# Evaluation of Biomarkers Associated with Pulmonary Hypertension in Dogs Infected with *Dirofilaria immitis*

Evaluación de Biomarcadores Asociados a Hipertensión Pulmonar en Perros Infectados por *Dirofilaria immitis*

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Doctorado de Investigación en Biomedicina Tesis Doctoral con Mención Internacional
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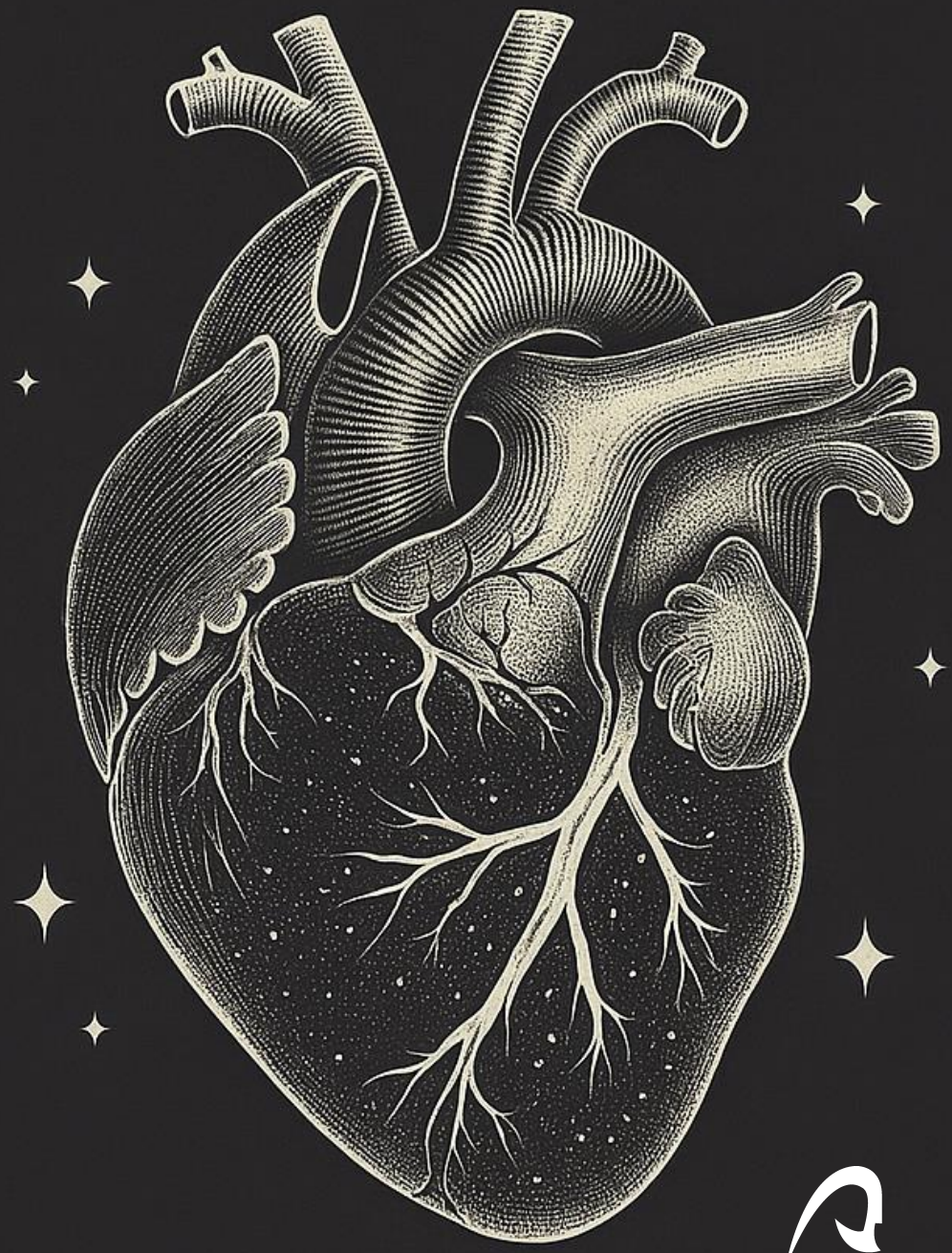

UNIVERSIDAD DE LAS PALMAS DE GRAN CANARIA
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# PROGRAMA DE DOCTORADO DE INVESTIGACIÓN EN BIOMEDICINA

# TESIS DOCTORAL

## EVALUACIÓN DE BIOMARCADORES ASOCIADOS A HIPERTENSIÓN PULMONAR EN PERROS INFECTADOS POR *DIROFILARIA IMMITIS*

## EVALUATION OF BIOMARKERS ASSOCIATED WITH PULMONARY HYPERTENSION IN DOGS INFECTED WITH *DIROFILARIA IMMITIS*


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# ÍNDICE

# 1. STATE OF THE ART; *DIROFILARIA IMMITIS*

## 1.1 Historical and Taxonomical Overview

*Dirofilaria immitis* is a filarioid nematode within the family Onchocercidae, phylum Nematoda, class Chromadorea, order Spirurida, genus *Dirofilaria* (Balmori de La Puente et al., 2024). This parasite was first described as a new species of *Filaria* by Joseph Leidy in 1856 after its identification in a dog from the United States (Leidy, 1856). Morphologically, adult worms are cylindrical and elongated, with pronounced sexual dimorphism (Barnes, 1987; Brusca & Brusca, 2003; Soulsby, 1968). Females are 100 to 170 mm in length and 4.6 to 6.3 mm in diameter, and males are 50 to 70 mm in length and 3.7 to 4.5 mm in diameter (Manfredi et al., 2007). Adult females are ovoviviparous, with the vulva located near the anterior end, where larval eggs hatch in the uterus, leading to the release of L1 (microfilariae) into the host's circulation (Manfredi et al., 2007). Males possess two unequal spicules and lack a gubernaculum (Kradin & Mark, 2010).

**Figure 1.** Macroscopic view of several adult worms of *Dirofilaria immitis* removed from the necropsy of a dog with severe cardiopulmonary dirofilariosis.

Although initially identified as a pathogen of domestic dogs, *D. immitis* has been reported in a wide range of hosts, including wild canines, domestic and wild felines, mustelid, rodents and even in humans (Simón et al., 2005; Montoya-Alonso et al., 2022). Over time, it has evolved from a parasitological curiosity into a globally significant veterinary concern (Montoya et al., 1998), despite its broader host range, dogs and other canids remain the definitive hosts and act as the principal reservoirs of infection (Hidaka et al., 2003). Its complex life cycle, vectorial transmission, and organ-specific pathology position it as a model for studying chronic parasitic disease (Bowman & Atkins, 2009).

Transmission occurs via hematophagous mosquitoes of diverse genera as *Culex*, *Aedes* and *Anopheles* (Morchón et al., 2022).

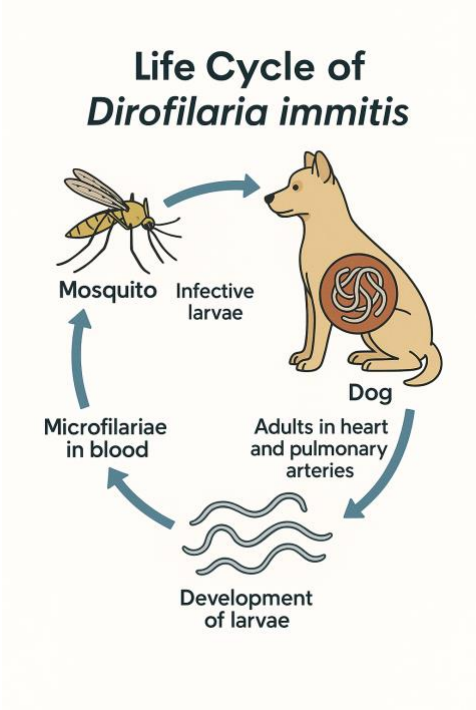
After transmission to a susceptible host, *D. immitis* migrates to the pulmonary arteries and establishes itself. Sometimes, they can also be present in the right ventricle of the heart. The parasite first enters the vascular and pulmonary systems, manifesting early lesions in the walls of the pulmonary arteries and lungs; this plays an important role in the subsequent development of pulmonary and cardiac complications (Martín, et al., 2012). This vascular colonization plays a central role in the pathogenesis of cardiopulmonary complications. The presence of adult nematodes in direct contact with the vascular endothelium leads to progressive alterations in the pulmonary arteries, generating intimal thickening, luminal narrowing, and increased rigidity. These changes result in a loss of arterial elasticity, tortuosity of vessels, and ultimately proliferative pulmonary endarteritis and endothelial damage. Over time, this condition predisposes to chronic pulmonary hypertension and can progress to right-sided heart failure (Carretón et al., 2016).

As the disease advances, the pathophysiological changes begin to manifest clinically. Infected animals often remain asymptomatic or exhibit only mild clinical signs during the early stages of infection. However, once clinical manifestations arise, the condition can become life-threatening if not promptly addressed. Adults of *D. immitis* worms contribute to the inflammatory damage in the lungs, leading to clinical sings such as persistent coughing, dyspnea, exercise intolerance, and in more advanced cases, weight loss, ascites, syncope and occasionally epistaxis (Carretón et al, 2017; Ames et al., 2020).

## 1.2 Life Cycle and Transmission

The parasite's life cycle is indirect, requiring mosquitoes as vectors. The cycle begins when a female mosquito ingests microfilariae (L1) from the blood of an infected microfilaremic dog. Inside the mosquito, the microfilariae develop into second-stage larvae (L2) and subsequently into infective third-stage larvae (L3) over a period of 8 to 19 days, depending on environmental conditions. These L3 larvae migrate to the mosquito’s proboscis, from where they are transmitted to a new host during a subsequent blood meal (Bowman & Atkins, 2009).

**Figure 2.** Illustration of the full life cycle of *Dirofilaria immitis*.




For transmission to be successful, a mosquito carrying infective L3 larvae must feed on a susceptible host. Once L3 are deposited onto the host's skin and enter via the mosquito bite wound, it migrates to the subcutaneous tissue where it molts to L4 in 3-4 days. The environmental development of microfilariae within the mosquito vector is temperature-dependent; as demonstrated by Slocombe et al. (1989), larval development ceases at temperatures below 14ºC, progressing optimally at 27-30ºC.

Afterward, the L4 larvae migrate through muscle fibers and molt into L5 after 45-65 days post infection. These L5 stage larvae enter the venous circulation and are carried via bloodstream until they reach the pulmonary arteries between 90 and 120 days, with the arrival of immature adults (Villanueva et al., 2022). Adult worms predominantly reside in the pulmonary arteries, although in cases of high worm burden, they may extend into the right ventricle (McCall et al., 2008).

By approximately 8 months post-infection, adult females can measure 20 to 30 cm in length, while males are shorter. The average lifespan of adult *D. immitis* worms in dogs is 5 to 7 years, and microfilariae may persist in circulation for up to 30 months (Bowman & Atkins, 2009).

Although transmission occurs primarily via mosquitoes, alternative transmission routes have been documented. Transplacental transmission of microfilariae from infected mothers to their puppies has been documented; however, L1 larvae transmitted this way are not infective. Similarly, blood transfusions from microfilaremic donors may introduce microfilariae into a recipient, but these too lack the capacity to develop further without

the mosquito vector (Mantovani and Jackson, 1966; Brinkmann et al., 1976; Todd & Howland, 1983; Menda, 1989).

Cardiopulmonary dirofilariosis in dogs involves a remarkable case of endosymbiosis in which *D. immitis* and *Wolbachia pipientis* share a phenomenal relationship (Manoj et al., 2021). This gram-negative intracellular bacterium of the family *Rickettsiaceae* is essential in the reproductive and long-term survival of filarial worms (Slatko et al., 2010; Taylor et al., 2013). Although its exact biological function is not yet fully understood, the inhibition of *Wolbachia* population is thought to suppress parasite development and represents a critical target in heartworm disease management (Taylor et al., 2013).

The presence of *Wolbachia* in filarial nematodes was first observed in 1970s, through electron microscopy (Kozek et al., 1977), but the bacteria was not conclusively identified until the 1990s (Sironi et al., 1995). *Wolbachia* is predominantly located in the lateral chords of adult worms, and within the reproductive tract of females, including oocytes, morulae, and microfilariae. This enables vertical transmission of the bacteria to offspring, maintaining the symbiosis across generations (Kramer et al., 2003; Kozek et al 2005).

Hosts are continuously exposed to *Wolbachia* antigens, primarily through secretion from the uterus of adult females and following the death of parasites, whether due to natural causes, host immune reponse, or therapeutic intervention. When adult worms, larvae, or microfilariae die, they release *Wolbachia* into host tissues. This bacterial release contributes significantly to the pathogenesis of heartworm disease (Bazzocchi et al., 2003; Morchón et al., 2007; McCall et al., 2008).

A major factor in the inflammatory response is the *Wolbachia* surface proteins (WSP), which elicits strong innate and adaptative immune activation. The release of WSP stimulates the recruitment of neutrophils and other immune cells, contributing to vascular inflammation and tissue damage (Genchi et al., 2012). In addition, infected animals develop a robust antibody response against WSP (Bazzocchi et al., 2000; Morchón et al., 2004; Kramer et al., 2005a).

The kinetics of anti-WSP antibody production have been evaluated in different host species (e.g., dogs and cats) and appear to vary depending on the number of infective

larvae, the phase of infection, and the host species itself (Bazzocchi et al., 2000; Morchón et al., 2004; Kramer et al., 2005a; Montoya-Alonso et al., 2020). Interestingly, these antibodies can be detected independently of the presence or severity of clinical signs, suggesting a consistent immunological exposure to *Wolbachia* antigens even in subclinical infections (Kramer et al., 2005b).

## 1.3 Epidemiological Relevance

The transmission dynamics of *D. immitis* in Europe are closely influenced by climatic and ecological variables, which directly affect the distribution, density and activity of vectors. Among these factors, temperature and relative humidity are critical parameters for the survival, and maturation of mosquito larvae are indispensable for the development of the infective third-stage larvae (L3) within the vector (Genchi et al., 2005; Kartashev et al., 2011). Warmer temperatures accelerate this development process within mosquitoes and prolong the transmission season, a phenomenon particularly evident in Southern and Mediterranean regions (Simón et al., 2012). Vector such as *Culex pipiens*, *Aedes albopictus*, and *Anopheles maculipennis* exhibit activity patterns that are closely linked to circadian rhythms and environmental cues. While *Culex* and *Anopheles* species are predominant nocturnal, *Aedes* is active during both, which increases the risk of transmission in urban and peri-urban enviroments (Madlock et al., 2012; European Centre for Disease Prevention and Control [ECDC] 2023).

Building on these ecological determinants, the geographic presence of *D. immitis* has expanded markedly in recent decades, with the parasite established itself in regions previously deemed non-endemic. Countries in Eastern and Central European, such as Hungary, Romania, and Serbia, have reported significant increases in autochthonous cases, likely driven by vector adaptation and climate variability (Genchi et al., 2011; Fuehrer et al., 2016). In traditionally endemic areas of Southern Europe, including Italy, Portugal, and Spain, the disease continues to show a rising prevalence, despite increased awareness and the implementation of prophylactic measures (Morchón et al., 2012; Genchi et al., 2020).

In Spain, recent epidemiological surveys estimate a national average prevalence of canine heartworm infection between 6% and 7% with higher values recorded in certain autonomous communities. The Canary Islands and Balearic Islands have consistently

reported prevalence rates above 10%, which is attributed to favorable climatic conditions and the abundance of competent mosquito vectors throughout the year (Montoya-Alonso et al., 2020; Montoya-Alonso et al., 2022). It is worth nothing in the Canary Islands, particularly in Gran Canaria, historical data indicate prevalence levels above 15% in dogs. However, more recent trends suggest a partial reduction in some areas, potentially due to intensified control strategies and veterinary education programs (Montoya-Alonso et al., 2011).

This regional heterogeneity highlights the dynamic nature of heartworm distribution within Spain. However, the detection of new foci, including previously unaffected islands such as Lanzarote, underscores the persistent risk of spread and the necessity of continuous surveillance (Montoya-Alonso et al., 2024). These emerging patterns reinforce the importance of integrating ecological monitoring with targeted public health strategies to effectively manage and mitigate the burden of this vector-borne disease.

## 1.4 Veterinary Relevance and Zoonotic Risk

In addition to its expanding geographic and ecological footprint, *D. immitis* represents a major concern in veterinary medicine due to its insidious clinical progression and the potential for life-threatening cardiopulmonary complications. The chronic nature of the disease often results in delayed diagnosis, as early signs such as mild coughing or fatigue are nonspecific and easy overlooked. As the infection advances, more severe clinical manifestations such as syncope, dyspnea, and signs of right-sided heart failure, tend to emerge, typically reflecting irreversible pulmonary vascular and cardiac damage (McCall et al., 2008; Simón et al., 2012).

From a public health perspective, *D. immitis* is recognized as a zoonotic agent. Although humans are considered aberrant hosts, in whom the parasite rarely reaches adult form, infection may still lead to the development of pulmonary granulomas, which are frequently mistaken for malignant nodules on thoracic imaging (Simón et al., 2005). This highlights the importance of accurate differential diagnosis to avoid unnecessary invasive procedures, such as biopsies or thoracotomies (Genchi et al., 2011).

Zoonotic cases are more likely to occur in endemic areas where mosquito vectors are abundant and control programs are inconsistently applied. The risk of human infection is closely linked to environmental and ecological factors that support mosquito proliferation

including urbanization, climate change, and the presence of untreated canine reservoirs (Fuehrer et al., 2016). Although human infection does not contribute to the parasite's life cycle, its occurrence serves as a sentinel indicator of intense transmission within the local canine population.

In veterinary practice, the significance of heartworm disease extends far beyond the individual patient. It encompasses responsibilities related to epidemiological surveillance, client education, and the implementation of preventive strategies, especially in regions experiencing ecological changes that facilitate vector expansion. In this context, the One Health approach takes on particularly relevance, promoting interdisciplinary collaboration among veterinarians, physicians, ecologists, and public health professionals to address vector-borne disease with zoonotic potential (Simón et al., 2012; Genchi et al., 2020).

## 1.5 Clinical Signs

The clinical presentation of *D. immitis* infection in dogs is highly variable and depends on several factors, including worm burden, duration of infection, host's immune response, and the presence of concurrent cardiopulmonary pathology. While some dogs remain asymptomatic, especially during early stages of infection or in cases with a low parasite load, others may exhibit progressive and potentially life-threatening signs as the disease advances.

Mild to moderate infections are often characterized by nonspecific respiratory signs such as a soft, chronic cough, occasional dyspnea, and exercise intolerance. These clinical features reflect the onset vascular inflammation and the development of mild pulmonary hypertension, which impair oxygen exchange without yet producing sings of overt heart failure (European Society of Dirofilariosis and Angiostrongylosis, 2017; American Heartworm Society, 2024).

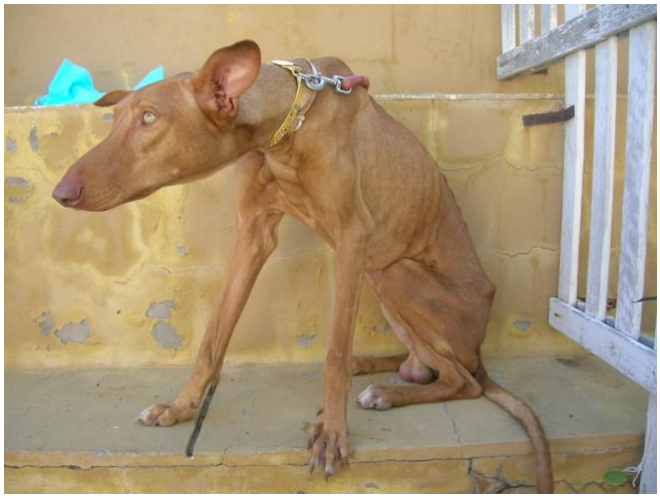


**Figure 3.** Dog infected with *Dirofilaria immitis* prior to receiving treatment showing signs of cachexia, poor body condition, and generalized muscle wasting.

As the disease progresses, especially in cases with moderate to high worm burdens, clinical signs become more pronounced. Affected dogs may present with tachypnea, syncope, decreased activity levels, and frequent harsh cough (McCall et al., 2008). These symptoms are primarily the result of advanced proliferative endarteritis, pulmonary parenchymal injury, and worsening pulmonary hypertension due to chronic vascular compromise (McCall et al., 2008; Simón et al., 2012).

In severe cases, particularly when right-sided heart failure develops, classic signs of congestive heart failure may be seen. These include ascities, hepatomegaly, peripheral edema, and jugular venous distention. In advanced stages, dogs may also present with cyanosis and cachexia. On auscultation, a right-sided systolic murmur may be detected, often due to tricuspid regurgitation, along with splitting of the second heart sound due to delayed closure of the pulmonic valve (McCall et al., 2008; Carretón et al., 2016).

Once of the most acute and dramatic manifestations of heartworm disease is caval syndrome, a rare but often fatal complication characterized by migration of a large number of adult worms into the right atrium and caudal vena cava. This results in mechanical obstruction of venous return severe hemolysis, and cardiogenic shock. Clinical signs include sudden onset of weakness, dyspnea, hemoglobinuria (dark reddish-brown urine), pale mucous membranes, and collapse. Without immediate surgical removal of the worms, the prognosis is extremely poor (Atkins et al., 2001).

