U.PORTO
ICBAS | INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
SCHOOL OF MEDICINE AND BIOMEDICAL SCIENCES

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Annexin A1, as a Potential Biomarker in Canine Heartworm Disease

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Instituto de Ciências Biomédicas de Abel Salazar
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Annexin A1, as a Potential Biomarker in Canine Heartworm Disease

Área científica: Ciências Clínicas - Medicina e Cirurgia de Animais de Companhia

Orientadora: Prof. Doutora Ana Patrícia Nunes Fontes de Sousa (ICBAS-UP)

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ABSTRACT

Background & Aim: Annexin A1 (AnxA1), also known as lipocortin 1, is a calcium-dependent, glucocorticoid-regulated protein that plays a key role in the immune response and resolution of inflammation. In humans, AnxA1 has been identified as a biomarker for inflammatory, autoimmune, and cardiovascular diseases, highlighting its potential as a diagnostic and prognostic tool (Sheikh & Solito, 2018). Given the inflammatory nature of heartworm disease (HWD), this study aimed to assess AnxA1 levels in dogs with HWD, both with and without concurrent pulmonary hypertension (PH).

Methods: A total of 129 dogs were enrolled and underwent physical examination, complete blood count, and heartworm testing using the Uranotest Dirofilaria®. In dogs that tested positive, serum concentrations of NT-proBNP, C-reactive protein (CRP), and cortisol were measured, and echocardiography was performed to determine the presence of pulmonary hypertension (PH). Based on these findings, dogs were allocated into three groups: control (CTRL; n=45), HWD without PH (HWD; n=43), and HWD with PH (HWD+PH; n=41). Serum AnxA1 concentrations were measured using BioSource® ELISA kits. Statistical analyses were conducted using GraphPad Prism Software®, with a 95% confidence interval and significance set at $p < 0.05$.

Results: No statistically significant differences in serum AnxA1 concentrations were observed between the study groups. However, higher AnxA1 levels were noted in females within the CTRL and HWD groups, as well as in older dogs from the CTRL group. The HWD+PH group showed significantly elevated leukocyte and red blood cell counts, NT-proBNP concentrations, and parasite burden. Moderate correlations were identified between AnxA1 and leukocyte count in the CTRL group, red blood cell count in the HWD group, age in the CTRL group and cortisol in the HWD+PH group.

Conclusions: These results indicate that AnxA1 is not a reliable independent biomarker for the diagnosis of canine HWD or secondary PH. However, additional studies are warranted to elucidate its potential utility when assessed in combination with other inflammatory mediators.

Keywords: Annexin A1; Biomarker; Heartworm disease; Pulmonary hypertension; Canine

RESUMO

Introdução e objetivos: A anexina A1 (AnxA1), também conhecida como lipocortina 1, é uma proteína regulada por glucocorticoides e dependente de cálcio que desempenha um papel fundamental na resposta imunitária e na resolução da inflamação. Em humanos, a AnxA1 foi identificada como um biomarcador para doenças inflamatórias, autoimunes e cardiovasculares, destacando o seu potencial como ferramenta de diagnóstico e prognóstico (Sheikh & Solito, 2018). Dada a natureza inflamatória da dirofilariose, este estudo teve como objetivo avaliar os níveis de AnxA1 em cães com esta doença, com e sem hipertensão pulmonar (HP) concomitante

Materiais e Métodos: Foram recrutados 129 cães, que foram submetidos a exame físico, hemograma e teste de despiste de dirofilariose utilizando o Uranotest Dirofilaria®. Nos cães que testaram positivo foram ainda avaliados os níveis séricos de NT-proBNP, proteína C-reactiva (PCR) e cortisol, bem como realizada uma ecocardiografia para determinar a presença de HP. Com base nestes resultados, os cães foram divididos em três grupos: controlo (CTRL; n=45), dirofilariose sem HP (n=43) e dirofilariose com HP (n=41). As concentrações séricas de AnxA1 foram avaliadas utilizando um Kit ELISA BioSource®. As análises estatísticas foram efetuadas com o software GraphPad Prism®, com um intervalo de confiança de 95% e um nível de significância de $p < 0,05$.

