneuritis in crop biopsies.

### iii) Conclusions and Discussion

In our study, we observed, for the first time, the correlation in PDD positive parrots of a decrease number of PCNA positive cells into the basal layer of affected crop’s epithelium (Fig. 3), associate with a reduction in thickness of superficial pre-keratinized layer (Fig. 5). Those may represent an unspecific early sign, strictly related to the dystrophic status of the crop’s wall, generated by the non suppurative ganglioneuritis. The progressive and dramatic thinning and atrophy of the crop’s wall, sometime associated with the organ dilatation, could suggest to be a late consequence of these early histological alterations.

For what concern the periganglia infiltrate, our preliminary results demonstrate a high percentage of CD3+ cells in the infiltrate, with large presence of CD8+ T cells and macrophages in affected and symptomatic birds (Fig. 2). These immunophenotypic findings correlate with morphologic data that indicate a microscopic changes characterized by non suppurative ganglioneuritis with gliosis, cuffing, spots of demyelination and ganglia swelling (photos) (Graham, 1984; Joyner et al., 1989; Graham, 1991; Gregory et al., 1996, Shivaprasad et al., 1997, Berhane et al., 2001), in our opinion, can suggest that an immunomediate - autoimmune mechanism can be correlated with the pathogenesis of histological lesions during the PDD processes.

The evidence of a seroprevalence of 70% in histologically and clinically positive parrots, supported to the similar results obtained in experimental inoculated animals by ganglioside extracting pool, confirms the possible roles of the antiganglosides antibodies in the pathogenesis of PDD. The hight negativity correspondence of 85% between both serological and histological tests show clearly how the low value of the blood antigangliosides antibodies can be an indication of the healthy status of the examined parrot. Moreover these evidences show the good predictivity of this non invasive test if compared with the 60-70% of the diagnostic golden standard method rappresented by the more invasive histological evaluation of crop biopsy.
Furthermore even if the case histories is very low to extrapolated a reliable results, the 3 of the 5 parrots (60%) that were histologically/clinically positive and serologically negative after a period of 1 year have changed their PDD status begane apparently (histologically/serologically/clinically) healthy animals. This observation appear very interesting for different aspects: the first one is that none Authors have described any case of recovery for this disease, and the second one, that the serological value can help the clinician to formulated a future prognosis for an affected patient. To explain why the 40% of this 5 parrots died rapidly (2-30 days) with low serological values we can formulate two different hypotesis:

- a high prevalence of a cell mediated immunoreaction to respect the humoral response during the early phases of the lesion development
- an acute and fastly lethal neural damage, induced by an unidentified neurotropic biological agent, precluding a serological conversion or an effective cell mediated response before the death.

Analising the serological results obtained from 1140 plasma samples is possible to see haw the gender positivity confirm the same susceptibility of male and female described by other Authors (Gregory, 1995; Phalen, 2006). Furthermore the rare increasing of the antiganglioside antibodies values observed in the young subject is the same observed by (Gregory, 1995; Phalen, 2006) by the histological examination. At the end the high 15% and the low 2% relative positivity showed respectively by the “Group Cackatoo” and “Group Lory” reflect the natural susceptibility to this disease for of these genera like describe previously by (Gregory, 1995; Phalen, 2006). The highest relative value 23% observed in the “Group Amazon” obtained to all appearance from healthy birds can be explained by a possible background interference induced by the hight level of lipoproteins that normaly can be present in the plasma of these Genera.

In conclusion the autoimmune mechanism mediated by antiganglioside antibodies appear very interesting, because places the proventricular dilatation disease PDD in the rare group of the avian autoimmune diseases. These kind of pathologies even if described more accuracy in the poultry not present a same equivalent disease in the avian exotic medicine. Moreover this new and fascinating theory not exclude, but can complete, the other aetiological hypotesis that saw in Avianbornavirus (Kistler et al., 2008, Honkavuori et al., 2008, Gancz, et al.,2009, Liertz et al., 2009) or in other biological agents infection not still recognized the causes of these disease like happened in subsequently of bacterial and viral infections in human Guillain Barrè syndrome.
Figure 6: Characteristic periganglia lymphoplasmacytic infiltrate (EE, 10X).

Figure 7: Characteristic periganglia lymphoplasmacytic infiltrate (EE, 20X).
Figure 8: Cellular population in infiltrate, LinT C3+ in blu, macrophage in brown (IHC 40X).

Figure 9: Demyelination of crop nervous structure (LFB 40X).
Figure 10: Reduce of thickness in pre-keratinized epithelial layer of the crop observed in positive sample for PDD (DHTS10X).

Figure 11: Thickness in pre-keratinized epithelial layer of the crop observed in negative sample for PDD (DHTS 10X).
Figure 12: Reducing mitotic activity in basal crop epithelium observed in positive sample for PDD (PCNA 10X).

Figure 13: Normal mitotic activity in basal crop epithelium observed in negative sample for PDD (PCNA 10X).

Figure 14: Antiganglioside extraction of CNS and PNS of parrots, the gangliosides (clear matrix) separated from the other lipids (dark matrix) and the aqueous methanol phase (upper liquid).
REFERENCES