Beyond cardiopulmonary signs, systemic effects can develop as the disease becomes chronic. These include weight loss, lethargy, and poor body condition, particularly in

dogs with long-standing infections. Additionally, protein-losing nephropathy may occur as a result of immune-complex glomerulonephritis, manifesting clinically as hypoalbuminemia and peripheral edema (McCall et al., 2008; Venco et al., 2011).

In summary, the clinical spectrum of *D. immitis* infection ranges from subclinical cases to fulminant cardiopulmonary failure, underscoring the importance of early detection, staging and risk stratification to guide appropriate treatment and improve long-term prognosis.

## 1.6 Diagnosis

The diagnosis of *Dirofilaria immitis* infection in dogs requires a multifaceted approach, aiming to assess disease severity and inform therapeutic decisions by combining clinical suspicion, antigen test, microfilaria detection, and diagnostic imaging. Early and accurate identification is essential, as clinical signs may be nonspecific or even absent in the initial stages of infection.

Among available methods, antigen testing remains the cornerstone of diagnosis. Commercially available enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic test detect circulating antigens, primarily derived from mature female worms, and typically become positive 5 to 7 months post-infection. These tests are highly specific, although false-negative results may occur in dogs with low worm burdens, all-male infections, or when immune complexes mask circulating antigens. Heat pretreatment of serum samples has been proposed to dissociate these complexes and thereby improve sensitivity, although the use of this technique remains controversial, as an increase in false-positive results has also been reported (Little et al., 2014; Venco et al., 2017).

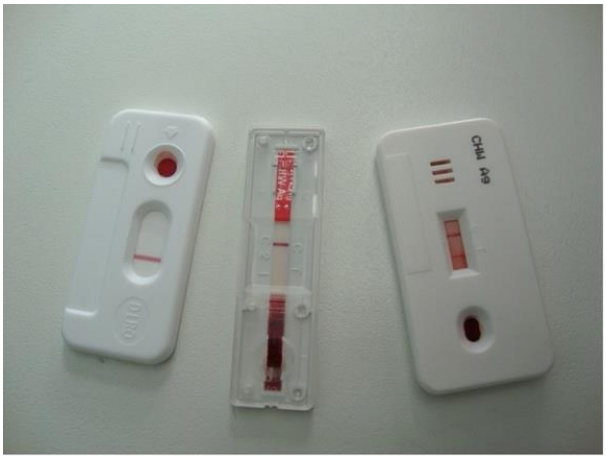


**Figure 4.** Different commercial rapid antigen tests for detecting *Dirofilaria immitis* infection in canine blood samples.

Microfilaria detection is a valuable complementary tool. The modified Knott test is considered the most sensitive technique for detecting circulating microfilariae. However, 20-30% of infected dogs may be amicrofilaremic due to factors such as single-sex infections, host immune responses, or prepatency infections (American Heartworm Society, 2024).

To evaluate the extend of cardiopulmonary involvement, thoracic radiography is indispensable. Common radiographic findings include enlargement of the main pulmonary artery, truncation or tortuosity of peripheral pulmonary arteries and right ventricular enlargement or hypertrophy. Additionally, pulmonary parenchymal infiltrates observed particularly in the caudal lung lobes, may indicate pneumonitis or thromboembolism (Falcón-Cordón et al., 2024).

Echocardiography plays a key role in cases where direct visualization of adult heartworms is possible, especially when worms are located in the right atrium or proximal pulmonary arteries. This imaging modality is especially useful diagnosing caval syndrome, as well as for assessing right heart function, tricuspid valve competence, and the presence of pulmonary hypertension (Vezzosi et al., 2018).

Routine laboratory testing, including complete blood count (CBC) and serum biochemistry may reveal eosinophilia, mild anemia, and elevated liver enzyme activity, particularly in dogs with congestive heart failure or hepatic congestion. Furthermore, urinalysis may uncover proteinuria, which is suggestive of immune-complex-mediated glomerulonephritis (Carretón et al., 2020).

In selected cases, advanced imaging techniques, such as computed tomography angiography, can provide detailed visualization of pulmonary vascular lesions and help evaluate suspected thromboembolic disease. Futhermore, cardiovascular biomarkers, notably NT-proBNP and D-dimer, are gaining importance in staging and prognostication, particularly in dogs suspected of having pulmonary hypertension or cardiac involvement (Vezzosi et al., 2018).

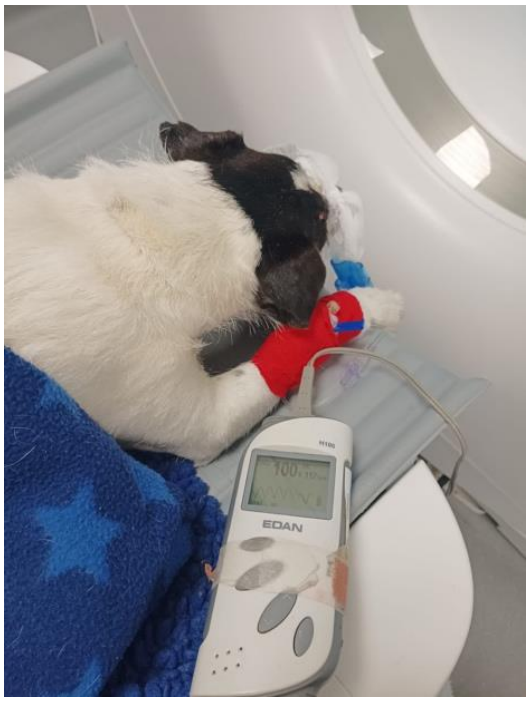



**Figure 5.** Computed tomography scan of a dog diagnosed with *Dirofilaria immitis*.

Ultimately, a comprehensive diagnostic approach that integrates serological, parasitological, imaging, and laboratory findings is essential to confirm infection, determine disease stage, and develop an appropriate treatment plan to the individual patient.

## 1.7 Diagnostic Challenges and the Role of Biomarkers

The diagnosis of *D. immitis* infection continues to pose clinical and epidemiological challenges, particularly in subclinical cases or during the early stages of disease. Conventional diagnostic modalities including antigen detection assays, the modified Knott's test, and thoracic imaging, each offer specific strengths, but also exhibit notable limitations. Antigen tests, widely adopted for their speed and accessibility, rely on the detection of uterine antigens from mature adult females, which may lead to false-negative

results in cases of low parasite burden, single-sex infections, or antigen-antibody complex formation (Little et al., 2014; European Society of Dirofilariosis and Angiostrongylosis, 2017; American Heartworm Society, 2024).

Similarly, the modified Knott test, a concentration technique for detecting circulating microfilariae, offers high sensitivity under ideal conditions. However, it may not identify occult infections, which are increasingly observed in dogs receiving chemoprophylaxis or in those with immune-mediated suppression of microfilaremia (Bowman et al., 2009). Imaging techniques such as thoracic radiography and echocardiography provide valuable structural and functional information; however, they are typically reserved for advanced stages of the disease, and their interpretation requires specialized equipment and clinical experience.

These diagnostic limitations underscore the importance of complementary strategies, especially in endemic areas or in patients with nonspecific or ambiguous clinical signs. In this context, biomarkers have emerged as a promising complement to traditional tools. They allow for the detection of early pathophysiological alterations in the cardiac, pulmonary, or renal systems, often preceding the appearance of detectable antigens or radiographic lesions (Carretón et al., 2012; Nabity et al., 2012).

Among these, NT-proBNP and cardiac troponins have demonstrated clinical utility in detecting myocardial stress or injury, particularly in dogs developing pulmonary hypertension or right-sided heart failure as a consequence of heartworm disease (Kellihan & Stepien, 2010; Oyama & Sisson, 2004). Aditionally, urinary neutrophil gelatinase-associated lipocalin (uNGAL) has proven to be a sensitive marker for early renal tubular injury, a common but often underrecognized complication in advanced heartworm infections (Segev et al., 2008; Palm et al., 2016).

In relation to the earlier discussed concerns, the American Heartworm Society (2024) ascertains the need of a complete clinical evaluation, which includes the patient's history, physical examination, standard laboratory tests, as well as molecular and biomarker analysis. This multi-modal approach improves early detection, prognosis, and enables more individualized therapeutic decision, particularly in high-risk cases.

The incorporation of biomarkers into veterinary practice allows clinicians not only to rely on routine diagnostic findings, but also to follow disease progression over time.
As scientific evidence continues to grow and diagnostic assays become standardized, use of biomarkers for screening, staging, and therapeutic monitoring is likely to increase; yields of such use would benefit patient and public health.

## 1.8 Treatment

Management of *Dirofilaria immitis* infection in dogs is a challenge and should be individualized to the stage of the infection, the presentation disease, and the risk of treatment-associated complications. The therapeutic goals are the eradication of all parasites, management of cardiopulmonary sequelae, and avoidance of complications, specially pulmonary thromboembolism, often seen, where a profound symptomatic portrait can motivate a dangerous consequence.

Treatment typically follows a patient-specific, step-by-step protocol, that combines pharmacological therapy, inflammation control, and, when indicated, surgical intervention. At the core of this therapeutic plan is adulticidal therapy, as recommended by the American Heartworm Society (AHS, 2024): three-dose protocol of melarsomine dihydrochloride administered intramuscularly in the lumbar epaxial muscles at dose of 2.5 mg/kg on days 60, 90, and 91. This regimen optimizes effectiveness while minimizing the risk of developing severe pulmonary complications associated with the death of adult worms.

A stabilization period is essential before the adulticide administration. This includes the administration of doxycycline at 10 mg/kg BID for 28-30 days aimed *Wolbachia spp*. A decrease in bacterial load results in a lower antigenic burden and in a decreased inflammatory response associated to worm death (Bazzocchi et al., 2008). Simultaneously, after a diagnosis is made, monthly preventive treatment with macrocyclic lactones (e.g., ivermectin, milbemycin oxime) are initiated to eliminate early larval stages (L3 and L4) and to prevent reinfection. Although prolonged use of macrocyclic lactones with doxycycline may result in slow-kill effects on adult worms, this approach is discouraged due to prolonged disease progression and the risk of drug resistance (McCall et al., 2014).

Anti-inflammatory therapy with glucocorticoids (e.g., prednisone 0.5–1 mg/kg/day for one week, followed by tapering) is frequently indicated to reduce pulmonary inflammation, especially in dogs exhibiting clinical or radiographic signs of pneumonitis or pulmonary hypertension. Additional supportive therapies may include bronchodilators, diuretics, vasodilators, oxygen supplementation, or inotropes, depending on the patient's cardiovascular status (Buriko et al., 2025)

Strict exercise restriction is a critical component throughout the treatment course. Increased physical activity raises pulmonary blood flow, thereby heightening the risk of embolization from dead worm fragments. Dogs should remain on restricted activity from the time of diagnosis and for at least 4-6 weeks after the final melarsomine injection (European Society of Dirofilariosis and Angiostrongylosis, 2017; American Heartworm Society, 2024).

## Treatment of Pulmonary Thromboembolism

Pulmonary thromboembolism is a frequent and potentially letal complication, especially during or after adulticide treatment. It occurs when dead worms or thrombotic debris embolize into the pulmonary vasculature, provoking acute inflammation, hypoxia, and hemodynamic compromise (European Society of Dirofilariosis and Angiostrongylosis, 2017; American Heartworm Society, 2024).

Clinical manifestations of pulmonary thromboembolism include acute dyspnea, tachypnea, cyanosis, hemoptysis, syncope, and in some cases, sudden death. Diagnosis is based on clinical suspicion, a recent history of adulticide administration, and supportive findings, thoracic radiographs and/or elevated D-dimer concentrations (McCall et al., 2008; Carretón et al., 2017).

Management of pulmonary thromboembolism is primarily supportive, and may include:

- Oxygen therapy to alleviate hypoxia.
- Corticosteroids (e.g., dexamethasone 0.1–0.2 mg/kg IV) to reduce pulmonary edema and inflammation.

- Anticoagulants (low molecular weight heparin) or antiplatelet agents (clopidogrel) in severe or recurrent cases, although their use in veterinary medicine must be carefully evaluated on a case-by-case basis.
- Intravenous fluids, administered judiciously to avoid volume overload, especially in dogs with right-sided heart failure.
- Analgesics or sedatives, which may be required in animals experiencing distress.

Prognosis depends on the extent of embolism and the dog's cardiopulmonary reserve. Mild cases can improve conservatively but at the same time, massive embolisms can result in sudden death despite treatment.

## Surgical Treatment: Caval Syndrome

Caval syndrome is a life-threatening, acute complication caused by adult worms migrating into the right atrium and caudal vena cava, which results in not only the obstruction of venous return, but also severe hemolysis, cardiogenic shock, and multiorgan dysfunction. Signs are those of acute collapse, with signs of hemoglobinuria, pale mucous membranes, jugular venous distension, and rapid clinical decline. Without immediate surgical intervention, death commonly occurs within 48–72 hours (Atkins et al., 1987; Hidaka et al., 2003).

Only emergency surgical removal of the worms through jugular venotomy has been proven to treat caval syndrome. It is typically performed under echocardiographic or fluoroscopic guidance using specialized devices such as flexible alligator forceps or basket catheters, as previously described (Yoon et al., 2011). Successful treatment results in normalization of hemodynamics and decrease in intravascular hemolysis.

The therapy is based on fluid therapy, blood transfusion, corticosteroids and oxygen therapy in the postoperative period. Following stabilization of the dog, adulticide treatment should be completed to eliminate any remaining worms within the pulmonary vessels. This carefully staged therapeutic approach is essential not only to improve prognosis and prevent fatal complications, but also to preserve long-term cardiopulmonary function in affected dogs.

## 1.9 Prophylaxis

Prophylaxis is a cornerstone in the control of *D. immitis* infection and the prevention of its potentially life-threatening consequences. Given the complexity and significant risks associated with adulticide treatment -such as pulmonary thromboembolism and caval syndrome- preventive strategies are widely regarded as the most effective and cost-efficient approach to managing heartworm disease.

Preventive protocols are designed to eliminate infective third-stage larvae (L3) transmitted by mosquitoes before they mature into adult worms. This goal is primarily achieved through the regular administration of macrocyclic lactones, which are highly effective in killing L3 and early L4 larval stages within 30–45 days post-infection (Noack et al., 2021). These agents include:

- Ivermectin (oral, monthly)
- Milbemycin oxime (oral, monthly)
- Moxidectin (oral, monthly; topical monthly; or injectable sustained-release)
- Selamectin (topical, monthly)

Monthly administration is critical, as macrocyclic lactones are ineffective against later-stage larvae and adult worms once development has progressed beyond a specific threshold (McCall et al., 2005). Therefore, strict compliance and consistency in administration are essential to ensure efficacy.

Whilst it is not a standard prophylaxis regimen, doxycycline prophylaxis has been considered, particularly if in an endemic area or in at-risk animals with repeated exposure (American Heartworm Society, 2024). If associated with macrocyclic lactones, doxycycline might beneficially reduce *Wolbachia* load and interfere with development and transmission potential of larvae (Kramer et al., 2018). However, the latter method is still being studied and is not commonly used as a preventive measure.

In addition to pharmacological prevention, environmental control of mosquito populations and reduction of vector exposure serve as valuable complementary strategies, especially in endemic regions. Recommended measures include:

- Minimizing outdoor activity during peak mosquito hours (dawn and dusk).
- Applying mosquito repellents and using insecticide-treated environments (when safe for dogs).
- Eliminating standing water to reduce breeding sites.

Routine screening is another key intervention in preventive programs. Even in dogs consistently receiving prophylaxis, annual antigen testing is recommended, as no preventive method is entirely fail-proof (European Society of Dirofilariosis and Angiostrongylosis, 2017). Early identification of breakthrough infections enables timely intervention before severe disease manifests.

In puppies, prophylactic treatment should begin at 6–8 weeks of age, regardless of prior exposure history, and should continue year-round throughout life in endemic areas. For adult dogs with unknown or inconsistent preventive histories, heartworm testing is essential before initiating prophylaxis, in order to avoid complications from undiagnosed pre-existing infections (American Heartworm Society, 2024).

In addition, to reduce the risk of infection and the possibility of environmental amplification of the parasite, in multi-dog households or shelters adoption of population level preventive measures is crucial. Finally, prevention, in addition to protecting individual dogs, is essential to reduce the overall prevalence of heartworm infection in dog populations.

## 1.10 Prognosis

The prognosis of *D. immitis* infection in dogs varies widely and is influenced by multiple factors, including the parasite burden, duration of infection, the degree of cardiopulmonary involvement, presence of complications such as pulmonary hypertension or thromboembolism, and the timeliness and appropriateness of therapeutic intervention (McCall et al., 2008).

In asymptomatic or mildly affected dogs, (usually in dogs with few worms and minimal vascular or parenchymal changes), prognosis is generally favorable, particularly if a prompt diagnosis is followed by treatment in accordance to currently available and

suggested guidelines (European Society of Dirofilariosis and Angiostrongylosis, 2017; American Heartworm Society, 2024). The majority of these patients fully recover with minimal residual cardiovascular compromise, as long as complete exercise restriction is enforced during the course treatment (Simón et al., 2012).

In contrast, dogs with moderate to severe disease, characterized by radiographic evidence of vascular lesions, right-sided heart enlargement, or clinical signs such as coughing, tachypnea, or exercise intolerance, have a more guarded prognosis. Although many of these animals respond well to a complete adulticide protocol combined with supportive care, they may develop long-term sequelae, including persistent pulmonary hypertension, reduced exercise capacity, or chronic pulmonary fibrosis (McCall et al., 2008).

Prognosis is significantly worse in complicated cases, particularly those involving pulmonary thromboembolism, right-sided heart failure, or caval syndrome. In such scenarios, the risk of sudden deterioration or death increases substantially, especially when diagnosis is delayed, or treatment is incomplete. Even with aggressive medical and surgical intervention, full recovery is not guaranteed, and residual cardiopulmonary compromise may persist (American Heartworm Society, 2024).

The development of caval syndrome is one of the most important prognostic factors. Survival is nearly impossible without surgical removal of the intravascular worms. Even with intervention, the mortality rate is substantial, especially in patients who are in hemodynamic shock or advanced organ dysfunction.

The long-term prognosis is also determined by follow-up and preventive treatment. Regular follow-up evaluations, including antigen testing and imaging when appropiate, are necessary for verification of adult worm clearance and for cardiopulmonary follow up. Dogs that successfully respond to treatment and start lifelong prophylaxis can return to a good quality of life; but many will need to manage several sequelae in the long term. Finally, prognosis is dramatically increased by averting infection and prophylaxis and regular screening remain the cornerstone of clinical practice.

# 2. INVASION AND LESIONS CAUSED BY THE PARASITE

## 2.1. Vascular Invasion and Endoarteritis

Once adult *Dirofilaria immitis* worms establish themselves in the pulmonary arteries, they induce a chronic, progressive proliferative endarteritis. This vascular lesion results from a combination of mechanical irritation caused by the presence and movement of the worms, the release of parasite-derived antigens, and the immunostimulatory effects of the endosymbiotic bacteria *Wolbachia spp*. These symbionts contribute to endothelial activation and significantly amplify the local inflammatory response, acting as potent adjuvants that enhance host immune reactivity (Furlanello et al., 1998; Morchón et al., 2004; Kramer et al., 2008).

The vascular endothelium suffers substantial damage, as evidenced by histopathological findings such as medial smooth muscle hypertrophy, intimal proliferation with myofibroblast activation and extensive perivascular and transmural inflammatory infiltrates composed predominantly of eosinophils, macrophages, and lymphocytes. Locally released inflammatory cytokines and reactive oxygen species further contribute to endothelial dysfunction and progressive fibrosis (Rawlings, 1982; Venco et al., 2014). Over time, these pathological changes lead to a loss of vascular compliance, increased pulmonary vascular resistance, and impaired oxygen exchange due to ventilation-perfusion mismatch.

In addition, endothelial injury facilitates platelet aggregation and the formation of microthrombi, predisposing affected dogs to pulmonary thromboembolism, which is a common and potentially life-threatening complication of heartworm disease. The chronic nature of endarteritis also promotes long-term remodeling of the pulmonary vasculature, thereby creating the conditions for the development of pulmonary hypertension and, eventually, right-sided heart failure (Atkins et al., 2001; Nelson & Couto, 2014).

As a whole, they constitute a key pathogenic mechanism of the cardiopulmonary disease induced by dirofilariosis and emphasizes the relevance of an early diagnosis and an effective treatment for mitigate irreversible lesions.

## 2.2. Pulmonary Hypertension and Right Ventricular Remodeling

Sustained vascular injury caused by the chronic presence of *D. immitis* within the pulmonary arteries inevitably leads to the development of pulmonary hypertension, a key pathophysiological consequence of canine heartworm disease. The progression of proliferative endarteritis, combined with vascular occlusion by adult worms and the formation of microthrombi, results in elevated pulmonary arterial pressure due to increased vascular resistance and impaired pulmonary perfusion (Calvert et al., 1999; Venco et al., 2014).