Resultados: Não foram observadas diferenças estatisticamente significativas nas concentrações séricas de AnxA1 entre os grupos avaliados. No entanto, foram registados níveis mais elevados de AnxA1 nas fêmeas dos grupos CTRL e com dirofilariose sem HP, bem como nos cães mais velhos do grupo CTRL. No grupo com HP, a contagem de leucócitos e glóbulos vermelhos, bem como as concentrações de NT-proBNP e a carga parasitária foram mais elevados. Foram identificadas correlações moderadas entre a AnxA1 e a contagem de leucócitos no grupo CTRL, a contagem de glóbulos vermelhos no grupo de dirofilariose sem HP, a idade no grupo CTRL e o cortisol no grupo de dirofilariose com HP.

Conclusões: Estes resultados indicam que a AnxA1 não é um biomarcador independente fiável para o diagnóstico da dirofilariose canina ou da HP secundária. No entanto, são necessários estudos adicionais para elucidar a sua potencial utilidade quando avaliada em combinação com outros mediadores inflamatórios.

Palavras-chave: Anexina A1; Biomarcador; Dirofilariose; Hipertensão pulmonar; Cão

CASUÍSTICA E ATIVIDADES REALIZADAS

Ao longo dos últimos oito meses, realizei um estágio no Hospital Veterinário do Porto, sendo que apenas as 16 semanas finais foram contabilizadas para efeitos de estágio curricular. Esta experiência permitiu aplicar, em contexto clínico, os conhecimentos teóricos adquiridos ao longo da formação académica, proporcionando contacto direto com a rotina hospitalar. Durante o estágio, participei em rotações semanais pelos diversos serviços, incluindo cirurgia, internamento, consultas gerais, dermatologia, oncologia, medicina interna e cardiologia. Paralelamente, tive a oportunidade de acompanhar consultas de especialidades não incluídas no plano de rotações, como oftalmologia, ortopedia e neurologia, acompanhar a evolução clínica dos animais internados e integrar a equipa de serviço de urgência, com participação ativa em turnos noturnos.

A Figura 1 apresenta um resumo dos casos acompanhados durante o estágio, distribuídos por especialidade. No total, foram registados 544 casos, dos quais 481 corresponderam à área de Medicina (88,4%) e 63 à área de Cirurgia (11,6%).