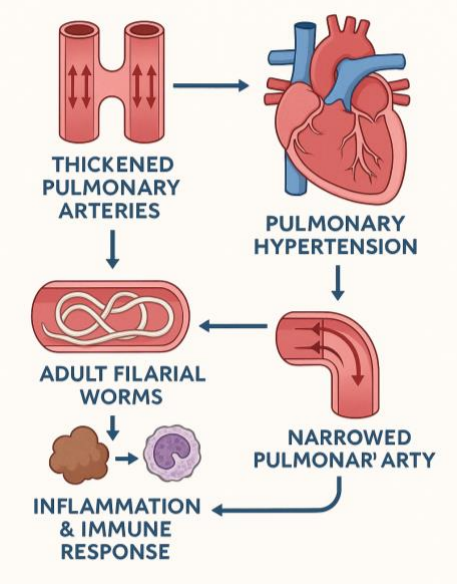



**Figure 6.** Diagram of the pathophysiology of pulmonary hypertension secondary to *Dirofilaria immitis* infection.

This chronic increase in afterload imposes a significant load on the right ventricle, leading to a compensatory remodeling response. It initially presents as concentric hypertrophy, but with disease progression, it transitions to right ventricle dilation and systolic dysfunction. Frequent echocardiographic findings in affected dogs consist of a thickened right ventricular wall, flattening or paradoxical septal motion and dilatation of the main pulmonary artery (Atkins et al., 2001; Vezzosi et al., 2018). Furthermore, secondary tricuspid regurgitation can be due to annular dilation, which adds to right-sided volume overload.

From a clinical standpoint, dogs with pulmonary hypertension often exhibit nonspecific yet progressively worsening signs such as tachypnea, exercise intolerance and syncope. As the condition advances, signs of right-sided congestive heart failure may develop, including ascites, hepatomegaly, and jugular vein distension. In severe cases, pulmonary

hypertension may culminate in cor pulmonale, a terminal stage of right ventricular failure resulting from chronic pulmonary disease (Nelson & Couto, 2014).

Importantly, the presence of pulmonary hypertension constitutes a major clinical risk factor during adulticide therapy. The sudden death of adult worms following treatment can precipitate acute pulmonary thromboembolism, which, when superimposed on pre-existing pulmonary hypertension, may lead to acute decompensation and fatal cardiovascular collapse (European Society of Dirofilariosis and Angiostrongylosis, 2017; American Heartworm Society, 2024). Consequently, through assessment and appropriate management of pulmonary hypertension are essential elements of therapeutic planning in dogs infected with heartworm.

## 2.3. Pulmonary Parenchymal Injury

The pulmonary parenchyma undergoes both direct and indirect injury as a result of *D. immitis* infection. Although primary lesions are localized within the pulmonary arteries, the adjacent lung tissue is inevitably affected due to the chronic inflammatory milieu, vascular compromise, and immune-mediated processes triggered by the parasite and its endosymbiotic bacteria, *Wolbachia spp.* (Kramer et al., 2011).

Inflammatory infiltrates are frequently observed surrounding terminal arterioles and small-caliber vessels, often accompanied by perivascular edema and multifocal alveolar hemorrhage (McCall et al., 2008). This inflammation arises not only from mechanical damage but also from immune responses directed against parasite antigens and *Wolbachia*-derived surface proteins. These proteins, especially abundant during worm death or molting, act as a pathogen-associated molecular patterns (PAMPs) that activate toll-like receptors on host immune cells, thereby inducing the release of pro-inflammatory cytokines and recruitment of leukocytes (Taylor et al., 2005).

Histologically, pneumonitis is characterized by mononuclear and eosinophilic infiltration, thickening of alveolar septa, and hyperplasia of type II pneumocytes. If the inflammatory process persists without proper resolution, it can lead to interstitial fibrosis, a condition that significantly impairs pulmonary compliance and gas exchange (Venco et al., 2011). The resulting fibrotic remodeling reduces oxygen diffusion capacity and

contributes to systemic hypoxia, thereby exacerbating pulmonary hypertension and right ventricular overload.

Moreover, recurrent microembolic events, stemming from worm fragments, immune complexes or thrombotic material, can induce aeas of parenchymal ischemia, pulmonary infarction, and localized necrosis. Over time, these lesions may envolve into chronic fibrotic nodules, further compromising lung function (Rawlings et al., 1993).

Altogether, these structural and functional alterations of the pulmonary parenchyma add to the hemodynamic burden imposed by vascular lesions, playing a pivotal role in the pathogenesis of respiratory compromise associated with canine heartworm disease.

## 2.4. Systemic Consequences

Systemic effects are common with severe or chronic *D. immitis* infection, although the primary pathology is confined to the cardiopulmonary system. These factors are due to a magnitude of chronic inflammation, immune complex deposition, hypoxia, and hemodynamic derangements.

One of the most prominent systemic manifestations is chronic inflammatory and immune-mediated disease, driven by continuous antigenic stimulation from both the parasite and its endosymbiont *Wolbachia*. Circulating immune complexes can deposit in distant organs, most notably the kidneys, resulting in membranous or membranoproliferative glomerulonephritis. Proteinuria, azotemia, and progressive renal dysfunction have been documented even in the absence of overt clinical signs of renal failure (McCall et al., 2008).

Hepatic congestion is an additional frequent complication that occurs secondary to right heart failure. Increased central venous pressure results in passive hepatic congestion, centrilobular sinusoidal dilation, and, in chronic phases, hepatic fibrosis. Dogs affected with congestive hepatopathy show clinical abnormalities of hepatocellular enzymes and functions (Álvarez et al., 2011).

Additionally, hypoxia-related organ dysfunction may develop due to impaired pulmonary gas exchange. Organs with high metabolic demands, such as the brain and skeletal muscles, are especially vulnerable. This can manifest as lethargy, exercise intolerance, and in advanced cases, neurologic signs such as disorientation or syncope (Bhutta et al., 2024).

Heavy worm burdens also can cause hemolysis and anemia in dogs, especially in those with caval syndrome, that are related to the mechanical destruction of erythrocytes by worms in the right atrium and vena cava. Clinical indicators are acute anemia, hemoglobinuria, and icterus, suggesting a life-threatening situation, which can be fatal without any immediate therapy (Atkins et al., 2001).

In addition, there may be a systemic pro-inflammatory state associated with heartworm disease that may predispose affected dogs to coagulopathies, such as disseminated intravascular coagulation. This risk is particularly elevated during adulticide treatment or after abrupt death of many worms. Accordingly, an accurate assessment of disease severity, and stabilization of the patient if same is required, is crucial before treatment is initiated so the likelihood of complications is minimized (American Heartworm Society, 2024).

In conclusion, the systemic consequences of heartworm disease reflect the multifactorial impact of chronic parasitism, immunopathology, and cardiopulmonary dysfunction. These complex interactions necessitate a comprehensive diagnostic and therapeutic approach to optimize clinical outcomes.

# 3. BIOMARKERS IN CANINE CARDIOPULMONARY DISEASE

Biomarkers have emerged as pivotal tools in the early detection, staging, and prognostic assessment of cardiopulmonary diseases in dogs. As molecular indicators of physiological or pathological states, they can enhance diagnostic precision, especially in complex clinical scenarios such as heartworm-associated pulmonary hypertension. In canine medicine, their integration into clinical practice supports objective monitoring of disease evolution and therapeutic efficacy, often complementing imaging modalities and clinical scoring systems (Oyama & Sisson, 2004; Nabity et al., 2012). Biomarkers provide measurable, reproducible, and often cost-effective means of evaluating disease status. Unlike conventional diagnostics, which may be limited by interobserver variability or procedural invasiveness, biomarkers enable serial monitoring with minimal stress to the patient. This makes them especially valuable in chronic conditions such as cardiopulmonary dirofilariosis, where disease progression and therapeutic response must be assessed longitudinally (Atkins et al., 2009; Carretón et al., 2013).

## 3.1. Rationale and Classification

Biomarkers are defined as any quantifiable molecular evidence to indicate normal biologic processes, pathological changes, or responses to therapeutic interventions. In the context of veterinary cardiology, they have been invaluable in the identification of subclinical alterations, monitoring disease burden, and guiding clinical decision-making (Segev et al., 2008; Nabity et al., 2012).

The canine biomarkers for cardiopulmonary pathology and their classification are based on pathophysiological relevance and diagnostic applications:

- Markers of myocardial injury: The cardiac-specific troponins, especially cTnI and cTnT, are released in the peripheral blood as result of cardiomyocyte damage. Increased levels are observed in myocardial infarction, myocarditis, and any type of cardiac injury as chronic heartworm disease (Winter et al., 2017).
- Hemodynamic stress markers: B-type natriuretic peptide (BNP) and its inactive fragment N-terminal pro-B-type natriuretic peptide (NT-proBNP) are produced due to ventricular wall stretching and pressure overload. These markers are instrumental in assessing congestive heart failure and pulmonary hypertension,

for prognostic information and for distinguishing between cardiac and respiratory etiologies of dyspnea (Oyama & Sisson, 2004; Kellihan et al., 2011).

- Thromboembolic markers**:** D-dimer, a degradation product of fibrin, is a sensitive, but non-specific, indicator of thrombus formation and degradation. It is especially useful in diseases suspected of pulmonary embolism as that can be associated with the disease condition such as severe cases of heartworm disease with associated thrombosis (Goggs et al., 2014).
- Renal injury markers**:** Urinary neutrophil gelatinase-associated lipocalin (uNGAL) is a marker of tubular damage and an early, sensitive biomarker of AKI. Since renal dysfunction frequently accompanies right-sided heart failure and pulmonary hypertension in dogs, uNGAL could provide useful information on cardiorenal interactions (Segev et al., 2008; Palm et al., 2016).

## 3.2 Physiopathological Basis of Biomarkers in Canine Cardiopulmonary Disease

From a pathophysiological perspective, each biomarker class provides molecular evidence of organ dysfunction that directly reflects disease mechanisms:

**Figure 7.** Infographic of the key biomarker categories used in the diagnosis and monitoring of canine heartworm disease

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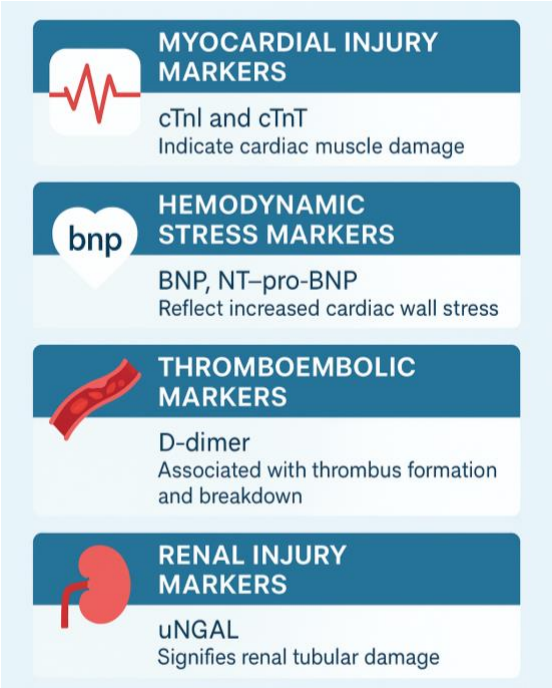

## Myocardial Injury Markers (Cardiac troponins I and T)

Cardiac troponins I (cTnI) and T (cTnT) are regulatory proteins located in the contractile cardiomyocyte myofibrils. They have an essential function in calcium-mediated actin-myosin interactions in cardiac contraction. Under normal physiological conditions, troponins are intracellular and undetectable in circulation. In physiological conditions, troponins are intracellular and are not present in the circulation. However, when the myocardial cell membrane integrity is compromised troponins are released systemic circulation (Oyama & Sisson, 2004).

Cardiomyocyte damage associated with heartworm disease is typically due to chronic right heart pressure overload, inflammation, or embolic damage. The right ventricular myocardium is susceptible to ischemic or mechanical stress, especially when pulmonary hypertension has been established, leading to leakage of troponins into the circulation (Carretón et al., 2017). Elevated levels of cTnI or cTnT in this context indicate ongoing myocardial cell damage, making them essential for detecting silent or early-stage cardiac compromise.

## Hemodynamic Stress Markers (BNP and NT-proBNP)

BNP and NT-proBNP are natriuretic peptides synthesized and stored in the ventricles, especially the myocardial cells of the right ventricle in dogs with pulmonary pathology. Their release is triggered by myocardial wall stretch, often caused by increased intracardiac pressure or volume overload (Oyama & Singletary). Once released, BNP exerts physiological effects such as vasodilation, natriuresis, and inhibition of the renin-angiotensin-aldosterone system, all aimed at reducing cardiac workload (Fu et al., 2018).

In heartworm disease, progressive obstruction of pulmonary arteries increases pulmonary vascular resistance, forcing the right ventricle to work harder. The resulting pressure overload leads to ventricular remodeling and hypertrophy, both of which stimulate the release of NT-proBNP. Thus, elevated NT-proBNP values are physiological evidence of right ventricular strain, reflecting the compensatory mechanisms of the heart under chronic pressure. It's especially helpful in distinguishing cardiac versus pulmonary causes of clinical signs like coughing or exercise intolerance (Oyama et al., 2009).

## Thromboembolic Markers (D-dimer)

D-dimer is a fibrin degradation product, specifically generated when cross-linked fibrin clots are broken down by plasmin. Its presence in circulation is evidence of active fibrinolysis, which typically follows thrombus formation (Nelson & Andreasen, 2003). In diseases involving vascular injury or stasis—both of which occur in heartworm disease—thromboembolic events are common (Carretón et al., 2017).

In particular, adult *D. immitis* can cause endothelial trauma, turbulent flow, and vascular inflammation, which predispose to in-situ thrombus formation in the pulmonary arteries. These events often trigger secondary fibrinolysis, leading to elevated D-dimer levels (Carretón et al., 2013). While not specific, D-dimer can support the suspicion of pulmonary thromboembolism and serves as a valuable rule-out test, potentially guiding clinicians toward additional imaging such as angiography or echocardiography (Nelson & Andreasen, 2003).

## Renal Injury Markers (uNGAL)

Urinary NGAL is a small glycoprotein produced by renal tubular epithelial cells in response to ischemic, inflammatory, or toxic injury. In normal physiology, NGAL is minimally expressed, but when renal tubules are damaged, NGAL is quickly induced and successfully excreted in the urine. This makes it one of the earliest biomarkers for acute kidney injury —often detectable before changes in creatinine or urea (Palm et al., 2016; Nabity et al., 2015).

Renal perfusion is also compromised in dogs with heartworm disease or right-sided heart failure, likely due to low cardiac output, venous congestion, or systemic inflammation. These mechanisms contribute to a type I or II cardiorenal syndrome, in which cardiac dysfunction precipitates renal injury (Pouchelon et al., 2015). By identifying early tubular injury, uNGAL provides a window of opportunity for therapeutic intervention before overt azotemia develops. Additionally, it helps in monitoring nephrotoxic effects of treatments such as adulticidal drugs or diuretics (Segev et al., 2008).

These biomarkers vary in sensitivity and specificity; however, being non-invasive and possibly being used to perform serial measurements makes them particularly

advantageous in veterinary practice (Oyama & Singletary, 2010; Segev et al., 2008). When used in combination with clinical assessment and imaging, as well as functional tests, they provide a comprehensive approach toward disease processes and guide evidence-based clinical decisions (Palm et al., 2016). The following subsections provide a focused evaluation of two biomarkers of particular clinical interest in canine cardiopulmonary dirofilariosis: NT-proBNP and uNGAL.

## 3.3 NT-proBNP in Pulmonary Hypertension

Building upon the physiological framework previously described, NT-proBNP represents a cornerstone biomarker for detecting early myocardial stress in canine cardiopulmonary disease. It is the inactive amino-terminal fragment of the prohormone released from cardiac myocytes in response to increased myocardial wall tension, particularly in conditions involving pressure overload or volume expansion (Yamamura et al., 2009). In canine medicine, NT-proBNP has emerged as a reliable biomarker for assessing cardiac strain, especially in diseases affecting the right heart and pulmonary circulation such as pulmonary hypertension secondary to *D. immitis* infection.

Heartworm disease results in progressive damage to the pulmonary vasculature, including endarteritis, perivascular inflammation, and thrombosis, which in turn leads to increases in pulmonary vascular resistance and pulmonary artery pressures (Atkins et al., 2009; Simón et al., 2012). The right ventricle struggles to maintain pulmonary perfusion, which causes compensatory hypertrophy and dilation, being the principal stimuli for NT-proBNP secretion (Gan et al., 2006). Elevated NT-proBNP concentrations, therefore, are used as a surrogate marker of right ventricular load and dysfunction in affected dogs (Kellihan & Stepien, 2010).

Numerous studies have confirmed the diagnostic and prognostic utility of NT-proBNP in dogs with pulmonary hypertension. Kellihan et al. (2011) demonstrated that NT-proBNP levels were significantly higher in dogs with moderate to severe pulmonary hypertension compared to healthy controls, and that values correlated positively with echocardiographic estimates of pulmonary arterial pressure. In heartworm-infected dogs, Carretón et al. (2013) showed that NT-proBNP concentrations increased proportionally

with disease severity and were significantly associated with radiographic and echocardiographic findings of right heart enlargement.

Moreover, NT-proBNP has shown value in detecting early myocardial involvement before clinical signs become evident. This makes it a powerful tool for screening and risk stratification, particularly in endemic areas where heartworm disease may be underdiagnosed until advanced stages. It also assists clinicians in distinguishing cardiac from respiratory etiologies of dyspnea, a frequent diagnostic challenge in canine patients (Oyama & Sisson, 2004).

The biomarker's stability in plasma, availability of validated canine-specific assays, and non-invasive collection protocols render NT-proBNP a practical option for serial monitoring. It can be used to evaluate therapeutic response, detect disease progression, and support decisions regarding the initiation or adjustment of pharmacologic interventions such as sildenafil or pimobendan in management of pulmonary hypertension (Guglielmini et al., 2010; Kellihan & Stepien, 2010).

Nevertheless, clinicians must interpret NT-proBNP values to make clinical judgements. Factors such as age, body condition, renal function, and concurrent cardiac disease can influence peptide concentrations (Boswood et al., 2016). Therefore, combination with echocardiography and clinical scoring maximize diagnostic accuracy.

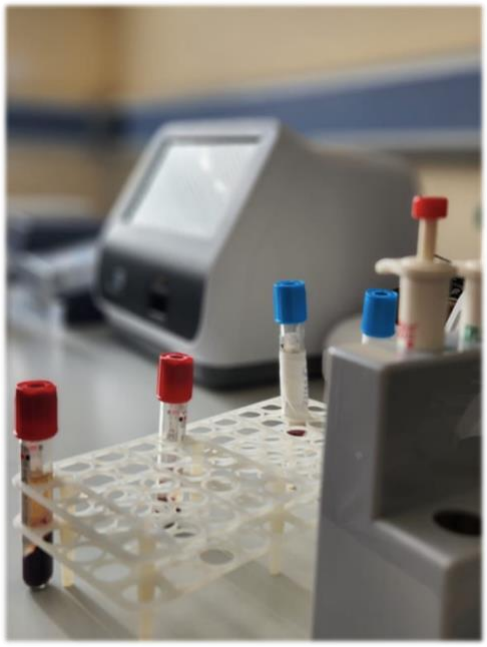

**Figure 8.** Measurement of NT-proBNP and D-dimer concentrations in the Animal Pathology Laboratory at ULPGC.

## 3.4 uNGAL as a Marker of Cardiorenal Interaction

The interplay between cardiac and renal dysfunction, termed cardiorenal syndrome, is increasingly recognized in both human and veterinary medicine. In dogs with cardiopulmonary disorders such as heartworm-induced pulmonary hypertension, renal perfusion may be compromised due to low cardiac output, increased venous pressure, and systemic inflammation. Identifying early renal impairment in this context is critical, as it worsens prognosis and complicates treatment (Segev et al., 2008; Palm et al., 2016).

Urinary neutrophil gelatinase-associated lipocalin (uNGAL), a 25-kDa protein, is expressed at low levels in normal renal tissue, but it is highly upregulated in response to tubular injury. In contrast to classical renal markers like creatinine or urea, which only increase significantly after loss of the substantial part of the nephron mass, uNGAL levels increases rapidly in response to tubular injury, demonstrating high sensitivity for early acute kidney injury (Mishra et al., 2003; Palm et al., 2016).

In the context of heartworm disease and pulmonary hypertension, uNGAL may be useful in identifying the presence of subclinical renal damage associated with chronic venous congestion and systemic hypoperfusion. Based on preliminary studies in canine patients, elevated uNGAL concentrations may occur in dogs with right-sided heart failure, even without the presence of announced azotemia (Segev et al., 2008). This aligns with human medicine findings, where uNGAL serves as a marker of poor outcome in patients with decompensated heart failure (Damman et al., 2014).

Furthermore, uNGAL has been investigated in combination with cardiac biomarkers such as NT-proBNP to characterize the multifactorial pathophysiology of cardiorenal syndrome. Concomitant elevations in both markers might suggest a higher risk profile, warranting closer monitoring and potentially aggressive treatment strategies (Palm et al., 2016).

An additional strength of uNGAL is the feasibility of serial measurements through urine sampling, reducing the need for repeated venipuncture and the lack of compliance seen in long-term monitoring. Despite these benefits, the application in veterinary practice

remains relatively limited, and further research is needed to establish reference ranges, standardize assay methodologies, and validate its prognostic value in cardiopulmonary diseases like heartworm-associated pulmonary hypertension.

In summary, uNGAL may be a potential tool in the early detection of renal component in canine cardiopulmonary disease. Its incorporation into diagnostic panels may contribute to the refinement of clinical decision-making and improve outcomes by identifiying patients at risk for progressive renal compromise in the context of advanced heart disease.

# 4. OBJECTIVES // OBJETIVOS

**4.1** Serum concentrations of N-terminal pro B-type natriuretic peptide (NT-proBNP) may reflect cardiovascular alterations associated with pulmonary diseases. In this context, its usefulness was proposed as a diagnostic and prognostic tool for detecting precapillary pulmonary hypertension in dogs naturally infected with *Dirofilaria immitis*, particularly in advanced clinical stages.

**4.1** Las concentraciones séricas del péptido natriurético tipo B N-terminal (NT-proBNP) pueden reflejar alteraciones cardiovasculares asociadas a enfermedades pulmonares. En este contexto, se propuso evaluar su utilidad como herramienta diagnóstica y pronóstica para la detección de hipertensión pulmonar precapilar en perros con infección natural por *Dirofilaria immitis*, especialmente en fases clínicas avanzadas.

**4.2** Pulmonary hypertension induced by *Dirofilaria immitis* may evolve during adulticide treatment, showing significant clinical and hemodynamic variations. The dynamics of NT-proBNP were proposed for analysis as a non-invasive biomarker for monitoring this condition, in relation to parasite burden, clinical signs, and echocardiographic severity.

**4.2** La hipertensión pulmonar inducida por *Dirofilaria immitis* puede evolucionar a lo largo del tratamiento adulticida, con variaciones clínicas y hemodinámicas significativas. Se planteó analizar la dinámica del NT-proBNP como biomarcador no invasivo para el seguimiento de esta condición, en relación con la carga parasitaria, los signos clínicos y la gravedad ecocardiográfica.

**4.3** Early detection of renal dysfunction is essential in chronic or systemic processes. In this regard, urinary neutrophil gelatinase-associated lipocalin (uNGAL) was proposed for evaluation as an early biomarker of kidney injury in dogs infected with *Dirofilaria immitis*, with or without pulmonary hypertension, even when conventional renal parameters remain within normal ranges.

**4.3** La detección precoz de la disfunción renal resulta esencial en procesos crónicos o sistémicos. En este sentido, se propuso evaluar la lipocalina asociada a gelatinasa de neutrófilos urinaria (uNGAL) como biomarcador temprano de daño renal en perros infectados con *Dirofilaria immitis*, con o sin hipertensión pulmonar, incluso en ausencia de alteraciones en los parámetros renales convencionales.

# 5. SCIENTIFIC PUBLICATIONS // PUBLICACIONES CIENTÍFICAS

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# Usefulness of NT-proBNP in dogs with heartworm: could this biomarker be useful to evaluate pulmonary hypertension?

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## Abstract

**Background** In recent years, the usefulness of echocardiography and serum biomarkers in the diagnosis of pulmonary hypertension (PH) in dogs with heartworm disease has been studied. Previously, N-terminal pro B-type natriuretic peptide (NT-proBNP) has shown high concentrations in dogs with heart disease and/or PH as well as its usefulness as a prognostic indicator, but it has never been evaluated in the diagnosis and prognosis of PH in dogs with heartworm disease. The aim was to evaluate the serum concentrations of NT-proBNP in dogs infected by *Dirofilaria immitis* to determine its usefulness as a tool to detect precapillary PH.

**Methods** NT-proBNP was determined in 50 heartworm-infected dogs. Presence/absence of PH was determined echocardiographically, using the Right Pulmonary Artery Distensibility Index (RPAD Index) and the systolic flow of tricuspid regurgitation mainly, together with other echocardiographic measurements following the guidelines of the American College of Veterinary Internal Medicine (ACVIM) for the diagnosis of PH. Other epidemiological parameters (breed, age, sex, status: client-owned or shelter dogs) and clinical parameters (microfilaremia, parasite burden, presence of symptoms, body condition) were collected as well.

**Results** Moderate-severe PH was present in 40% of the dogs (RPAD Index < 29.5%), NT-proBNP concentrations being significantly higher compared with dogs that did not have PH. A cutoff for NT-proBNP of ≥ 1178.45 pmol/l showed a sensitivity of 64.3% and a specificity of 95.5% for the presence of moderate-severe PH. Older dogs and dogs from shelters showed significantly higher NT-proBNP concentrations. Dogs with symptoms and low body condition presented significantly higher NT-proBNP concentrations as well.

**Conclusions** The determination of NT-pro-BNP concentration can be a useful tool in the diagnostic work-up of dogs with heartworm disease and associated PH and can help to identify animals in the more advanced stage of this disorder.

**Keywords** Natriuretic peptides, NT-proBNP, Pulmonary hypertension, Biomarker, Heartworm, *Dirofilaria immitis*, Dogs, Animal diseases, Endarteritis

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## Background

Heartworm disease, caused by the nematode *Dirofilaria immitis*, is a vector-borne disease and is widely distributed. While dogs and wild canids are definitive hosts, other species, including humans, can also be infected [1]. Infected dogs can remain asymptomatic in less severe infections or may display a mild cough, while in severe infections, they usually present severe clinical signs such as weight loss, dyspnea, ascites, and syncope, being a life-threatening disease [1, 2]. The adult worms are in the pulmonary arteries, causing proliferative endarteritis; these vascular changes start very soon after the arrival of the worms and manifest as vascular inflammation, endothelial damage, sloughing, and villous proliferation of the intima, among others [3, 4]. This situation causes a decrease in the elasticity and increase in the resistance of the arteries that, chronically, can cause the development of pulmonary hypertension (PH) and, in some cases, also right-sided congestive heart failure [1, 2].

PH is a hemodynamic and pathophysiological state present in a wide variety of cardiovascular, respiratory, and systemic diseases [5]. Specifically, heartworm disease generates PH of precapillary origin, which is defined as the result of pathological abnormalities on the arterial side of the pulmonary vascular system. Although the gold standard of PH diagnosis is the right-heart catheterization directly measuring the systolic pulmonary arterial pressure, in veterinary and human medicine, the diagnosis is currently echocardiographycally determined [6].

However, echocardiography is still an indirect method of PH estimation, and there are limitations that make the diagnosis of this condition difficult [7]. Therefore, different non-invasive diagnostic methods for approaching the determination of PH are continually being sought, such as the determination of different serological biomarkers [8, 9]. Among these biomarkers, the concentrations of N-terminal pro-B-type natriuretic peptide (NT-proBNP) have been evaluated in dogs and humans [9, 10]. Brain natriuretic peptide (BNP) exists as a prohormone that is cleaved into the inactive N-terminal fragment and biologically active hormone BNP prior to release into the blood circulation in response to ventricular myocyte stretch [10]. NT-proBNP has a longer lifetime and is typically used as a surrogate marker for the biologically active form [11]. Previous investigations showed that serum concentrations of NT-proBNP act as an excellent biomarker for the diagnosis and monitoring of congestive heart failure and, indirectly, the myocardial function of dogs and cats [12, 13], and elevated NT-proBNP concentrations have been described in dogs with post-capillary PH due to left heart failure secondary to severe mitral regurgitation [14]. Historically, the left ventricle has been considered the major source of BNP, with the right ventricle having a smaller contribution [15]. However, in humans and dogs, increased NT-proBNP concentrations have been described in presence of precapillary PH, which could be useful to stratify the severity of the disease, monitor the response to treatment and serve as an indicator of prognosis [16–18]. Therefore, the aim of this study was to evaluate the serum concentrations of NT-proBNP in dogs infected by *D. immitis* to determine its usefulness as a tool to detect precapillary PH.

## Methods

This prospective study included 50 heartworm-infected dogs brought to the Veterinary Teaching Hospital of the University of Las Palmas de Gran Canaria, located in a hyperendemic region, during the period from September 2021 to July 2022 [19]. Heartworm diagnosis was determined using a commercial ELISA test kit to detect circulating antigens (Uranotest Dirofilaria, Urano Vet SL, Barcelona, Spain) following the manufacturer's instructions. The dogs were further evaluated for the presence/absence of circulating microfilariae using a modified Knott test.

A complete record was kept for each animal, including demographic and epidemiological data (breed, sex, age, status: client-owned or shelter dogs). All dogs were subjected to history and a complete physical exam. Infected dogs were considered symptomatic if presence of one or more symptoms related to heartworm disease were observed (dyspnea, cough, exercise intolerance, weakness, loss of weight and syncope) as well as symptoms related to right-sided congestive heart failure (ascites, jugular venous distension and hepatomegaly). Their body condition was determined based on the nine-scale body condition score (BCS) system [20], always carried out by the same researcher.

Inclusion criteria were: the dogs had not previously received heartworm chemoprophylaxis, the dogs had not started any treatment against *D. immitis*, and the animal owners provided informed consent to include the dogs in the study. Dogs with concomitant cardiorespiratory diseases (i.e. left heart disease, dilated cardiomyopathy, congenital diseases, chronic respiratory diseases), detected though clinical history, anamnesis, clinical findings and additional diagnostic tests, were excluded from the study. Moreover, to rule out any influence of azotemia, anemia or systemic hypertension in the concentrations of NT-proBNP [7, 21], dogs with abnormal concentrations of serum creatinine and/or blood urea nitrogen (BUN), abnormalities in hematology analysis indicating signs of anemia or systolic blood pressure > 160 mmHg (as measured by oscillometric methods) were also excluded from the study.

All dogs were echocardiographically evaluated, using ultrasound equipment with spectral and color Doppler and multifrequency probes (2.5–10 MHz, Viviq Iq®, General Electric, Boston, MA, USA). All dogs were conscious, gently restrained in right and left lateral recumbency and under electrocardiographic monitoring during the exam. All recordings were made by the same researcher. The presence/absence of PH was determined following the guidelines of the American College of Veterinary Internal Medicine (ACVIM) [5]. The Right Pulmonary Artery Distensibility Index (RPAD Index) and tricuspid regurgitation systolic flow, among other measures taken routinely, were used as previously described [22–24]. Other echocardiographic measurements carried out were tricuspid regurgitation pressure gradient (TRPG), pulmonary trunk to aorta ratio (PT:Ao), global tissue Doppler imaging (G-TDI) index, tricuspid annular plane systolic excursion (TAPSE), pulmonary vein to pulmonary artery ratio (PV:PA), right ventricular acceleration time (AT), right ventricular ejection time (ET), AT:ET ratio, right atrial area index (RAAi) and right ventricular end-diastolic area index (RVEDAi).

For the RPAD index and PV:PA ratio, the dogs were placed in right lateral recumbency; the transducer was placed in the third intercostal space, and the beam was directed caudally and dorsally. The systolic dimension of the pulmonary artery was measured at the maximum diameter and diastolic diameter at its smallest dimension. Diameters of the pulmonary artery were calculated by using the method according to previous authors [25]. For the measurement of tricuspid regurgitation systolic flow, TRPG, PT:Ao ratio, G-TDI, TAPSE, AT, ET, AT:ET ratio, RAAi and RVEDAi, apical four-chamber view and left cranial transverse view were used [26].

For each measurement, three continuous cardiac cycles were recorded. The animals were classified with the presence of moderate-severe PH when presented values of a RPAD Index < 29.5%, a tricuspid regurgitation systolic flow > 3.4 m/s, TRPG < 30 mmHg, PA:Ao ratio > 1.05–1.23, AT > 5.50 ± 0.31, ET > 10.6 ± 0.14, AT:ET ratio < 0.30, G-TDI > 11.8 ± 8.50, RVEDAi > 4.9–10.9 $cm^2/m^2$, RAAi > 4.2–10.2 $cm^2/m^2$ and TAPSE > 4.78–7.64. The parasite load was echocardiographically determined according to previous guidelines [27], scoring the load from 1 to 4, scores 1 and 2 being considered as showing low parasite burden and scores 3 and 4 high parasite burden.

For the determination of NT-proBNP, blood samples were collected from the cephalic vein, placed in serum tubes and centrifuged at 1432 g for 10 min. The samples were kept refrigerated until analysis, always within the next 2 h. NT-proBNP was measured with the VCHECK immunochromatography analyzer (Bionote, Big Lake, MN, USA), previously validated for canine species [28]. Reference values for healthy dogs were established by the manufacturer as < 900 pmol/l, based on values suggestive of cardiac disease [28].

Statistical analyses were performed using commercially available software (BM SPSS® Statistics 25.0, New York, USA). For categorical variables (breed, sex, legal status, BCS, symptoms, presence/absence of microfilariae, parasite load, presence/absence of PH), frequencies and percentages were analyzed; for continuous variables (age, NT-proBNP concentrations), standard deviation, median and interquartile range are shown. For continuous variables, the differences in the parameters between groups were evaluated by means of Mann-Whitney/Kruskall-Wallis tests (non-parametric) or t-Student/ANOVA (parametric) based on the normality of the variables to be evaluated by means of Shapiro-Wilks test. For categorical variables, the differences were evaluated with Pearson's non-parametric chi-square test. For multiple comparisons, when significant differences were identified, post hoc pairwise comparisons were made using Pearson's *P* test with Bonferroni corrections. All contrasts were accompanied by the effect size estimator to complete the interpretation of the results. For categorical variables, Cramer's V was used and for continuous variables, Cohen's D. Receiver-operating characteristic (ROC) curve analyses were performed to determine the optimal cutoff values for the prediction of RPAD index < 29.5% (moderate or severe PH). For all analyses, $P < 0.05$ was considered statistically significant. The results of the ROC curves of the NT-proBNP concentrations were used to estimate the event of suffering from PH (RPAD index < 29.5%), and the cut-off points of the parameters that maximize sensitivity and specificity (through of the Youden index) were evaluated.

For this study, no ethical approval was required, since all blood samples were routinely collected for official diagnostic and monitoring purposes and subsequently made available for this study. The study was carried out in accordance with the current Spanish and European legislation on animal protection.

## Results

Most of the studied dogs were mongrels (58%, $n = 29$) while 42% ($n = 21$) were pure-bred dogs. The most representative pure-bred dogs were Canarian hound, German shepherd, Siberian husky and Chihuahua. The proportion of females was higher than that of males (54% versus 46%, respectively). The mean age of the studied dogs was 4.6 years, ranging from 1 to 14 years. Based on their legal status, 72% ($n = 36$) were client-owned dogs, and 28% ($n = 14$) were from animal shelters. Regarding BCS, dogs were classified as very thin/

underweight (BCS 1 to 3) (24%, $n=12$), normoweight (BCS 4–5) (64%, $n=32$) or overweight/obese (BCS 6 to 9) (12%, $n=6$) [17].

Symptoms were present in 38% ($n=19$) of the dogs. When microfilaremia was assessed, 48% ($n=24$) were microfilaremic and 52% ($n=26$) amicrofilaremic. Regarding parasite load, this was established as high in 50% ($n=25$) of the dogs and low in 50% ($n=25$).

Dogs were classified into two groups according to presence or absence of PH. Moderate-severe PH was present in 40% ($n=20$) of the dogs and mild or absent in 60% ($n=30$). Mean RPAD index in dogs with PH was 20.1%, and mean RPAD index in dogs without PH was 40.6%. All dogs with tricuspid regurgitation systolic flow > 3.4 m/s already showed RPAD indexes < 29.5%. No significant differences were observed by breed or sex regarding PH status although the results showed that dogs with moderate-severe PH were significantly older (Mann-Whitney test, $U=456.50$, $Z=-3.82$, $P=0.002$). Based on their legal status, significant differences were observed between both groups when compared with the PH status (ANOVA, $F=14.84$, $P=0.034$). Neither microfilaremia (Chi-square test, $X^2=1.09$, $df=1$, $P=0.297$) nor parasite load (Chi-square test, $X^2=1.33$, $df=1$, $P=0.248$) was statistically influenced by the presence/absence of PH ($P>0.05$).

No significant differences were observed by breed or sex regarding NT-proBNP concentrations. Dogs with pathological concentrations of the biomarker (> 900 pmol/l) were significantly older [8.1 (7.2–8.9) years] compared with dogs with NT-proBNP concentrations within reference values [6.5 (5.7–7.4) years] (ANOVA, $F=13.52$, $P=0.002$). Neither microfilaremia nor parasite load was statistically influenced by the results of NT-proBNP concentrations. NT-proBNP was significantly higher in dogs with PH (2004.5 pmol/l, 1249.5–2759.4 pmol/l) compared to dogs without PH (689.9 pmol/l, 528.5–851.2 pmol/l) (ANOVA, $F=13.28$, $P=0.001$). Moreover, the concentrations of NT-proBNP in dogs with PH were significantly above the reference values, while NT-proBNP did not differ significantly from the reference values in dogs with mild or absence of PH.

NT-proBNP concentrations were significantly higher in dogs with symptoms (1749.8 pmol/l, 1176.5–2323.1 pmol/l), being more than three times higher than in dogs without symptoms (573.1 pmol/l, 233.8—912.4 pmol/l) (ANOVA, $F=13.50$, $P=0.001$). Significant differences were also found in NT-proBNP concentrations in dogs with lower BCS (1478.8 pmol/l, 1105.2–1852.3 pmol/l) compared to dogs with normoweight (923.5 pmol/l, 631.4–1215.6 pmol/l) or overweight/obesity (819.2 pmol/l, 602.26–1036.24 pmol/l) (ANOVA, $F=5.83$, $P=0.02$).

Using as cutoff the reference value of NT-proBNP for healthy dogs provided by the manufacturer (900 pmol/l), the sensitivity of NT-proBNP to detect PH was 64.3%, with a specificity of 86.4%. For the determination of presence of moderate-severe PH, a cutoff of ≥ 1178.45 pmol/l showed a sensitivity of 64.3% and a specificity of 95.5% for the presence of moderate-severe PH. The area under the ROC curve (AUC) of the NT-proBNP concentrations to estimate the presence of PH was moderate (0.747, 95% IC: 0.543, 0.950, Youden's J statistic 0.597).

## Discussion

Many of the biomarkers evaluated in dogs with cardiac disease have also been evaluated as tools in diagnosing PH, including NT-proBNP. Many studies have explored NT-proBNP concentrations in dogs with left ventricular dysfunction and/or postcapillary PH [16, 29], although there are only a few studies in veterinary medicine briefly describing an elevation in NT-proBNP concentration in dogs with precapillary PH [7, 16]. However, it is well studied in humans that NT-proBNP concentrations are significantly elevated in the presence of precapillary PH, which causes right ventricular stress and pressure overload with the release of BNP from the right ventricular myocardium [10, 17, 18]. Moreover, in humans, increases in NT-proBNP concentrations have been correlated to presence and severity of precapillary PH, and NT-proBNP concentration serves as a diagnostic support, as a tool to monitor the response to treatment and as a prognostic predictor of mortality [17, 18, 30].

The results of the present study demonstrate the utility of this biomarker in helping to determine the presence of PH in dogs infected with *D. immitis*, being consistent with the results reported by other authors in dogs with precapillary PH caused by other pathologies of various origins [7]. Although PH is a serious and frequent phenomenon in heartworm infection, and this may be irreversible even once the parasites are eliminated [31], the usefulness of this biomarker for detection and assessment of PH had never been evaluated for this disease to our knowledge. There is only one published study in which NT-proBNP was evaluated in dogs with heartworm, pathological concentrations in the chronic stages of the disease (classes III and IV) being described, being useful to help establish severity status [32]. Although PH was not evaluated in that study, which could be the key piece that explains the increases in NT-proBNP, the authors argued that presence of PH, cardiac damage and pulmonary thromboembolisms may be the cause of the increases of this biomarker. Likewise, an older study established a relationship between elevations of another natriuretic peptide, atrial natriuretic peptide (ANP), and the presence of mild heartworm infection in dogs [33].

Moreover, other biomarkers have shown their usefulness in the determination of PH in dogs with *D. immitis*, such as, endothelin-1, C-reactive protein and other acute phase proteins [34–37].

The cutoff points that maximize the sensitivity and specificity for the detection of PH were established at 1178.75 pmol/l (64.3% and 95.5%, respectively), the sensitivity and specificity being lower for the cutoff of 900 pmol/l established by the laboratory (64.3% and 86.4%, respectively). These results differ from those shown by other authors, in which the sensitivity and specificity of NTproBNP concentration was 91.7% and 62.5%, respectively, for an NT-proBNP concentration cutoff of 900 pmol/l [7]. This may be due to the different methodology used, highlighting the use of a different NT-proBNP analyzer and the estimation of the presence of PH using other echocardiographic indicators. Given that the reference values provided by laboratories are based on the diagnosis of cardiac damage, the value of 1178.75 pmol/l could be interesting in helping to determine the presence/absence of PH, especially because of the high specificity achieved in this research for that cutoff.

The results showed that older dogs had a higher incidence of PH and therefore significantly higher values of NT-proBNP, probably due to older dogs having more chronic infections and therefore more severe and advanced vascular damage. Similarly, dogs from shelters presented higher NT-proBNP values, possibly because they mostly had had a poorer quality of life and usually came from rural areas, where they are often used as working animals (i.e. hunting or guard dogs) and housed outdoors without prevention against mosquito vector bites [38].

However, no significant changes in natriuretic peptide concentrations and presence/absence of PH were observed between microfilaremic and non-microfilaremic dogs. These results were similar to previous studies in which no relationship was observed between the presence of microfilariae and PH [23]. These results apply equally to the parasite load, there being no direct relationship with the presence of PH or elevations of NT-proBNP. Although there are still discrepancies in this regard, these results coincide with previous studies that indicate that the parasite load is not a determining factor in the development of a severe proliferative endarteritis [23, 27, 39].