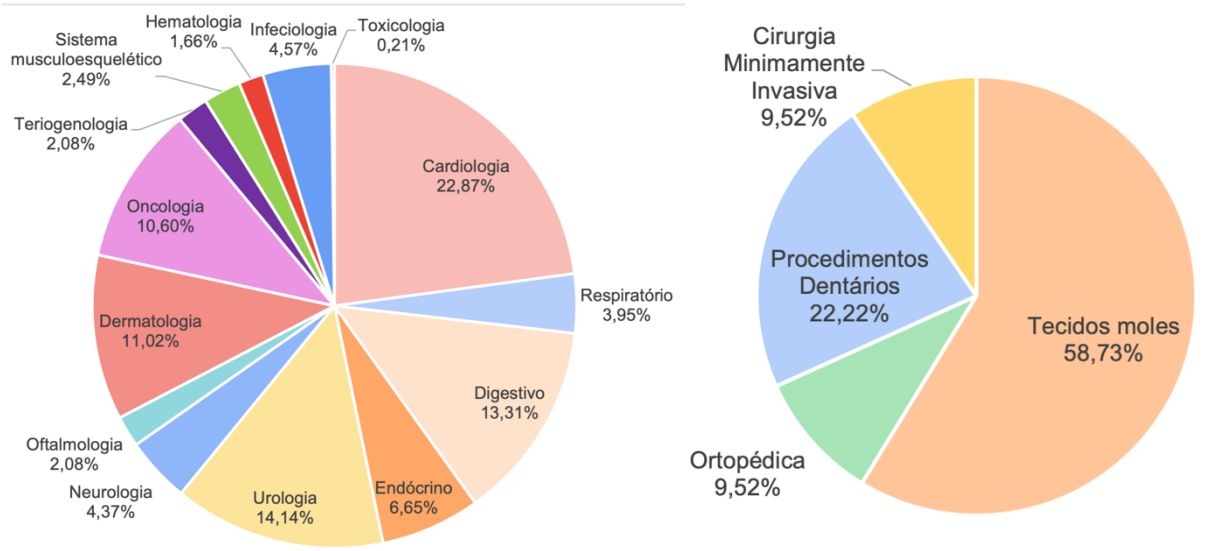

Figura 1: Distribuição dos casos de Medicina (à esquerda) e de Cirurgia (à direita) observados no Hospital Veterinário do Porto, por especialidades com as respetivas percentagens

Relativamente aos casos de Medicina, foram acompanhados 110 de cardiologia (22,87%), 68 de urologia (14,14%), 64 do aparelho digestivo (13,31%), 53 de dermatologia (11,02%), 51 de oncologia (10,60%), 32 de endocrinologia (6,65%), 22 de infeciologia (4,57%), 21 de neurologia (4,37%), 19 do aparelho respiratório (3,95%), 12 do sistema musculoesquelético (2,49%), 10 de oftalmologia (2,08%), 10 de teriogenologia (2,08%), 8 de hematologia (1,66%) e 1 de toxicologia (0,21%). A distribuição detalhada dos casos clínicos por especialidade médica está apresentada na Tabela 1.

No que diz respeito aos casos de Cirurgia, foram realizados 37 procedimentos de tecidos moles (58,73%), 14 intervenções dentárias (22,22%), 6 cirurgias ortopédicas (9,52%) e 6 cirurgias minimamente invasivas (9,52%). A discriminação dos procedimentos cirúrgicos encontra-se na Tabela 2.

Paralelamente à componente hospitalar acima descrita, tive a oportunidade de integrar um projeto de investigação intitulado "Diagnostic potential of annexin A1 in dogs with pulmonary hypertension secondary to heartworm disease", o qual deu origem à presente dissertação de mestrado. No âmbito deste projeto, colaborei na quantificação dos níveis séricos de AnxA1, bem como na análise e discussão dos resultados obtidos. O estudo contou com a colaboração da Faculdade de Medicina Veterinária da Universidade de Las Palmas de Gran Canaria, Espanha, e do Departamento de Farmacologia e Terapêutica da Faculdade de Medicina da Universidade do Porto. Com base em resultados preliminares, apresentei uma comunicação em formato de póster no IJUP'25 (Encontro de Investigação Jovem da Universidade do Porto): Reis-Ferreira A, Costa-Rodriguez N, Silva-Pereira C, Castanheira-Moreira J, Vera-Rodriguez D, Brás-Silva C, Lobo L, Sousa T, Montoya-Alonso JA, Fontes-Sousa AP. "Potential influence of sex and age on serum annexin A1 levels in dogs: implications for inflammatory regulation".

Adicionalmente, tive a oportunidade de colaborar noutro projeto de investigação que utilizou um modelo experimental de diabetes *mellitus* induzido por estreptozotocina em ratazanas Wistar. Este estudo, desenvolvido em parceria com o Departamento de Farmacologia e Terapêutica da Faculdade de Farmácia da Universidade do Porto, teve como objetivo avaliar os efeitos do tratamento com losartan e finerenona na morfologia e função cardíaca. A minha participação centrou-se na recolha e conservação de amostras biológicas. Para além da avaliação cardíaca, o estudo procurou ainda investigar os efeitos destes fármacos noutros sistemas, nomeadamente no trato gastrointestinal. Esta investigação resultou na elaboração de um artigo científico, atualmente publicado, no qual consto como coautora pelo contributo prestado: Esteves-Monteiro M, Vitorino-Oliveira C, Castanheira-Moreira J, Ferreira-Duarte M, Dias-Pereira P, Costa VM, Morato M, Duarte-Araújo M (2025). "Differential Effects of Losartan and Finerenone on Diabetic Remodeling, Oxidative Stress and ACE Activity in the Gastrointestinal Tract of Streptozotocin-Induced Diabetic Rats". *International Journal of Molecular Sciences*, *26*(13), 6294. https://doi.org/10.3390/ijms26136294.