Symptomatic patients showed NT-proBNP values three times higher than those observed in asymptomatic patients. This could be because symptomatic dogs usually present more advanced and chronic stages of the disease; therefore, a greater presence of PH severity was to be expected. These results also apply to dogs with low BCS, which also showed significantly higher NT-proBNP values, probably due to the characteristic weight loss suffered by dogs with HW in more advanced stages. However, another study did not find differences in the presence or absence of PH according to body score, so it is necessary to do more studies in this regard [37].

## Conclusions

The determination of NT-pro-BNP concentration can be a useful tool in the diagnostic work-up of dogs with heartworm disease and associated PH and can help to identify animals in the more advanced stage of this disorder. It is necessary to evaluate the cardiopulmonary status of the dog as a determining factor to choose a specific treatment protocol and provide an accurate prognosis. The determination of NT-proBNP could be very helpful in this regard. Further studies with a large number of animals are necessary to determine optimal cut-off values for diagnosis with greater strength as well as to evaluate the utility at the prognostic level, as has already been established in humans.

#### Abbreviations

PH Pulmonary hypertension
NT-proBNP N-terminal pro B-type natriuretic peptide

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Not applicable.

#### Author contributions

SNGR, NCR and JIM collected the samples and performed the experiments. JAMA, EC and RM designed the study. NCR, EC and YFC analyzed the results. NCR, EC and RM wrote the manuscript. JAMA coordinated the entire study. All authors corrected, read and approved the final manuscript.

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#### Availability of data and materials

All data generated or analysed during this study are included in this article. The datasets used and/or analysed during the present study are available from the corresponding author upon reasonable request.

## Declarations

#### Ethics approval and consent to participate

All owners of the participating dogs were informed and consented to participate. The project was carried out in accordance with the current Spanish and European legislation on animal protection.

#### Consent for publication

All owners of the participating dogs were informed and consented to the publication of the results.

#### Competing interests

The authors declare no conflict of interest.

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## Publisher's Note

# 1.2 Pulmonary Hypertension and NT-proBNP Dynamics During the Course of Adulticide Treatment in Naturally Infected Dogs

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RESEARCH

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# Pulmonary hypertension and NT-proBNP dynamics during the course of adulticide treatment in dogs naturally infected by *Dirofilaria immitis*

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## Abstract

**Background** Pulmonary hypertension (PH) is a frequent complication in dogs with heartworm disease caused by *Dirofilaria immitis*. Although echocardiography remains the main diagnostic tool, its operator- and preload-dependence may limit accuracy. N-terminal pro-B-type natriuretic peptide (NT-proBNP) is a cardiac biomarker that increases in response to ventricular wall stress and may be useful for monitoring right-sided heart disease. This study aimed to evaluate NT-proBNP concentrations in dogs with precapillary PH due to heartworm disease during adulticide treatment.

**Methods** In total, 90 dogs diagnosed with heartworm disease were prospectively enrolled and classified according to the presence of PH based on echocardiographic criteria. NT-proBNP concentrations were measured on days 0, 30, 60, and 90 of adulticide treatment. Additional data collected included the presence/absence of microfilariae, clinical signs, parasite burden, and renal values. Dogs received adulticidal therapy following current international guidelines. Statistical analyses assessed correlations between NT-proBNP levels, epidemiological, clinical and echocardiographic classification, and treatment progression.

**Results** Dogs with PH had significantly higher NT-proBNP concentrations at baseline compared with those without PH (2038 ± 1671 versus 583 ± 185 pmol/L, $P < 0.001$). NT-proBNP levels were also positively correlated with parasite burden ($r = 0.530$, $P < 0.05$), presence of clinical signs ($r = 0.456$, $P < 0.05$), and age ($r = 0.29$, $P < 0.05$). During treatment, a progressive decrease in NT-proBNP concentrations was observed in dogs with PH, while levels remained stable in dogs without PH. Receiver operating characteristic (ROC) analysis identified a cut-off of 1524.8 pmol/L for detecting moderate-to-severe PH (sensitivity: 99%, specificity: 87%).

**Conclusions** NT-proBNP is a valuable noninvasive biomarker for detecting and monitoring PH in dogs with heartworm disease. Its concentrations seem to reflect parasite burden, clinical status, and echocardiographic severity, and decline progressively with adulticide therapy. Integration of NT-proBNP into diagnostic and therapeutic protocols may enhance management of heartworm-infected dogs with suspected PH.

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**Keywords** Heartworm, NT-proBNP, Pulmonary hypertension, Heartworm disease, Biomarker, Adulticide treatment, Canine cardiology, Echocardiography

## Background

Pulmonary hypertension (PH) is defined as increased pressure within the pulmonary vasculature. It is a hemodynamic and pathophysiologic condition present in a wide variety of cardiovascular, respiratory, and systemic diseases, such as heartworm disease [1]. It has been previously reported that *Dirofilaria immitis* causes vascular changes in dogs, including proliferative endarteritis of the pulmonary arteries, which begins very soon after the arrival of the adult worms [2]. The reduction in arterial lumen, along with the loss of elasticity and compliance of the arteries, can chronically affect the arterial side of the pulmonary vascular system and is referred to as precapillary PH. If not controlled, precapillary PH can lead to right-sided congestive heart failure and become potentially life-threatening [2, 3].

The diagnosis can be challenging. The gold standard for determining PH is right heart catheterization; however, the cost and technical requirements of this direct evaluation of pulmonary arterial pressure make it a limited technique in veterinary medicine [1, 4]. Conversely, echocardiography has been demonstrated to be a viable alternative to invasive methods, particularly using transthoracic Doppler echocardiography [5]. Estimating the peak systolic pulmonary arterial pressure by measuring the tricuspid regurgitation peak velocity (TRV) and calculating the right ventricular to right atrial pressure gradient (RV:RA PG) can help assess the probability that a dog has PH [1, 6, 7]. In addition, assessment of the pulmonary regurgitation (PR) jet can offer an alternative approach for estimating mean or diastolic pulmonary arterial pressure [8–10]. When tricuspid regurgitation and/or PR are not present, the right pulmonary artery distensibility index (RPAD Index) has been validated as a valuable method to estimate the presence and severity of PH in heartworm-infected dogs [1, 11, 12].

Echocardiography is operator-dependent, requiring optimal three-dimensional alignment, and is preload-dependent, in contrast to cardiac biomarkers, whose concentrations can be measured independently of the operator using a blood test [13]. One of the most studied biomarkers is brain natriuretic peptide (BNP). In humans, it has been used in cases of precapillary PH and is recommended for initial risk stratification [13–16]. BNP exists as a prohormone that is cleaved into the inactive N-terminal fragment (NT-proBNP) and the biologically active hormone BNP prior to its release into the bloodstream in response to ventricular myocyte stretch [17]. The half-life of NT-proBNP is longer than that of the active hormone BNP, and it exhibits greater stability in circulating blood and after sampling [18–20]. Several studies in dogs have suggested that serum NT-proBNP levels can increase in the presence of precapillary PH, making this biomarker a valuable diagnostic tool when used alongside historical data, physical examination, and diagnostic imaging [21–23]. In addition, a previous study in dogs infected with *D. immitis* evaluated NT-proBNP concentrations, reporting an increase in this biomarker in dogs with precapillary PH [24].

Given the relevance of NT-proBNP as a potential biomarker for determining precapillary PH in dogs with heartworm disease, this study aimed to evaluate its response throughout adulticidal treatment in dogs infected with this parasite, with or without PH.

## Methods

### Animals studied

This prospective study included 90 dogs diagnosed with heartworm disease, all of which were presented to the Veterinary Teaching Hospital at the University of Las Palmas de Gran Canaria. The research was conducted in a region with a high prevalence of the disease [25] between September 2021 and July 2023. Diagnosis was confirmed using an immunocromatographic test kit for detection of *D. immitis* antigens (Uranotest Dirofilaria, Urano Vet SL, Barcelona, Spain), in accordance with the manufacturer's instructions. In addition, the modified Knott test was performed to assess the presence of circulating microfilariae.

A detailed record was maintained for each dog, including breed, sex, and age. All dogs underwent a complete physical examination and clinical history review. Animals exhibiting one or more clinical signs commonly associated with heartworm disease—such as dyspnea, coughing, exercise intolerance, weakness, weight loss, syncope, or signs of right-sided congestive heart failure (e.g., ascites, jugular venous distension, and hepatomegaly)—were classified as symptomatic.

Inclusion criteria required that dogs had not received previous heartworm prophylaxis or undergone any previous treatment for *D. immitis*. Informed consent was obtained from all owners prior to participation. Dogs were excluded if they presented with concurrent cardiopulmonary conditions (e.g., left-sided heart disease, dilated cardiomyopathy, congenital abnormalities,

chronic respiratory diseases), as determined by clinical evaluation, medical history, and further diagnostic testing (e.g., thoracic X-rays, blood work). Dogs with abnormal serum creatinine or blood urea nitrogen (BUN) concentrations, evidence of anemia, or systemic hypertension were also excluded to minimize potential interference with NT-proBNP concentrations [27, 28].

Dogs received adulticide therapy following the general principles outlined in the guidelines established by international heartworm societies, particularly the American Heartworm Society and European Society of Dirofilariosis and Angiostrongylosis, while incorporating modifications based on recently published clinical protocols [29–31]. Specifically, treatment began on day 0 with a 4-week course of doxycycline (10 mg/kg, BID) to target *Wolbachia pipientis* endosymbionts. Concurrently, a monthly oral combination of ivermectin (≥ 6 µg/kg) and pyrantel pamoate (≥ 5 mg/kg) was administered for macrocyclic lactone-based heartworm prevention and larvae elimination. Following the protocol described by Carretón et al. [31], a three-dose melarsomine regimen was initiated with a single injection on day 30, followed by two injections on days 60 and 61 (2.5 mg/kg each). Dogs were reassessed on day 90, and discharge was granted in the absence of abnormalities, such as adult heartworms detected via echocardiography, radiographic changes, or cardiorespiratory signs. A final antigen test was conducted on day 270 (6 months post-discharge) to confirm the effectiveness of the adulticide protocol. Physical activity was restricted throughout the treatment, with particular emphasis on the period between the first melarsomine injection and discharge.

#### Echocardiographic evaluation

All dogs underwent echocardiographic evaluation on days 0, 30, 60, and 90, using a system equipped with spectral and color Doppler and multifrequency transducers (2.5–10 MHz, Vivid Iq®, General Electric, Boston, MA, USA). Examinations were performed on conscious dogs positioned in right and left lateral recumbency under electrocardiographic monitoring. All echocardiographic measurements were performed by a single operator. The presence or absence of PH was assessed following the guidelines of the American College of Veterinary Internal Medicine (ACVIM) [1]. Tricuspid regurgitation systolic flow was assessed using color-flow Doppler superimposed on real-time 2D images, and continuous-wave spectral Doppler was used to measure tricuspid regurgitant velocity (TRV), as previously described [22].

The right pulmonary artery distensibility index (RPAD index) was also evaluated owing to its relevance in heartworm disease [11, 32–34]. Other echocardiographic parameters measured included tricuspid regurgitation pressure gradient (TRPG), pulmonary trunk to aorta ratio (PT:Ao), global tissue Doppler imaging (G-TDI) index, tricuspid annular plane systolic excursion (TAPSE), pulmonary vein to pulmonary artery ratio (PV:PA), right ventricular acceleration time (AT), right ventricular ejection time (ET), AT:ET ratio, right atrial area index (RAAi), right ventricular end-diastolic area index (RVEDAi), and right ventricular to right atrial pressure gradient (RV:RA PG). The modified Bernoulli equation (pressure gradient [mmHg] = $4 \times \text{velocity}^2$ [$m/s^2$]) was applied to TRV to calculate RV:RA PG. An RV:RA PG > 40 mmHg, in the absence of pulmonary valve stenosis or right ventricular outflow tract obstruction, was considered diagnostic of PH [22, 26].

For RPAD index and PV:PA ratio measurements, dogs were positioned in right lateral recumbency, and the transducer was placed in the third intercostal space with the beam directed caudally and dorsally. Pulmonary artery measurements were obtained at maximal systolic and minimal diastolic dimensions following previously established methodologies [35]. TRPG, PT:Ao ratio, G-TDI, TAPSE, AT, ET, AT:ET ratio, RAAi, and RVEDAi were measured using apical four-chamber and left cranial transverse views [36].

Each echocardiographic parameter was recorded over three consecutive cardiac cycles. Dogs were classified as having moderate-to-severe PH if they met any of the following echocardiographic criteria, according to the guidelines of the American College of Veterinary Internal Medicine (ACVIM) [1]: RPAD index < 29.5%, tricuspid regurgitation systolic flow > 3.4 m/s, TRPG < 30 mmHg, PA:Ao ratio > 1.05–1.23, AT > 5.50 ± 0.31 ms, ET > 10.6 ± 0.14 ms, AT:ET ratio < 0.30, G-TDI > 11.8 ± 8.50, RVEDAi > 4.9–10.9 $cm^2/m^2$, RAAi > 4.2–10.2 $cm^2/m^2$, and TAPSE > 4.78–7.64 mm (Table 1).

Furthermore, on day 0, parasite burden was assessed by echocardiography using a previously established scoring system [11], categorizing relative parasite loads from 1 to 4, with scores 1–2 considered low (parasites not visible or only a few echoes in the distal part of the right pulmonary artery) and scores 3–4 considered high (worm echoes occupying the right pulmonary artery or extended to the main pulmonary artery).

#### Systemic blood pressure determination

Blood pressure was measured on days 0, 30, 60, and 90, using a high-definition oscillometric device (Vet HDO Monitor®, S + B medVet GmbH, Babenhausen, Germany). The cuff size was selected on the basis of the diameter of the forelimb or, alternatively, the tail, depending on the dog. The final blood pressure value was calculated as the mean of three consecutive measurements

**Table 1** Echocardiographic parameters measured in dogs with and without pulmonary hypertension on days 0, 30,60 and 90

| Parameter | Day 0 | Day 30 | Day 60 | Day 90 |
|---|---|---|---|---|
| Dogs with pulmonary hypertension ($n=31$) | | | | |
| RPAD index (%) | 22.3 (19.8–23.7) | 25.5 (23.8–26.1) | 30.8 (28.7–31.2) | 34.6 (32.8–35.6) |
| TRPG (mmHg) | 111.8 (78.0–128.5) | 68.7 (64.5–73.2) | 46.1 (45.0–52.0) | 27.2 (25.2–31.5) |
| PT:Ao ratio | 1.74 (1.62–1.78) | 1.54 (1.50–1.60) | 1.41 (1.36–1.47) | 1.29 (1.26–1.35) |
| G-TDI (m/s) | 7.6 (7.3–8.4) | 7.9 (7.5–8.5) | 8.0 (7.9–8.7) | 8.4 (8.2–8.9) |
| TAPSE (cm) | 1.02 (0.95–1.15) | 1.21 (1.15–1.25) | 1.38 (1.32–1.45) | 1.59 (1.50–1.65) |
| PV:PA | 0.68 (0.65–0.78) | 0.81 (0.78–0.90) | 0.91 (0.85–0.99) | 0.97 (0.96–1.05) |
| AT (ms) | 59 (55–70) | 78 (70–90) | 98 (90–110) | 120 (115–135) |
| ET (ms) | 211 (195–225) | 225 (210–240) | 236 (220–250) | 250 (230–265) |
| AT:ET | 0.27 (0.28–0.34) | 0.34 (0.32–0.40) | 0.41 (0.39–0.47) | 0.49 (0.45–0.54) |
| RAAi ($cm^2/m^2$) | 18.7 (16.0–20.0) | 15.9 (14.8–18.5) | 14.1 (13.5–17.0) | 13.5 (12.0–15.5) |
| RVEDAi ($cm^2/m^2$) | 20.2 (17.0–21.5) | 16.5 (15.9–19.8) | 15.1 (14.4–18.3) | 14.3 (13.2–17.0) |
| RV:RA PG (mmHg) | 8.7 (7.0–9.3) | 6.5 (5.2–6.8) | 4.2 (3.8–5.2) | 2.9 (2.5–3.7) |
| Dogs without pulmonary hypertension ($n=59$) | | | | |
| RPAD index (%) | 36.1 (34.0–36.5) | 35.2 (34.8–37.1) | 35.8 (35.4–37.5) | 36.3 (36.0–38.2) |
| TRPG (mmHg) | 21.3 (20.5–27.0) | 20.9 (19.8–25.0) | 19.6 (18.0–23.0) | 19.2 (17.2–22.0) |
| PT:Ao ratio | 1.17 (1.10–1.20) | 1.12 (1.05–1.15) | 1.08 (1.00–1.10) | 1.03 (0.95–1.05) |
| G-TDI (m/s) | 8.0 (7.8–8.4) | 8.5 (8.0–8.6) | 8.7 (8.2–8.8) | 8.6 (8.3–8.9) |
| TAPSE (cm) | 1.58 (1.50–1.70) | 1.66 (1.55–1.75) | 1.72 (1.58–1.78) | 1.75 (1.60–1.80) |
| PV:PA | 0.97 (0.95–1.05) | 1.08 (1.00–1.10) | 1.06 (1.03–1.13) | 1.11 (1.05–1.15) |
| AT (ms) | 115 (100–120) | 119 (110–130) | 135 (120–140) | 138 (125–145) |
| ET (ms) | 219 (210–230) | 228 (220–240) | 235 (230–250) | 243 (235–255) |
| AT:ET | 0.48 (0.45–0.55) | 0.51 (0.48–0.56) | 0.57 (0.50–0.58) | 0.54 (0.51–0.59) |
| RAAi ($cm^2/m^2$) | 13.9 (12.0–15.5) | 12.8 (11.5–14.2) | 11.9 (11.0–13.8) | 11.5 (10.5–13.0) |
| RVEDAi ($cm^2/m^2$) | 15.4 (13.0–16.0) | 13.6 (12.5–15.5) | 13.4 (12.0–15.0) | 12.7 (11.5–14.5) |
| RV:RA PG (mmHg) | 3.1 (2.2–3.4) | 2.4 (2.1–3.1) | 2.2 (2.0–2.8) | 2.1 (1.8–2.6) |

RPAD index, right pulmonary artery distensibility index; TRPG, tricuspid regurgitation pressure gradient; PT:Ao ratio, pulmonary trunk to aorta ratio; G-TDI, global tissue Doppler imaging; TAPSE, tricuspid annular plane systolic excursion; PV:PA, pulmonary vein to pulmonary artery ratio; AT, right ventricular acceleration time; ET, right ventricular ejection time; RAAi, right atrial area index; RVEDAi, right ventricular end-diastolic area index; RV:RA PG, right ventricular to right atrial pressure gradient

taken at 5-min intervals. Dogs exhibiting signs of stress likely to affect blood pressure readings were allowed time to rest, adjust, or calm down. Systemic arterial hypertension was defined as sustained elevations in systolic blood pressure (SBP) exceeding 160 mmHg in accordance with established consensus guidelines [37]. The results were not evaluated in the statistical study, since only normotensive dogs were included in the study and this test was only performed to exclude the presence of hypertensive dogs from the study.

### Serum analysis

For NT-proBNP analysis, a minimum of 2 mL of blood was collected from the cephalic vein of each dog into serum tubes. Samples were collected on days 0, 30, 60, and 90, centrifuged at 1432 *g* for 10 min and analyzed within 2 h. NT-proBNP concentrations were measured using the VCHECK immunochromatographic analyzer (Bionote, Big Lake, MN, USA), which has been validated for use in dogs [38]. According to the manufacturer, reference values for healthy dogs are < 900 pmol/L. Renal parameters (creatinine and BUN) were also evaluated to identify any renal impairment that could affect NT-proBNP levels [27].

### Statistical analyses

Statistical analyses were performed using SPSS® Statistics 25.0 (IBM, New York, USA). Categorical variables (e.g., breed, sex, clinical signs, microfilariae presence, parasite burden, PH status) were expressed as frequencies and percentages. Continuous variables (e.g., age, NT-proBNP concentrations) were reported as means ± standard deviation, and medians and interquartile range. Comparisons of continuous variables were made using Mann–Whitney or Kruskal–Wallis tests for nonparametric data and *t*-tests or analysis of variance (ANOVA) for parametric data, with normality assessed via the Shapiro–Wilk test. Differences in categorical variables were assessed using

Pearson's Chi-squared test. Post hoc pairwise comparisons were performed with Bonferroni correction when applicable. Effect sizes were calculated using Cramer's V for categorical variables and Cohen's D for continuous variables. Receiver operating characteristic (ROC) curve analysis was used to determine the optimal NT-proBNP cut-off value for predicting an RPAD index < 29.5%, with the Youden index applied to maximize sensitivity and specificity. A *P*-value < 0.05 was considered statistically significant.

## Results

The group of dogs with PH on day 0 ($n=31$) included 16 mixed-breed dogs, 3 Andalusian Terriers, 3 Labrador Retrievers, 2 American Pit Bull Terriers, and 1 each of the following breeds: Pug, Smooth Fox Terrier, Canary Hound, Canary Dog, French Bulldog, Pointer, and Boxer. The group of dogs without PH on day 0 ($n=59$) consisted of 42 mixed-breed dogs, 4 Canary Dogs, 4 German Shepherds, 2 Canary Hounds, 2 Majorero Dogs, and 1 each of the following breeds: Rottweiler, Miniature Pinscher, English Water Spaniel, Xoloitzcuintle, and American Pit Bull Terrier.

NT-proBNP was pathologically increased in 23 dogs on day 0. Of these, 19 dogs (61.3%) were classified within the PH group, whereas 4 dogs (6.8%) belonged to the normotensive group. The results showed that sex, breed, and renal values were not significantly different between dogs with/without PH. However, dogs with PH were significantly older (8.22 ± 3.09 years) than those without PH (5.32 ± 2.89 years) (*U* [456.5], *P* 0.002, *d* 0.898). Analysis of epidemiological variables in relation to NT-proBNP concentrations revealed significant differences in biomarker levels only with respect to the age of the dogs studied ($r=0.29$, $P=0.007$).

At baseline (day 0), the presence of circulating microfilariae did not significantly influence NT-proBNP concentrations ($r_s=0.077$, $P=0.476$). Microfilariemic dogs ($n=48$) had a mean NT-proBNP concentration of 1212.66 ± 1392.79 pmol/L, compared with 932.68 ± 913.94 pmol/L in amicrofilariemic dogs ($n=42$). In contrast, parasite burden was significantly associated with NT-proBNP levels. A moderate positive correlation was observed between worm burden and NT-proBNP concentrations ($r_s=0.530$, $P<0.05$). When parasite burden was categorized as low (scores 1–2; $n=40$) or high (scores 3–4; $n=50$), dogs in the high-burden group exhibited significantly higher NT-proBNP concentrations (1478.79 ± 1526.73 pmol/L) than those with a low burden (634.77 ± 283.16 pmol/L) ($r_s=0.370$, $P<0.001$).

A significant positive correlation was found between NT-proBNP concentrations and the presence and severity of PH. Both Pearson's ($r_{(123)}=0.576$, $P<0.05$) and Spearman's ($r_s=0.509$, $P<0.05$) correlation coefficients indicated that elevated biomarker levels were associated with increased severity PH. Moreover, dogs classified as having moderate-to-severe PH on day 0 had significantly higher NT-proBNP concentrations (2038 ± 1671.36 pmol/L) compared with dogs without PH (583.27 ± 185.18 pmol/L) ($P<0.001$).

At baseline, 44.44% ($n=40$) of the dogs showed clinical signs. Dogs presenting with symptoms such as dyspnea, coughing, and exercise intolerance had significantly higher NT-proBNP levels (1696.69 ± 1591.62 pmol/L) than asymptomatic dogs (594.69 ± 248.72 pmol/L). This association was supported by Pearson's ($r_{(123)}=0.456$, $P<0.05$) and Spearman's ($r_s=0.473$, $P<0.05$) correlations.

Significant changes in NT-proBNP concentrations were observed over the evaluation period (days 0, 30, 60, and 90), particularly in the PH group. Among dogs with PH, a progressive decrease in NT-proBNP values was recorded: 1665.45 ± 1512.97 pmol/L on day 30, 1543.23 ± 1428.69 pmol/L on day 60, and 1462.14 ± 1355.46 pmol/L on day 90. In contrast, NT-proBNP concentrations in the non-PH group remained stable, with values of 512.98 ± 176.43 pmol/L, 501.36 ± 169.85 pmol/L, and 489.14 ± 160.72 pmol/L on days 30, 60, and 90, respectively (Fig. 1).

Repeated-measures ANOVA did not reveal a significant overall trend over time (PH group: $F=1.16$, $P=0.331$; non-PH group: $F=0.36$, $P=0.779$). In the group with PH, pairwise comparisons revealed a significant decrease in NT-proBNP levels between day

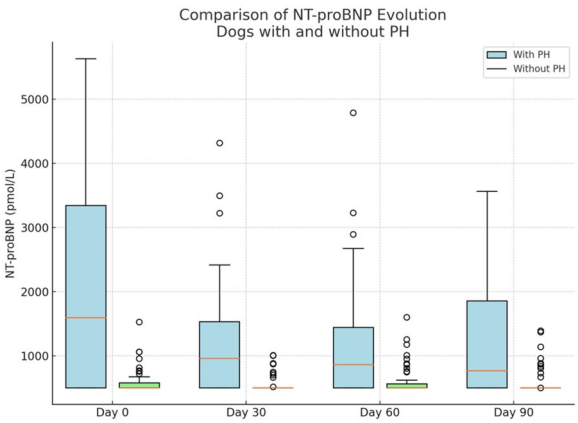


**Fig. 1** Box-and-whisker plots showing the distribution of NT-proBNP concentrations (pmol/L) in dogs with pulmonary hypertension (PH) and without PH at days 0, 30, 60, and 90. Blue boxes represent dogs diagnosed with PH, and green boxes represent those without PH. The horizontal line within each box indicates the median; the box spans the interquartile range (IQR), and whiskers extend to 1.5 × IQR. Outliers are shown as individual points. Dogs with PH exhibited consistently higher NT-proBNP values across all time points

0 and day 90 ($U_{(18)}=3.13$, $Z=1.77$, $P=0.016$). No significant differences were observed between day 0 and day 30 ($U_{(18)}=3.14$, $Z=1.58$, $P=0.446$), day 0 and day 60 ($U_{(18)}=3.42$, $Z=0.61$, $P=0.627$), or day 30 and day 90 ($U_{(18)}=3.26$, $Z=0.23$, $P=0.345$). However, a significant reduction was found between day 30 and day 60 ($U_{(18)}=3.01$, $Z=0.99$, $P=0.022$). In the group without PH, none of the pairwise comparisons reached statistical significance (all $P>0.05$), confirming the stability of NT-proBNP concentrations in these dogs.

Using a cutoff of 900 pmol/L, NT-proBNP yielded a sensitivity of 61.29% and a specificity of 93.85% for detecting PH. For identifying moderate-to-severe PH, a threshold of ≥ 1524.8 pmol/L provided a sensitivity of 99% and specificity of 87% (AUC = 0.95; $P<0.05$). This cutoff was validated by the Youden index (0.86), which confirmed optimal diagnostic performance.

## Discussion

The results of this study reinforce the usefulness of NT-proBNP as a biomarker for assessing the presence of PH in dogs infected with *D. immitis*, as shown in a previous study [24]. The association between NT-proBNP and precapillary PH can be explained by increased pulmonary arterial pressure and right ventricular overload, which lead to the release of this biomarker in response to myocardial wall stress [17]. Although the primary source of BNP is left ventricular myocardial stress, the right ventricle is also a source of BNP, and other studies have reported elevated NT-proBNP levels in canine diseases causing precapillary hypertension [21, 22, 28].

A positive correlation between parasite burden and NT-proBNP levels was also observed, suggesting that a higher number of adult parasites may exacerbate pulmonary vascular remodeling and right ventricular dysfunction, promoting the development and progression of PH, as previously suggested by other authors [39–41]. However, these results differ from those previously obtained by the same research team in a similar study [24]. The reason is unclear and may be due to confounding factors, such as the lifestyle habits of the infected dogs. For example, it is known that intense physical exercise worsens vascular pathology regardless of the parasite burden [42–44] and, in this study, such data were unavailable, as many of the animals had been recently adopted from shelters.

NT-proBNP values were significantly higher in older dogs. These results are consistent with previous reports [24], likely because in endemic areas older dogs are more prone to chronic infections and therefore more severe and long-standing vascular damage, leading to PH more frequently [25, 45]. Likewise, symptomatic dogs showed significantly higher NT-proBNP concentrations than asymptomatic ones, as in previous studies [24, 46], probably due to being in more advanced and chronic stages of the disease [2, 46]. Other studies emphasize the importance of including PH in the differential diagnosis of elevated cardiac biomarkers in dogs with respiratory signs, highlighting the role of NT-proBNP in both screening and monitoring disease progression [22]. This supports NT-proBNP as a severity marker in dogs with PH, as its elevation is associated with symptoms such as dyspnea, coughing, and exercise intolerance [6, 28]. However, the presence of elevated NT-proBNP in some asymptomatic dogs suggests that it may also detect subclinical changes, making it valuable for early identification of PH in its initial stages [13].

Another finding was the absence of a statistically significant relationship between the presence of microfilariae and NT-proBNP levels. This result aligns with previous studies [24, 33] indicating that microfilariae have a minor impact on pulmonary hemodynamics compared with adult worm burden.

Receiver operating characteristic (ROC) curve analysis identified an NT-proBNP cutoff value of 1524.8 pmol/L for predicting moderate-to-severe PH, with 99% sensitivity and 87% specificity. This cutoff differs from the one obtained previously in a similar study, which was established at 1178.75 pmol/L, but with lower sensitivity and higher specificity (64.3% and 95.5%, respectively) [24]. It is also considerably higher than the value reported by Kellihan et al. [28], who found an NT-proBNP cutoff of 900 pmol/L with 91.7% sensitivity and 62.5% specificity in 12 dogs with precapillary PH. The high sensitivity and specificity obtained in this study indicate that NT-proBNP may be a reliable marker for detecting PH in dogs with *D. immitis*, facilitating more accurate diagnosis and reducing operator dependence in echocardiographic assessments [7].

In addition, this study evaluated the evolution of this biomarker throughout adulticide treatment. A gradual decrease in mean NT-proBNP values was observed at each serial measurement, with a statistically significant reduction in biomarker concentrations between day 30 and day 60. While the first dose of melarsomine has been shown to induce the death of approximately 51.7% of adult heartworms over the subsequent weeks [47], it is important to note that neither complete parasite elimination nor a rapid resolution of pulmonary hypertension is expected at this stage. In fact, the onset of pulmonary thromboembolism resulting from worm death may transiently exacerbate vascular pathology [2, 3]. Nonetheless, the observed NT-proBNP decline may reflect early hemodynamic stabilization resulting from several overlapping factors. First, the reduction in adult worm load may already alleviate some degree of

mechanical obstruction and shear stress, contributing to decreased release of vasoconstrictive mediators such as endothelin-1 and parasitic metabolic byproducts [3, 44, 48]. Second, prior elimination of *Wolbachia pipientis* through doxycycline therapy has been reported to reduce pulmonary inflammation and endarteritis [49], thereby mitigating vascular injury and immune-mediated responses to worm death. Together, these effects may have contributed to a modest improvement in right ventricular load and myocardial stress, even in the continued presence of pulmonary hypertension. Thus, while echocardiographic indicators of pulmonary hypertension may not show immediate improvement, the decline in NT-proBNP likely reflects the early composite impact of reduced parasite-induced inflammation, partial worm clearance, and improved vascular homeostasis.

NT-proBNP values reached their lowest levels in PH dogs at the end of treatment, although they remained within pathological ranges. This finding agrees with previous reports stating that pulmonary endarteritis is not reversible, and once the parasites are eliminated, PH will persist [34]. However, according to those same authors, it may still be too early to determine the irreversibility of PH, as some worms may still be dying, and arterial inflammation may take time to subside [34, 50, 51], so it would be interesting to evaluate this biomarker in these animals at a later timepoint.

However, dogs without PH did not show significant alterations in NT-proBNP levels during adulticide treatment. For all these reasons, NT-proBNP is proposed as a highly useful serological marker for monitoring adulticide treatment in infected dogs [5].

In conclusion, the findings of this study strengthen the usefulness of NT-proBNP as a biomarker to detect, assess severity, and monitor treatment response in PH associated with canine heartworm disease. Given its high predictive value and clinical applicability, incorporating NT-proBNP into diagnostic protocols could improve early detection and management of this condition, ultimately enhancing patient outcomes.

#### Abbreviations

| | |
|---|---|
| PH | Pulmonary hypertension |
| NT-proBNP | N-terminal pro B-type natriuretic peptide |

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#### Author contributions

N.C.R., D.J.V.R., and S.F.C. collected the samples and performed the experiments. J.A.M.A., E.C., and R.M. designed the study. N.C.R., E.C., and B.R.M. analyzed the results. N.C.R., E.C., and R.M. wrote the manuscript. J.A.M.A. coordinated the entire study. All authors corrected, read and approved the final manuscript.

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#### Availability of data and materials

No datasets were generated or analyzed during the current study.

### Declarations

#### Ethics approval and consent to participate

All owners of the participating dogs were informed and consented to participate. The project was carried out in accordance with the current Spanish and European legislation on animal protection.

#### Consent for publication

All owners of the participating dogs were informed and consented to the publication of the results.

#### Competing interests

The authors declare no competing interests.

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## Publisher's Note

# 5.3 Urinary NGAL as an Early Marker of Renal Dysfunction in Dogs with Heartworm Disease and Pulmonary Hypertension

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# Urinary NGAL as an Early Marker of Renal Dysfunction in Dogs with Heartworm Disease and Pulmonary Hypertension

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## Simple Summary

Heartworm disease is a serious condition in dogs caused by the parasite *Dirofilaria immitis*. In advanced stages, it can lead to pulmonary hypertension (PH), a condition that affects both the lungs and heart. PH may also harm the kidneys, even before standard tests like creatinine or urea indicate any abnormalities. In this study, we examined a protein called urinary NGAL (uNGAL), which is released by kidney cells in response to injury, to determine whether it could help detect early kidney damage in dogs with heartworms. We found that dogs with PH had higher levels of uNGAL than dogs without PH, despite having normal results on standard kidney tests. These findings suggest that uNGAL may be a valuable tool for detecting early renal damage and improving disease monitoring in affected dogs.

## Abstract

Heartworm disease, caused by *Dirofilaria immitis*, often leads to pulmonary hypertension (PH), a serious cardiovascular complication in infected dogs. PH may impair renal function through hemodynamic and inflammatory mechanisms, even when traditional biomarkers such as serum creatinine and blood urea nitrogen (BUN) remain within normal ranges. This study aimed to assess urinary neutrophil gelatinase-associated lipocalin (uNGAL) levels in dogs naturally infected with *D. immitis*, with and without PH, to evaluate its potential as an early biomarker of renal dysfunction. Forty-two infected dogs were included and divided into two groups based on the presence (n = 14) or absence (n = 28) of PH, diagnosed via echocardiography. uNGAL concentrations were significantly higher in dogs with PH (mean 66.49 ± 6.67 ng/mL) compared to those without PH (mean 49.01 ± 14.48 ng/mL; $p < 0.0001$), despite normal creatinine and BUN values. No significant associations were found between uNGAL and sex, age, breed, or clinical signs. These findings suggest that uNGAL may serve as a sensitive biomarker of early renal impairment in dogs with heartworm disease and PH, even in the absence of overt azotemia, supporting its use in clinical evaluation and the monitoring of disease progression.

**Keywords:** *Dirofilaria immitis*; heartworm disease; canine; urinary NGAL; echocardiography; pulmonary hypertension; renal injury



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## 1. Introduction

Heartworm disease is a serious parasitic condition caused by the nematode *Dirofilaria immitis*. It is prevalent worldwide, particularly in regions with warm climates, and poses significant health risks to both domestic and wild canids [1,2]. The disease affects millions of dogs annually and, if left untreated, can lead to severe cardiovascular and pulmonary complications. Adult parasites primarily reside in the pulmonary arteries, where they induce pathological changes that reduce arterial elasticity and increase vascular resistance, ultimately resulting in precapillary pulmonary hypertension (PH) [3,4]. Precapillary PH is defined as elevated pressure within the pulmonary arterial vasculature and can arise as a complication of various underlying diseases [5]. Regardless of its etiology, sustained increases in pulmonary vascular pressure contribute to the progression of PH and eventually lead to varying degrees of right ventricular dysfunction and congestive heart failure (CHF) [6].

In addition to its cardiovascular effects, *D. immitis* infection is also associated with renal lesions, primarily caused by the deposition of immune complexes in the glomerular basement membrane. This leads to thickening and subsequent obstruction of the glomerular capillaries. Renal damage due to *Wolbachia* endosymbiont deposition and the presence of microfilariae have also been reported [7–10]. These processes damage the glomerular endothelium and promote the development of proliferative glomerulonephritis, which can manifest as proteinuria and, in some cases, progress to azotemia and hypoalbuminemia [11,12]. In human medicine, renal function is a strong, independent predictor of prognosis in patients with CHF [7]. In veterinary medicine, a similar relationship has been demonstrated in several studies [13–15]. However, in companion animals, the interplay between PH and renal injury remains complex and not yet fully understood [16]. Given that PH can progress to right-sided CHF if left untreated, and considering its high incidence in dogs with heartworm disease, further investigation into the potential relationship between these conditions is warranted.

Doppler echocardiography is the diagnostic method of choice for detecting PH in veterinary medicine, offering a non-invasive and accessible means of estimating pulmonary arterial pressure [5]. Regarding renal function, traditional markers such as serum creatinine and blood urea nitrogen (BUN) concentrations are still the most commonly used, despite their limitations. These markers are late indicators of kidney disfunction and cannot distinguish between functional and structural damage [17,18]. While creatinine and BUN levels typically rise only after approximately 60–70% of renal function has been lost, symmetric dimethylarginine (SDMA)—an endogenous biomarker of renal excretory function—can increase earlier, after about a 40% reduction in glomerular filtration rate, making it a more sensitive indicator for early detection [17,19].

Recent advances have identified neutrophil gelatinase-associated lipocalin (NGAL) as a promising biomarker of kidney injury in veterinary medicine. NGAL, a member of the lipocalin family, is expressed by neutrophils and various other cells, including renal tubular epithelial cells, pulmonary cells, and cardiomyocytes. It is rapidly released into the bloodstream and urine in response to tubular injury [20]. Although its role has been widely studied in other clinical contexts [20–24], the potential of urinary NGAL (uNGAL) as a biomarker in canine heartworm disease—and its association with PH—remains unexplored.

This study aimed to evaluate uNGAL concentrations as an early marker of renal dysfunction in dogs with heartworms, and to determine whether the presence of PH influences this parameter. If confirmed, uNGAL could serve as a valuable tool for assessing renal function and improving the clinical management of affected dogs.

## 2. Materials and Methods

This prospective study included 42 dogs naturally infected with *D. immitis* that presented to the Veterinary Teaching Hospital of the University of Las Palmas de Gran Canaria (ULPGC) between September 2022 and September 2023. A detailed record was maintained for each animal, including identification, age, sex, breed, and the presence or absence of clinical signs at diagnosis. All dogs resided in heartworm-endemic areas, ensuring homogeneity in exposure risk. Heartworm infection was diagnosed using a commercial immunochromatographic test kit (Uranotest Dirofilaria, Urano Vet SL, Barcelona, Spain). In addition, routine hematological and biochemical analyses were performed, including renal parameters (BUN and creatinine).

Inclusion criteria required dogs to test positive for *D. immitis* antigen without prior prophylactic or adulticidal treatment. Exclusion criteria ruled out animals with pre-existing cardiorespiratory conditions that could independently cause PH—such as chronic degenerative valve disease, dilated cardiomyopathy, congenital heart defects, or chronic respiratory diseases—as well as unrelated renal disorders.

All dogs underwent echocardiographic examination, including Doppler ultrasound, using multifrequency probes (2.5–10 MHz) on a Vivid Iq® system (General Electric, Boston, MA, USA). Examinations were performed by a member of the research team with six years of exclusive experience in small animal cardiorespiratory medicine, and were always conducted by the same investigator to ensure consistency. Dogs were examined while conscious and gently restrained in right and left lateral recumbency under continuous electrocardiographic monitoring. The presence or absence of PH was assessed following the guidelines of the American College of Veterinary Internal Medicine (ACVIM) [5]. Standard parameters such as maximum tricuspid regurgitation velocity (TRV) and systolic pulmonary artery flow were recorded. Additionally, the right pulmonary artery distensibility index (RPAD index) was measured as previously described [25], and pulmonary artery diameters were calculated accordingly [25–27]. TRV was measured using apical four-chamber and left cranial transverse views. Special care was taken to align the cursor parallel to the direction of flow, optimize gain settings, and measure the regurgitant jet at the dense outer edge of the velocity profile, avoiding fine linear signals (the "feathered edge") [5]. For each parameter, three consecutive cardiac cycles were recorded. Parasite burden was evaluated via echocardiography using a previously established scoring system [28], which classified relative worm burden on a scale from 1 to 4. Scores of 1–2 were considered low (indicating no visible parasites or only a few echoes in the distal segment of the right pulmonary artery), while scores of 3–4 were considered high (indicating worm echoes occupying the right pulmonary artery or extending into the main pulmonary artery).

Urine samples were collected via ultrasound-guided cystocentesis. Semi-quantitative analysis was performed using two different commercial test strips (Uranotest 11C and Uranotest 2AC; Urano Vet SL, Barcelona, Spain), which were analyzed with the automatic semi-quantitative analyzer Uranotest Reader® (Urano Vet SL, Barcelona, Spain), according to the manufacturer's instructions. The remaining urine was stored at −80 °C until further analysis. Urinary NGAL (uNGAL) concentrations were measured using a commercial sandwich ELISA kit (Dog NGAL ELISA Kit, BIOPORTO Diagnostics, Hellerup, Denmark), with reference values for healthy dogs reported as <60 ng/mL, according to the manufacturer's specifications. The assay was calibrated and validated in the laboratory of the Department of Animal Pathology, Faculty of Veterinary Medicine, ULPGC. For validation purposes, stored serum samples from 20 healthy dogs were also analyzed. Results were expressed as urinary NGAL concentrations (uNGAL) in ng/mL.