Tabela 1: Distribuição e especificação dos casos clínicos de medicina interna observados no Hospital Veterinário do Porto, separados por sistemas.

Cardiologia	Número	Percentagem
Bloqueio atrioventricular 3º grau	3	2,73%
Bloqueio atrioventricular de 2º Grau	1	0,91%
Cor triatriatum dexter	1	0,91%
Cor triatriatum sinister	1	0,91%
DDVM	34	30,91%
Displasia da válvula tricúspide	2	1,82%
Displasia válvula mitral	2	1,82%
Estenose pulmonar	1	0,91%
Estenose subaortica	2	1,82%
Extrassistoles ventriculares (Não investigado)	4	3,64%
Fenótipo CMH	28	25,45%
Fenótipo de cardiomiopatia restritiva	1	0,91%
Fistula sistémico-pulmonar	1	0,91%
Hemangiossarcoma AD (diagnóstico presuntivo)	4	3,64%
Hipertensão pulmonar	2	1,82%
Hipertensão sistémica	5	4,55%
Mesotelioma (diagnóstico presuntivo)	1	0,91%
Obstrução dinâmica trato de saída VE (TSVE)	10	9,09%
Rotura corda tendinosa	1	0,91%
Tromboembolismo arterial felino	6	5,45%
Total	110	100,00%

Endocrinologia	Número	Percentagem
Addison - hipoadrenocorticismo	2	6,25%
Cetoacidose diabética	5	15,63%
Cushing - hipoadrenocorticismo	4	12,50%
Diabetes insipidus	1	3,13%
Diabetes *mellitus*	9	28,13%

Respiratório	Número	Percentagem
Asma felina	5	26,32%
Bronquite crónica canina	2	10,53%
Colapso traqueal	2	10,53%
Derrame pleural	8	42,11%
Pneumonia por aspiração	2	10,53%
Total	19	100,00%

Urologia	Número	Percentagem
Cistite	3	4,41%
Doença renal aguda	2	2,94%
Doença renal crónica (DRC)	25	36,76%
Doença trato urinário inferior felino	9	13,24%
Infeção do trato urinário	7	10,29%
Massa renal	1	1,47%
Ureter ectópico	1	1,47%
Ureterocelo	1	1,47%
Ureterolitíase	2	2,94%
Uretrolitíase	17	25,00%
Total	68	100,00%

Digestivo	Número	Percentagem
Corpo estranho	12	18,75%
Dilatação gástrica	2	3,13%
Dilatação-torção gástrica	1	1,56%
Enteropatia com perda de proteína	3	4,69%
Fecaloma	2	3,13%
Gastroenterite inespecífica	11	17,19%
Hepatite crónica/Cirrose	2	3,13%
Hérnia perianal	1	1,56%

Hipertiroidismo	5	15,63%
Hipocálcemia pós-parto	1	3,13%
Hipoparatiroidismo	1	3,13%
Hipotiroidismo	4	12,50%
Total	32	100,00%

Neurologia	Número	Percentagem
Ataxia congénita (não investigada)	1	4,76%
Discoespondilite	2	9,52%
Epilepsia idiopática	3	14,29%
Hérnia discal	6	28,57%
Massa no pró-encafalo	1	4,76%
Massa no SNC	2	9,52%
Massa no tronco cerebral	1	4,76%
Meningite responsiva a corticos	2	9,52%
Meningoencefalite de origem desconhecida	1	4,76%
Sindrome vestiblar periférico	2	9,52%
Total	21	100,00%

Dermatologia	Número	Percentagem
Calcinose cutânea	1	1,89%
Dermatite atópica canina	19	35,85%
Dermatite por lambedura acral	1	1,89%
Dermatofitose	1	1,89%
Efluvio telogénico	1	1,89%
Esporotricose	1	1,89%
Intertrigo	1	1,89%
Lentigo	1	1,89%
Massa no ouvido médio (adenoma/adenocarcinoma)	2	3,77%
Micetoma	1	1,89%
Oniquite lupoide	1	1,89%