Data were analyzed using SPSS Base 29.0 software for Windows (SPSS Inc./IBM, Chicago, IL, USA). Categorical variables were summarized using frequencies and percent-

ages, while continuous variables were described using mean, standard deviation, median, and interquartile range. Differences in continuous variables between groups were evaluated using either the Mann–Whitney or Kruskal–Wallis tests (non-parametric), or Student's t-test or ANOVA (parametric), depending on the normality of the data as assessed by the Shapiro–Wilk test. Differences in categorical variables were analyzed using Pearson's Chi-squared test. All statistical comparisons were accompanied by effect size estimates to aid interpretation: Cramer's V for categorical variables and Cohen's d for continuous variables. When statistically significant differences were found using the Kruskal–Wallis test, post hoc pairwise comparisons were conducted using the Mann–Whitney U test with Bonferroni correction. Receiver operating characteristic (ROC) curve analyses were performed to determine the optimal cutoff values, using a reference threshold of 60 ng/mL. Sensitivity and specificity were assessed based on the cutoff points that maximized diagnostic accuracy. A $p$-value < 0.05 was considered statistically significant.

Ethical approval was not required for this study, as all blood samples were collected as part of routine diagnostic and monitoring procedures and were subsequently made available for research. All owners were informed about the study and provided written informed consent prior to participation. The study was conducted in accordance with current Spanish and European legislation on animal protection.

## 3. Results

Based on echocardiographic evaluation, dogs were divided into two groups: Group A (n = 28), consisting of dogs without PH, and Group B (n = 14), consisting of dogs with PH (Table 1).

Most of the studied dogs were purebred (52.4%, n = 22), while 47.6% (n = 20) were mongrels. The most common pure breeds included the Canarian Hound, German Shepherd, Cocker Spaniel, and American Pit Bull Terrier. Males represented 54.8% (n = 23) of the population, while females accounted for 45.2% (n = 19). The age range of the studied dogs was 1 to 10 years (mean 4.6 $\pm$ 2.7 years). No statistically significant differences were observed in breed or sex in relation to the presence or absence of PH. Similarly, age comparison between dogs with and without PH using the Mann–Whitney U test revealed no statistically significant difference between groups (U = 0.618; $p$ = 0.196).

uNGAL concentrations were significantly higher in Group B (66.49 $\pm$ 6.67 ng/mL) compared to Group A (49.01 $\pm$ 14.48 ng/mL), with a statistically significant difference ($p$ < 0.0001). Based on reference values, 78.5% (n = 11) of Group B dogs had uNGAL concentrations above the reference threshold, compared to only 7% (n = 1) in Group A. No statistically significant differences in uNGAL concentrations were found with respect to age, sex, or breed.

Receiver operating characteristic (ROC) curve analysis was conducted to evaluate the ability of uNGAL to discriminate between dogs with and without PH (Figure 1). The analysis yielded an area under the curve (AUC) of 0.857, indicating good diagnostic accuracy. The optimal cutoff value was identified as 60 ng/mL and was statistically significant ($p = 1.07 \times 10^{-7}$). Cohen's d was 46.20, indicating a very large effect size and supporting the strong discriminative power of uNGAL in this context.

Microfilaremia was detected in 66.7% (n = 28) of dogs, while 33.3% (n = 14) tested negative for circulating microfilariae. When stratified by PH status, 57.14% (n = 16) of dogs in Group A and 85.71% (n = 12) in Group B were microfilaremic. Although the proportion was higher in Group B, the difference was not statistically significant (U = 0.593; $p$ = 0.336). Additionally, no significant differences in uNGAL concentrations were observed between microfilaremic and amicrofilaremic dogs.

**Table 1.** Echocardiographic parameters measured in dogs with and without pulmonary hypertension. Values are expressed as mean (minimum–maximum). Abbreviations: FS: Fractional shortening; EF: Ejection fraction; LVIDd: Left ventricular internal diameter in diastole; RVDd: Right ventricular end-diastolic diameter; PA Vmax: Peak velocity of the pulmonary artery; PT:Ao: Pulmonary trunk-to-aorta ratio; TAPSE: Tricuspid annular plane systolic excursion; RAV: Right atrial volume; RPAD index: Right pulmonary artery distensibility index; AT: Right ventricular acceleration time; DT: Deceleration time; ET: Right ventricular ejection time; TRV: Maximum tricuspid regurgitation velocity.

| **Dogs with Pulmonary Hypertension (n = 14)** | |
|---|---|
| **Parameter** | **Values** |
| FS (%) | 36.34 (29.31–42.94) |
| EF (%) | 65.27 (54.70–72.47) |
| LVIDd (cm) | 0.55 (0.52–0.60) |
| RVDd (cm) | 0.92 (0.77–1.17) |
| IVSd (cm) | 0.92 (0.80–1.12) |
| PA Vmax (m/s) | 0.80 (0.68–0.88) |
| PT:Ao | 1.04 (1.00–1.12) |
| TAPSE (cm) | 1.46 (1.05–1.54) |
| RAV (ml) | 4.93 (1.61–6.41) |
| RPAD index | 25.67 (7.93–33.01) |
| AT (ms) | 60.30 (60.30–80.40) |
| DT (ms) | 126.65 (118.65–144.05) |
| ET (ms) | 160.80 (32.62–185.93) |
| AT/ET | 0.16 (0.11–0.18) |
| TRV m/s | 3.33 (3.00–3.56) |
| **Dogs Without Pulmonary Hypertension (n = 28)** | |
| **Parameter** | **Values** |
| FS (%) | 37.70 (31.81–39.77) |
| EF (%) | 65.90 (58.80–72.09) |
| LVIDd (cm) | 0.57 (0.47–0.69) |
| RVDd (cm) | 0.96 (0.83–1.09) |
| IVSd (cm) | 1.05 (0.94–1.15) |
| PA Vmax (m/s) | 0.82 (0.73–0.92) |
| PT:Ao | 0.99 (0.96–1.05) |
| TAPSE (cm) | 1.35 (1.13–1.50) |
| RAV (ml) | 5.55 (3.44–10.37) |
| RPAD index | 30.27 (21.65–44.57) |
| AT (ms) | 64.00 (60.30–85.42) |
| DT (ms) | 117.25 (100.50–132.32) |
| ET (ms) | 33.95 (24.95–152.43) |
| AT/ET | 0.14 (0.12–0.18) |
| TRV m/s | 1.10 (0.97–1.24) |

Parasitic burden was also evaluated, with 22 dogs classified as having a low burden (scores 1 and 2) and 20 dogs classified as having a high burden (scores 3 and 4). When stratified by PH status, 39.3% (n = 11) of dogs in Group A and 64.3% (n = 9) in Group B showed a high parasite burden. However, the difference between groups was not statistically significant ($\chi 2$ =1.444; $p$ = 0.230). When comparing uNGAL concentrations between high- and low-parasite-burden groups, although concentrations were higher in Group B, no statistically significant differences were found (Table 2). Nevertheless, the evaluation of uNGAL concentrations across individual parasite burden scores using the Kruskal–Wallis test revealed statistically significant differences (H = 12.087; $p$ = 0.007). To further explore these differences, a post hoc pairwise comparisons using the Mann–Whitney U test with Bonferroni correction was performed. These analyses showed that uNGAL

concentrations were significantly higher in dogs with a parasite burden score of 4 compared to those with a score of 3 ($p = 0.007$). No other pairwise comparisons between scores (1 vs. 2, 1 vs. 3, etc.) reached statistical significance (Figure 2). Dogs with a parasite burden score of 4 showed the highest mean uNGAL concentration (n = 6; 69.83 ng/mL), followed by scores of 3 (n = 14; 47.85 ng/mL), 2 (n = 19; 46.36 ng/mL), and 1 (n = 3; 41.08 ng/mL), suggesting a positive association between parasitic burden and uNGAL levels.

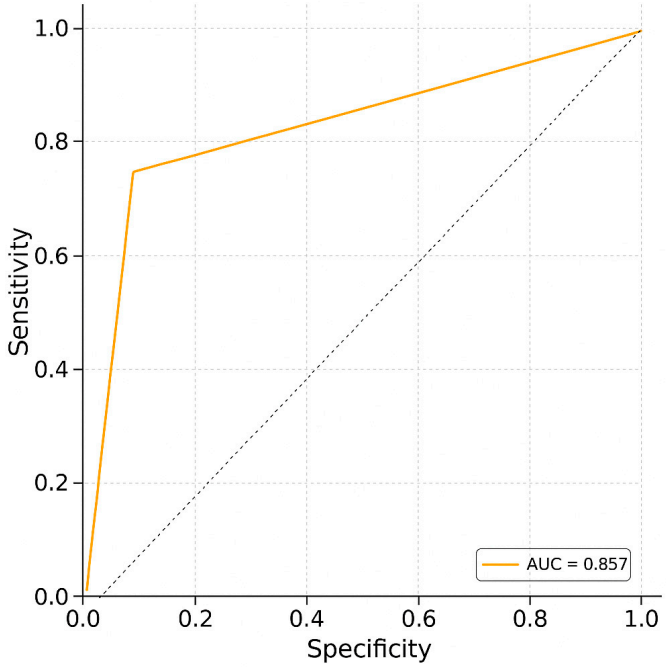


**Figure 1.** Receiver operating characteristic (ROC) curve analysis. The ROC curve illustrates the trade-off between sensitivity and specificity for the diagnostic test. The area under the curve (AUC) is 0.857, reflecting a strong ability to discriminate between positive and negative cases (sensitivity: 78.6%; specificity: 75%).

**Table 2.** uNGAL concentrations based on different classifications of the study dogs. Data are presented as mean ± standard deviation. Statistically significant results ($p < 0.05$) are marked with an asterisk. PH = pulmonary hypertension; Microfilaremia = presence of microfilariae in peripheral blood; Proteinuria/Borderline Proteinuria = presence of proteins in urine. Groups were categorized according to high or low parasite burden as described [28].

| | **Concentrations of uNGAL** | | ***p*-Value** |
|---|---|---|---|
| | **Present** | **Absent** | |
| PH | 66.49 ± 6.67 ng/mL (n = 14) | 49.01 ± 14.48 ng/mL (n = 28) | <0.0001 * |
| Microfilaremia | 51.41 ± 18.89 ng/mL (n = 28) | 46.66 ± 15.92 ng/mL (n = 14) | 0.588 |
| Symptoms | 62.09 ± 9.25 ng/mL (n = 19) | 43.70 ± 18.12 ng/mL (n = 23) | 0.427 |
| Proteinuria/Borderline Proteinuria | 62.09 ± 9.25 ng/mL (n = 14) | 43.70 ± 18.12 ng/mL (n = 28) | 0.076 |
| | High | Low | |
| Parasite Burden | 54.83 ± 16.41 ng/mL (n = 20) | 45.64 ± 19.97 ng/mL (n = 22) | 0.154 |

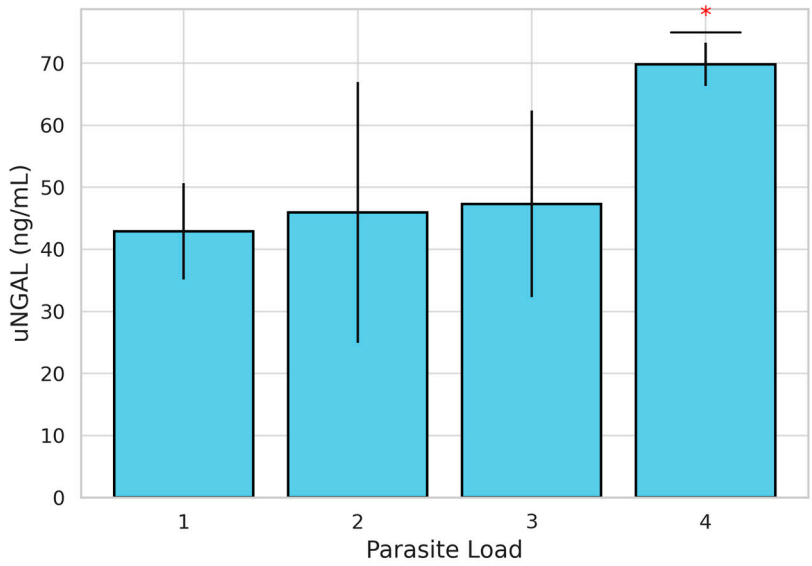


**Figure 2.** Bar graph showing mean uNGAL concentrations (ng/mL) ± standard deviation in dogs naturally infected with *Dirofilaria immitis*, stratified by parasite burden score (1 to 4). A statistically significant increase in uNGAL was observed in dogs with a score of 4 compared to those with a score of 3 (*) ($p$ = 0.007, Mann–Whitney U test with Bonferroni correction).

Clinical signs were observed in 19 dogs at the time of examination or as reported in their clinical history. The clinical signs included cough, dyspnea, weight loss, exercise intolerance, pale mucous membranes, syncope, and ascites. Only a single dog had R-CHF, which precluded additional subgroup analysis. Although 64.3% (n = 9) of dogs of Group B showed clinical signs vs. 35.7% (n = 10) in Group A, no statistically significant associations were found between the presence of clinical signs and PH, or between clinical signs and uNGAL concentrations (Table 2). None of the dogs presented with vena cava syndrome.

No dogs showed abnormal serum creatinine or BUN levels. Overall, 7.1% (n = 3) of dogs showed proteinuria (UPC > 0.5) and 26.2% (n = 11) had borderline proteinuria (UPC 0.2–0.5). When stratified by PH status, 21.43% (n = 6) of Group A dogs and 57.14% (n = 8) of Group B dogs showed proteinuria or borderline proteinuria. The relationship between proteinuria and PH was assessed using the Chi-squared test. Although uNGAL concentrations tended to be higher in proteinuric dogs, no statistically significant differences were observed ($\chi^2$ = 0.98; $p$ = 0.321) (Table 2). A moderate positive correlation was observed between proteinuria and uNGAL concentrations (Spearman's ρ = 0.489); however, this correlation did not reach statistical significance ($p$ = 0.076).

## 4. Discussion

Pulmonary hypertension is a common and serious complication of canine heartworm disease and one of the main contributors to clinical signs in infected dogs. If left untreated, it can progress to right-sided heart failure, severely compromising the animal's health [29]. The development of pulmonary microvascular dysfunction increases vascular resistance and right ventricular afterload, ultimately leading to progressive cardiac deterioration [29,30]. Although PH is more likely in chronic infections, typically in older dogs, no significant age differences were observed between dogs with and without PH in this study, consistent with previous reports [31–33].

As cardiac dysfunction progresses, central venous pressure rises, reducing effective renal perfusion and increasing interstitial and hydrostatic pressures in Bowman's capsule. These changes impair both glomerular and tubular function [34,35], potentially leading

to subclinical kidney injury. In such cases, tubular epithelial cells may release NGAL in response to damage and inflammation [22,23].

In human medicine, elevated NGAL levels have been associated with PH. For instance, in patients with acute coronary syndrome, higher uNGAL levels were found in those with PH, suggesting uNGAL as a useful biomarker for detecting cardiac complications, even in the absence of overt renal dysfunction [36]. In pediatric patients with congenital heart disease, NGAL has also been proposed as a marker of inflammation and vascular remodeling in the context of PH [37]. Experimental studies have shown that NGAL contributes to PH progression by inhibiting apoptosis in pulmonary artery smooth muscle cells, thereby promoting proliferation and vascular thickening [38]

The findings of this study may reflect early tubular injury resulting from venous congestion, as well as chronic systemic inflammation secondary to heartworm disease and PH. This pattern resembles that observed in other canine diseases such as leishmaniasis [22,32]. Although only one dog in our cohort showed signs of right-sided CHF, it is well documented that renal venous congestion and impaired perfusion can occur even without overt clinical signs (e.g., ascites), due to elevated right atrial and central venous pressures [39]. This can lead to subclinical renal injury before clinical CHF is apparent. Additionally, inflammation induced by heartworms likely contributes to pulmonary vascular remodeling and renal injury via immune complex deposition and systemic cytokine activation. Prior studies have identified acute-phase proteins in heartworm-infected dogs, particularly those with PH [40,41]. Moreover, *D. immitis* induces renal changes through immune complex deposition, microfilaremia, and the presence of *Wolbachia* bacteria [7,9]. Glomerular lesions associated with heartworm disease include glomerulosclerosis, chronic interstitial nephritis, and renal amyloidosis [10,42–44], and endothelial injury from immune complexes may result in proliferative glomerulonephritis [11,45]. Although this study cannot fully differentiate among these mechanisms, they likely act synergistically and should be jointly considered in the pathogenesis of renal injury in heartworm disease with PH.

Significantly, uNGAL levels were elevated in dogs with PH, even in the absence of abnormal serum creatinine or BUN, reinforcing its role as an early and sensitive biomarker of tubular injury. This suggests that uNGAL can detect renal involvement earlier than conventional functional markers. Additionally, a trend toward a positive association between uNGAL and proteinuria, although not statistically significant, may indicate combined tubular and glomerular damage. These findings support the potential clinical relevance of uNGAL for detecting early renal impairment in dogs with advanced *D. immitis* infection and PH.

A higher proportion of dogs with PH exhibited proteinuria or borderline proteinuria (57.1%) compared to those without PH (21.4%), although this difference was not statistically significant. Additionally, a moderate positive correlation was observed between uNGAL and proteinuria; while not reaching statistical significance, this trend may be relevant for future studies, especially considering the limited sample size. Moreover, it remains biologically plausible. Proteinuria primarily reflects glomerular permeability defects, while uNGAL indicates tubular cell damage. Sustained proteinuria can exert a toxic effect on proximal tubular cells through mechanisms like endocytic overload, oxidative stress, and cytokine induction, all of which promote NGAL expression and release [46]. Conversely, the hemodynamic and inflammatory environment associated with PH may simultaneously injure glomeruli and tubules [47,48]. In patients with chronic kidney disease, persistent proteinuria has been shown to induce tubulointerstitial inflammation and fibrosis [49,50]. Given the known interrelation between glomerular and tubular injury as well as the moderate correlation observed in this study, the authors postulate that the lack of statistical significance found in this study may be secondary to a small sample size and further

research with larger study populations is needed. Previous research has demonstrated that dogs with PH due to heartworm disease show greater proteinuria [11], reinforcing the idea that both hemodynamic and inflammatory mechanisms contribute to renal damage. Proteinuria often precedes overt signs of renal dysfunction, underscoring its value as an early marker [51]. Thus, the coexistence of proteinuria and elevated uNGAL likely reflects dual glomerular and tubular injury, driven by the inflammatory and hemodynamic stress of PH.

No significant differences in uNGAL levels were found between microfilaremic and amicrofilaremic dogs. While this study did not reveal an association, the limited sample size may have hindered the detection of true differences, and larger studies are needed to further assess the impact of microfilaremia on renal biomarkers like uNGAL.

Although SDMA is widely recognized as a sensitive marker of early glomerular filtration rate decline, it was not included in this study because its diagnostic performance had already been assessed in a previous study [11]. That study found no significant differences in SDMA between healthy dogs and those naturally infected with *D. immitis*, including those with PH. These results suggest that SDMA alone may have limited utility for identifying early renal dysfunction in this context. Nevertheless, future research incorporating both glomerular and tubular biomarkers—such as SDMA and uNGAL—in larger and longitudinally monitored populations may help clarify their combined diagnostic and prognostic utility. Therefore, the combined use of both markers may provide complementary insights into different stages and compartments of renal injury.

Parasite burden also influenced uNGAL concentrations. Although no significant differences were found using a simple high/low classification, statistically significant differences emerged when parasite burden was assessed using a 4-point scoring system [28]. Dogs with a score of 4 had the highest uNGAL levels, supporting the hypothesis that higher worm loads provoke more severe renal damage through greater antigenic stimulation, immune complex formation, and inflammation [52–55]. The parasites and their associated components (including *Wolbachia*) not only contribute to PH via vascular remodeling but also directly promote renal injury via persistent antigenic stimulation, immune complex formation and deposition, and the activation of systemic pro-inflammatory pathways [1,3,29]. These mechanisms may lead to both glomerular and tubular injury, independent of hemodynamic compromise, and should be considered as key contributors to renal pathology in heartworm-infected dogs, particularly in those with high worm burdens.

Interestingly, symptomatic dogs did not show statistically significant elevations in uNGAL levels. This discrepancy with previous studies, in which dogs with heartworm and PH were significantly more symptomatic [26,31,32], may again be explained by the small sample size and may also have influenced the lack of significance with the uNGAL results.

This study has several limitations. The relatively small number of dogs with PH (n = 14) may have reduced statistical power, especially for subgroup analyses (e.g., proteinuria, clinical signs, microfilaremia, and the small number of dogs with overt right-sided CHF). Though non-significant, observed trends—such as higher rates of proteinuria and symptoms in PH dogs—may still be clinically relevant. Future studies with larger, more balanced samples are necessary to confirm these findings and better assess the relationships between PH, renal damage, and systemic manifestations. Although the 60 ng/mL cutoff for uNGAL was based on manufacturer-provided values from healthy dogs, ROC curve analysis in this study supported its diagnostic value for identifying PH among heartworm-infected dogs. However, this threshold has not been validated across broader canine populations, and additional studies are needed to define disease-specific reference intervals. Lastly, infection chronicity was not directly measured, which may have influenced the results, as PH and renal complications evolve over time [4,11,56]. Furthermore, proteinuria

was assessed using a semi-quantitative automated dipstick analysis rather than UPC ratio. While standardized and machine-read to reduce variability, dipstick testing is less accurate and may misestimate proteinuria.

## 5. Conclusions

Dogs with heartworm disease and PH exhibited increased uNGAL concentrations, suggesting the presence of subclinical renal involvement even in the absence of elevations in conventional markers such as creatinine and BUN. The observed trends highlight the potential utility of uNGAL as an early biomarker of renal dysfunction in dogs with cardiovascular complications of *Dirofilaria immitis* infection. Therefore, uNGAL may serve as a valuable tool for the early detection of renal impairment in dogs infected with *D. immitis*, particularly in those with advanced cardiovascular involvement, thereby facilitating the implementation of preventive therapeutic strategies.

Given that renal involvement in heartworm disease remains a poorly explored area with no standardized clinical protocols for its diagnosis, staging, or treatment, further investigation is essential. Particular attention should be paid to renal function in dogs with PH, and the continued evaluation of the diagnostic and prognostic value of biomarkers such as uNGAL is strongly recommended.

**Author Contributions:** N.C.-R., D.J.V.-R., and S.F.-C. collected the samples and performed the experiments. J.A.M.-A., E.C., and R.M. designed the study. N.C.-R., E.C., and B.R.M. analyzed the results. N.C.-R. and E.C. wrote the manuscript. J.A.M.-A. coordinated the entire study. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** All owners of the participating dogs were informed and consented to the publication of the results.

**Data Availability Statement:** All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

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# 6. CONCLUSIONS // CONCLUSIONES

**6.1** The determination of NT-pro-BNP concentration can be a useful tool in the diagnostic work-up of dogs with heartworm disease and associated pulmonary hypertension and can help to identify animals in the more advanced stage of this disorder. It is necessary to evaluate the cardiopulmonary status of the dog as a determining factor to choose a specific treatment protocol and provide an accurate prognosis. The determination of NT-proBNP could be very helpful in this regard. Further studies with a large number of animals are necessary to determine optimal cut-off values for diagnosis with greater strength as well as to evaluate the utility at the prognostic level, as has already been established in humans.

**6.1** La determinación de la concentración de NT-proBNP puede ser una herramienta útil en el abordaje diagnóstico de perros con dirofilariosis y con hipertensión pulmonar asociada, y puede ayudar a identificar a los animales en fases más avanzadas de la enfermedad. Es necesario evaluar el estado cardiopulmonar del perro como un factor determinante para elegir un protocolo de tratamiento específico y proporcionar un pronóstico preciso. En este sentido, la determinación de NT-proBNP podría resultar de gran ayuda. Se requieren estudios adicionales con un mayor número de animales para determinar valores de corte óptimos con mayor solidez diagnóstica, así como para evaluar su utilidad a nivel pronóstico, como ya se ha establecido en medicina humana.

**6.2** NT-proBNP has proven to be a reliable biomarker for detecting, assessing the severity of, and monitoring treatment response in pulmonary hypertension associated with canine heartworm disease. Given its high predictive value and clinical applicability, incorporating NT-proBNP into diagnostic protocols could enhance early detection and clinical management of this condition, ultimately improving patient outcomes.

**6.2** El NT-proBNP ha demostrado ser un biomarcador fiable para detectar, evaluar la gravedad y monitorizar la respuesta al tratamiento en la hipertensión pulmonar asociada a la dirofilariosis canina. Dado su alto valor predictivo y aplicabilidad clínica, la incorporación del NT-proBNP en los protocolos diagnósticos podría

mejorar la detección temprana y el manejo clínico de esta condición, contribuyendo así a mejorar los resultados en los pacientes.

**6.3** Dogs with heartworm disease and pulmonary hypertension present increased uNGAL levels, suggesting the presence of subclinical renal damage in early stages. Although some variables did not reach statistical significance, likely due to the limited sample size, the findings support the hypothesis that pulmonary hypertension contributes to renal injury through both hemodynamic and inflammatory mechanisms. This is particularly relevant considering that other conventional markers such as creatinine and BUN remained within normal ranges. Therefore, uNGAL may serve as an early biomarker of renal dysfunction in dogs infected with *D. immitis,* especially in those with advanced cardiovascular involvement, facilitating the implementation of preventive therapeutic strategies. Given that renal involvement in heartworm disease remains a poorly explored area with no standardized clinical protocols for its diagnosis, staging, or treatment, further investigation is essential. Special attention should be paid to renal function in dogs with pulmonary hypertension, and continued evaluation of the diagnostic and prognostic value of biomarkers such as uNGAL is strongly recommended.

**6.3** Los perros con dirofilariosis e hipertensión pulmonar presentan niveles elevados de uNGAL, lo que sugiere la presencia de daño renal subclínico en fases tempranas. Aunque algunas variables no alcanzaron significación estadística, probablemente debido al tamaño limitado de la muestra, los hallazgos respaldan la hipótesis de que la HP contribuye al daño renal mediante mecanismos hemodinámicos e inflamatorios. Esto resulta particularmente relevante si se considera que otros marcadores convencionales como la creatinina y la urea permanecieron dentro de los rangos normales. Por tanto, uNGAL podría servir como biomarcador temprano de disfunción renal en perros infectados con *D. immitis*, especialmente en aquellos con afectación cardiovascular avanzada, lo que facilitaría la implementación de estrategias terapéuticas preventivas. Dado que la afectación renal en la dirofilariosis sigue siendo un área poco explorada, sin protocolos clínicos estandarizados para su diagnóstico, estadiaje o tratamiento, se considera esencial continuar con la investigación.

Se recomienda prestar especial atención a la función renal en perros con hipertensión pulmonar y seguir evaluando el valor diagnóstico y pronóstico de biomarcadores como uNGAL.

# 7. SUMMARY // RESUMEN

*Dirofilaria immitis* is a parasitic nematode responsable for canine heartworm disease, has seen increasing incidence worldwide, including in Spain, where the average prevalence is estimated at 6.25%. This rise is closely linked to climate change and the broader spread of mosquito vectors. Clinical signs in affected dogs often lack specificity, ranging from occasional coughing to more serious symptoms like difficulty breathing or exercise intolerance, which reflect progressive cardiopulmonary injury. The disease provokes chronic inflammation and damage to the pulmonary arteries, potentially leading to fibrosis, thrombosis, and pulmonary hypertension (PH). The presence of *Wolbachia pipientis,* a symbiotic bacterium, further amplifies the inflammatory response. In dogs with PH, the increased pulmonary vascular resistance leads to right heart strain, which can contribute to systemic complications. While echocardiography remains the primary diagnostic tool for PH, its results can vary depending on the operator's experience. This has encouraged the search for complementary, non-invasive diagnostic methods.

The thesis evaluated the utility of NT-proBNP, a cardiac biomarker, in detecting PH in dogs with heartworm disease. Higher NT-proBNP levels were found in dogs with echocardiographic signs of PH, helping to differentiate those with advanced cardiac involvement. NT-proBNP proved particularly useful when interpreted alongside imaging results, supporting its value in early identification of severe cases.

Additionally, the study monitored NT-proBNP concentrations during adulticide treatment with melarsomine. A notable and progressive decline in levels post-treatment suggested reduced cardiac stress and improved heart function. These findings highlight NT-proBNP's potential not just as a diagnostic tool but also for tracking treatment response.

Finally urinary neutrophil gelatinase-associated lipocalin (uNGAL) was explored as a marker of kidney injury. Increased uNGAL levels were detected in dogs with PH, even when standard renal markers were normal. Though some results lacked statistical significance, likely due to small sample size, uNGAL showed promise as a sensitive tool for detecting early kidney damage in heartworm infected dogs, supporting a more complete clinical evaluation.

*Dirofilaria immitis*, el nematodo que causa la dirofilariosis cardiopulmonar en perros, ha aumentado su presencia a nivel mundial. En España, la prevalencia media estimada es del 6,25 %, aunque varía según la región. Este incremento se relaciona con el cambio climático y la expansión de los mosquitos vectores. Los signos clínicos suelen ser inespecíficos, desde tos leve hasta dificultad respiratoria o intolerancia al ejercicio, reflejando el daño progresivo a nivel cardiopulmonar. La enfermedad desencadena inflamación crónica y lesión vascular, afectando sobre todo a las arterias pulmonares, lo que puede derivar en fibrosis, trombosis e hipertensión pulmonar (HP). Además, la bacteria simbiótica *Wolbachia pipientis* contribuye a intensificar la respuesta inflamatoria. La HP genera un aumento de la resistencia vascular pulmonar y sobrecarga del ventrículo derecho, con riesgo de afectar a otros órganos. Aunque la ecocardiografía es la técnica diagnóstica de referencia, su interpretación puede variar entre operadores, lo que ha impulsado la búsqueda de herramientas complementarias, no invasivas y objetivas.

Esta tesis evaluó la utilidad del biomarcador NT-proBNP para detectar HP en perros con dirofilariosis. Se observaron niveles significativamente más altos en aquellos con signos ecocardiográficos compatibles con HP, lo que permitió identificar casos con compromiso cardíaco avanzado. El NT-proBNP resultó especialmente útil al combinarse con parámetros de imagen, apoyando su papel en la detección temprana de los casos más graves.

También se estudió su evolución durante el tratamiento adulticida con melarsomina. Se observó un descenso progresivo y significativo tras completar el protocolo, lo que sugiere una mejoría hemodinámica del ventrículo derecho y del estado cardiovascular general. Estos datos respaldan su utilidad no solo como herramienta diagnóstica, sino también para el seguimiento terapéutico.

Por último, se exploró el potencial del uNGAL urinario como biomarcador precoz de disfunción renal. Se detectaron concentraciones elevadas en perros con HP, incluso cuando la creatinina y la urea eran normales. Aunque algunas diferencias no fueron estadísticamente significativas, probablemente por el tamaño reducido de la muestra, los resultados apoyan el uso del uNGAL como herramienta sensible para detectar daño renal subclínico en perros con filariosis, contribuyendo a una evaluación clínica más completa.

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# 9. SCIENTIFIC CONTRIBUTIONS ASSOCIATED WITH THIS THESIS

## 9.1 Participation in national and international congress

Costa-Rodríguez, N.; Carretón, E.; Falcón-Cordón, Y.; Matos, J.I.; Montoya-Alonso, J.A. Epidemiological profile of vector-borne canine diseases in Spain (Leishmania, Dirofilaria, Ehrlichia and Anaplasma). VIII Congreso de Investigación Biomédica (CIB), Febrero 05-07, 2020, Valencia, España.

Melián-Henríquez, A.; Matos-Rivero, J.I.; Montoya-Alonso, J.A.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Carretón, E. Echocardiographic evaluation of pulmonary hypertension in dogs with heartworm disease using the pulmonary vein to pulmonary artery ratio. XXXI Congresso SoIPa & 2021 ESDA Event. June 16-19, 2021, Teramo, Italy.

Falcón-Cordón, Y.; Montoya-Alonso, J.A.; Costa-Rodríguez, N.; Caro-Vadillo, A.; García-Rodríguez, S.N.; Matos-Rivero, J.I.; Carretón, E. Serum acute phase proteins in dogs with heartworm disease (*Dirofilaria immitis*) before and after adulticide treatment. XXXI Congresso SoIPa & 2021 ESDA Event. June 16-19, 2021, Teramo, Italy.

Falcón-Cordón, Y.; Carretón-Gómez, E.; Martínez, S.; Tvarijonaviciute, A.; Franco, L.; Costa-Rodríguez, N.; García-Rodríguez, S.N.; Matos-Rivero, J.I.; Montoya-Alonso, J.A. Evaluation of serum Endothelin-1 in dogs with heartworm disease (*Dirofilaria immitis*) before and after adulticide treatment. XXVIII International Conference of the World Association for the Advancement of Veterinary Parasitology. July 19-22, 2021, Dublin, Ireland.

Falcón-Cordón, S.; Carretón, E.; Matos-Rivero, J.I.; Costa-Rodríguez, N.; García-Rodríguez, S.N.; Montoya-Alonso, J.A. Utility of thoracic radiology as clinical indicator of pulmonary hypertension in dogs with heartworm disease (*Dirofilaria immitis*). XXVIII International Conference of the World Association for the Advancement of Veterinary Parasitology. July 19-22, 2021, Dublin, Ireland.

Matos-Rivero, J.I.; Falcón-Cordón, Y.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Carretón, E.; Montoya-Alonso, J.A. Evaluation of pulmonary hypertension in dogs with heartworm disease using the pulmonary trunk to descending aorta diameter ratio. World

Small Animal Veterinary Association Congress (WSAVA 2021). November 13-15, 2021, Dublin, Ireland.

Matos-Rivero, J.I.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Carretón, E.; Falcón-Cordón, Y.; Montoya-Alonso, J.A. Evaluación de la hipertensión pulmonar en perros con dirofilariosis cardiopulmonar mediante el uso del ratio broncoarterial. XVI Andalusian Congress of Veterinarians Specialists in small Animals. November 12-13, 2021, Almeria, Spain.

Costa-Rodríguez, N.; García Rodríguez, S.N.; Matos-Rivero, J.I.; Morchón, R.; Montoya-Alonso, J.A.; Carretón, E. Evaluación laboratorial de la hipercoagulabilidad en perros con *Dirofilaria immitis*. Andalusian Congress of Veterinarians Specialists in small Animals. November 12-13, 2021, Almeria, Spain.

Costa-Rodríguez, N.; García Rodríguez, SN.; Matos, J.I.; Montoya-Alonso, J.A.; Morchón, R.; Carretón, E. Utility of Serum Amyloid A (SAA) concentrations in cats with Aelurostrongylus abstrusus to determinate severity and parasite load. 15th International Congress of Parasitology (ICOPA 2022). August 21-26, 2022, Copenhagen, Denmark.

Matos-Rivero, J.I.; Falcón-Cordón, Y.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Carretón, E.; Montoya-Alonso, J.A. Assessment of pulmonary hypertension in dogs with heartworm by using the ratio between the right and left ventricle. 32nd ECVIM-CA Congress. September 1-3, 2022, Gothenburg, Sweden.

Matos-Rivero, J.I.; Pérez-Menchén D.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Carretón, E.; Montoya-Alonso, J.A. Usefulness of tissue doppler imaging in the evaluation of pulmonary hypertension in dogs with heartworm. 32nd ECVIM-CA Congress. September 1-3, 2022, Gothenburg, Sweden.

Matos-Rivero, J.I.; Falcón-Cordón, Y.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Carretón, E.; Montoya-Alonso, J.A. Usefulness of computed tomography pulmonary vein to pulmonary artery ratio in the assessment of pulmonary hypertension in dogs with heartworm disease. 15th International Congress of Parasitology (ICOPA 2022). August 21-26, 2022, Copenhagen, Denmark.

Costa-Rodríguez, N.; Hernández-Jiménez L.; Falcón-Cordón, Y.; García-Rodríguez, S.N.; Matos-Rivero, J.I.; Carretón, E.; Montoya-Alonso, J.A. Elevations in serum cortisol in dogs with heartworm disease as a biomarker of stress. 32nd ECVIM-CA Congress. September 1-3, 2022, Gothenburg, Sweden.

Matos-Rivero, J.I.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Falcón-Cordón, Y.; Carretón, E.; Montoya-Alonso, J.A. Importance of anamnesis and physical examination in canine heartworm disease. 7th ESDA Congress (ESDA 2022). September 22-24, 2022, Madrid, Spain.

Matos-Rivero, J.I.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Falcón-Cordón, Y.; Carretón, E.; Montoya-Alonso, J.A. Two-dimensional echocardiographic measurements for diagnosis and staging of heartworm disease. 7th ESDA Congress (ESDA 2022). September 22-24, 2022, Madrid, Spain.

Matos-Rivero, J.I.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Falcón-Cordón, Y.; Carretón, E.; Montoya-Alonso, J.A. Tricuspid Regurgitation Velocity/Pulmonary Artery Flow Velocity Time Integral Measured By Echocardiography In Canine Heartworm Disease. 7th ESDA Congress (ESDA 2022). September 22-24, 2022, Madrid, Spain.

Matos-Rivero, J.I.; García-Rodríguez, S.N.; Costa-Rodríguez, N.; Falcón-Cordón, Y.; Carretón, E.; Montoya-Alonso, J.A. Evaluation of thoracic computed tomography findings in dogs naturally infected by *Dirofilaria immitis*. 7th ESDA Congress (ESDA 2022). September 22-24, 2022, Madrid, Spain.

Carretón, E.; Costa-Rodríguez, N.; Falcón-Cordón, Y.; Morchón, R.; Montoya-Alonso, J.A. New biomarkers in heartworm disease. 7th ESDA Congress (ESDA 2022). September 22-24, 2022, Madrid, Spain.

Costa-Rodríguez, N.; Matos-Rivero, J.I.; Falcón-Cordón, Y.; Morchón R.; García-Rodríguez, S.N.; Montoya-Alonso, J.A.; Carretón, E. Measurement of cortisol in dogs infected by *dirofilaria immitis*. 7th ESDA Congress (ESDA 2022). September 22-24, 2022, Madrid, Spain.

Costa-Rodríguez, N.; García-Rodríguez, S.N.; Matos-Rivero, J.I.; Montoya-Alonso, J.A.; Morchón R.; Carretón, E. Usefulness of NT-proBNP in dogs with heartworm: Could this biomarker be useful to evaluate pulmonary hypertension? 2022 American Heartworm Society Triennial Symposium. September 8-11, 2022, New Orleans, USA.

Rodríguez-Trujillo, R.; Costa-Rodríguez, N.; Falcón-Cordón, S.; Batista-Arteaga, M. Neumotórax en neonatos recién nacidos por cesáreas distócicas. XVIII Congreso Andaluz de Veterinarios "Especialistas en Animales de Compañía". Noviembre 24-25, 2023, Córdoba, España.

Matos-Rivero, J.I.; García-Rodríguez, S.N.; García-Guasch L.; Costa-Rodríguez, N.; Falcón-Cordón, Y.; Carretón, E.; Montoya-Alonso, J.A. Análisis comparativo de la determinación del RPADi en la dirofilariosis cardiopulmonar canina. XXII Congress of Veterinary Specialties of AVEPA. April 21-22, 2023, Bilbao, Spain.

García-Rodríguez, S.N.; Gutiérrez-Ramos, C.; Encinoso-Quintana, M.; Matos, J.I.; Falcón-Cordón, Y.; Costa-Rodríguez, N.; Carretón, E.; Montoya-Alonso, J.A. Evaluación tomográfica de las relaciones bronquiales y vasculares pulmonares en gatos infectados de forma natural por *Dirofilaria immitis.* XXII Congress of Veterinary Specialties of AVEPA. April 21-22, 2023, Bilbao, Spain.

Matos-Rivero, J.I.; Abecassis, R.; García-Rodríguez, S.N.; Santana J.A.; Saavedra, D.; Costa-Rodríguez, N.; Carretón, E.; Montoya-Alonso, J.A. Clinical and echocardiographic findings in dogs with heartworm caval syndrome. 33rd ECVIM Congress. September 21-23, 2023, Barcelona, Spain.

García-Rodríguez, S.N.; González, M.; Matos, J.I.; Costa-Rodríguez, N.; Carretón, E.; Montoya-Alonso, J.A. Co-infections and risk factors in stray cats in Gran Canaria (Spain): *Dirofilaria immitis*, *Bartonella henselae*, feline leukemia and feline immunodeficiency. 33rd ECVIM Congress. September 21-23, 2023, Barcelona, Spain.

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**9.2 Q1 Scientific publications**

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**9.4 Final Project degrees**

Usefulness and sensitivity of blood smear for detecting microfilariae in dogs with dirofilariosis (*Dirofilaria immitis*). Presented by student Loretto Coderch Franchy. University of Las Palmas de Gran Canaria. Degree in Veterinary Medicine. Academic year 2020-2021.

Measurement of cortisol in dogs infected by *Dirofilaria immitis*. Presented by student Laura Hernández Jiménez. University of Las Palmas de Gran Canaria. Degree in Veterinary Medicine. Academic year 2021-2022.

Use of corticosteroids during treatment in dogs infected with *Dirofilaria immitis*. Is it a risk or a benefit? Presented by student Susana Flores Vervliet. University of Las Palmas de Gran Canaria. Degree in Veterinary Medicine. Academic year 2022-2023.

Neutrophil Gelatinase-Associated Lipocalin (NGAL) as a biomarker of kidney disease in dogs infected by *Dirofilaria immitis*. Presented by student Naiara Muñoz Rodríguez.

University of Las Palmas de Gran Canaria. Degree in Veterinary Medicine. Academic year 2023-2024.

Evaluation of doxycycline at different dosages in canine heartworm disease (*Dirofilaria immitis*): Are we administering too much antibiotic? Presented by student Alejandro Serrano Mesa. University of Las Palmas de Gran Canaria. Degree in Veterinary Medicine. Academic year 2023-2024.

Serum amyloid A and Haptoglobin as a prognostic marker in cats with respiratory parasitosis (*Aelurostrongylus abstrusus* and *Dirofilaria immitis*). Presented by student Isabel Ayala de la Cruz. University of Las Palmas de Gran Canaria. Degree in Veterinary Medicine. Academic year 2023-2024.

Procalcitonin measurements in dogs with dirofilariosis: An indicator of inflammatory response? Presented by student María del Pino Medina Sánchez. University of Las Palmas de Gran Canaria. Degree in Veterinary Medicine. Academic year 2024-2025.