IBD (diagnóstico presuntivo)	2	3,13%
Lipidose Hepática	5	7,81%
Megaesófago	1	1,56%
Mucocele Biliar	2	3,13%
Pancreatite	12	18,75%
Triadite	8	12,50%
Total	64	100,00%

Oftalmologia	Número	Percentagem
Cataratas	2	20,00%
Cherry eye	1	10,00%
Descolamento de retina	1	10,00%
Glaucoma	1	10,00%
Neurite ótica aguda	1	10,00%
Síndrome de degenerescência retinal adquirida súbita (SARDS)	1	10,00%
Úlcera da córnea	2	20,00%
Uveíte	1	10,00%
Total	10	100,00%

Teriogenologia	Número	Percentagem
Hiperplasia prostática	2	20,00%
Piómetra	6	60,00%
Prostatite	2	20,00%
Total	10	100,00%

Sistema musculoesquelético	Número	Percentagem
Displasia da anca	1	8,33%
Fratura colo do fémur	1	8,33%
Fratura fémur	3	25,00%
Fratura lombar	2	16,67%
Luxação femuro-tibial	1	8,33%
Miosite dos músculos mastigadores	1	8,33%

Otite externa	6	11,32%
Otite média	3	5,66%
Paniculite nodular estéril	1	1,89%
Pênfigo foliácio	2	3,77%
Pioderma	7	13,21%
Sindrome atópico felino (SAF)	4	7,55%
Total	53	100,00%

Oncologia	**Número**	**Percentagem**
Adenocarcinoma prostático (diagnóstico presuntivo)	2	3,92%
Carcinoma da glandula mamária	4	7,84%
Carcinoma de células de transição (CCT)	1	1,96%
Carcinoma de células escamosas (CCE)	8	15,69%
Fibrossarcoma	1	1,96%
Hemangioma	1	1,96%
Hemangiossarcoma	7	13,73%
Linfoma intestinal	16	31,37%
Mastocitoma	4	7,84%
Mesotelioma	2	3,92%
Osteossarcoma	1	1,96%
Sarcoma tecidos moles	4	7,84%
Total	51	100,00%

Toxicologia	**Número**	**Percentagem**
Rodenticidas	1	100,00%
Total	1	100,00%

Osteoartrite	1	8,33%
Politraumatismo	2	16,67%
Total	12	100,00%

Hematologia	**Número**	**Percentagem**
Anemia hemolítica imunomediada	3	37,50%
Displasia Medular	1	12,50%
Trombocitopenia imunomediada	4	50,00%
Total	8	100,00%

Infeciologia	**Número**	**Percentagem**
Calicivirose felina	3	13,64%
Coronavírus canino	2	9,09%
FeLV	1	4,55%
FIV	3	13,64%
Giardíase	1	4,55%
Leptospirose	2	9,09%
Panleucopénia	4	18,18%
Parvovirose	3	13,64%
PIF	3	13,64%
Total	22	100,00%

Tabela 2: Distribuição e especificação dos casos clínicos de cirurgia observados no Hospital Veterinário do Porto.

Cirurgia de Tecidos Moles	Número	Percentagem
Cistotomia	2	5,41%
Enterotomia	2	5,41%
Esplenectomia	3	8,11%
Gastropexia	1	2,70%
Gastrotomia	2	5,41%
Linfadenectomia (Gânglio poplíteo)	1	2,70%
Mastectomia	3	8,11%
Nodulectomia	8	21,62%
Orquiectomia	3	8,11%
Ovariohisterectomia	7	18,92%
Resolução de piómetra	2	5,41%
Toracotomia (remoção de timoma)	1	2,70%
Uretrostomia	2	5,41%
Total	37	100,00%

Cirurgia Minimamente Invasiva	Número	Percentagem
Bypass ureteral subcutâneo (SUB)	2	33,33%
Implantação pacemaker (Fluoroscopia)	2	33,33%
Resolução ducto arterial persistente (Fluoroscopia)	2	33,33%
Total	6	100,00%

Cirurgia Ortopédica	Número	Percentagem
Amputação membro	1	16,67%
Osteotomia de nivelamento do platô tibial (TPLO)	2	33,33%
Remoção de placa	2	33,33%
Resolução de fratura óssea	1	16,67%
Total	6	100,00%

Procedimentos Dentários	Número	Percentagem
Exodontia	1	7,14%
Higiene profissional da cavidade oral (HPCO) com exodontia	9	64,29%
Higiene profissional da cavidade oral (HPCO)	4	28,57%
Total	14	100,00%

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABREVIATIONS

μg/dL	Micrograms per deciliter
Ac2-26	Synthetic peptide acetyl-2-26
ACVIM	American college of veterinary internal medicine
AnxA1	Annexin A1
APP	Acute Phase Protein
CRP	C-reactive protein
cTnI	Cardiac troponin I
CTRL	Control
D. immitis	*Dirofilaria immitis*
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
FPR	Formyl peptide receptor
FPR2/ALX	Formyl peptide type 2/lipoxin A4 receptor
FSH	Follicle-Stimulating Hormone
Hp	Haptoglobin
HWD	Heartworm disease
IL-1	Interleukin 1
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IL-10	Interleukin 10
Kg	Kilogram
L1	First-stage larvae
L2	Second-stage larva
L3	Third-stage larva
L4	Fourth-stage larva
LH	Luteinizing Hormone
m/s	Metres per segund
mg/Kg	Milligrams per kilogram
mg/L	Milligrams per liter
mM	Milimolar
mmHg	Millimeters of mercury
mRNA	Messenger ribonucleic acid

NF-κB	Nuclear factor kappa B
NT-proBNP	N-Terminal pro-B-type Natriuretic Peptide
PAP	Pulmonary Arterial Pressure
PAWP	Pulmonary Arterial Wedge Pressure
PDE5i	Phosphodiesterase-5-inhibitor
pg/mL	Picograms per milliliter
PH	Pulmonary Hypertension
pmol/L	Picomoles per litre
POCUS	Point of Care Ultrasounds
PON-1	Paraoxonase-1
PVR	Pulmonary Vascular Resistance
RBC	Red Blood Cells
RPAd	Right pulmonary artery diameter in diastole
RPADi	Right pulmonary artery distensibility index
RPAs	Right pulmonary artery diameter in systole
TNF-α	Tumor necrosis factor alfa
TRV	Tricuspid valve regurgitation velocity
vel. AP	Pulmonary artery flow velocity
vel. RP	Pulmonary valve regurgitation velocity
ΔP PV	Pressure gradient across the pulmonary valve
ΔP TV	Pressure gradient across the tricuspid valve

1. INTRODUCTION

Inflammation is a physiological defense mechanism triggered by pathogens or tissue injury, aiming to eliminate the harmful stimulus, promote its neutralization, and support tissue repair and regeneration. This response is orchestrated by a tightly regulated interplay between pro-inflammatory and anti-inflammatory pathways, which collectively act to restore homeostasis (Perretti & Dalli, 2009). Under normal conditions, these mechanisms are sufficient to ensure an effective immune response and the successful resolution of inflammation (Flower, 1988).

In dogs, as in other mammals, the inflammatory response is activated when homeostasis is disrupted by invading pathogens. If this response is insufficient or dysregulated, the infection may persist and clinical signs become evident. Moreover, the inflammatory process itself can cause significant local tissue damage, further amplifying the response and perpetuating a cycle of inflammation and injury. Without therapeutic intervention, this self-sustaining cycle cannot be interrupted. Anti-inflammatory drugs are therefore essential to disrupt this cycle, promoting resolution by reducing inflammation, alleviating clinical signs, and preventing additional tissue damage (Flower, 1988).

In cardiac disease, inflammation plays a critical role, and in some cases, an excessive or unresolved inflammatory response can impair tissue repair and drive disease progression. In this context, inflammation is increasingly recognized as a key component in the pathophysiology of conditions such as heartworm disease (HWD) (Carretón et al., 2017a) and pulmonary hypertension (PH) (Liu et al., 2022), contributing to cardiac injury. Persistent inflammation in cardiac disorders may lead to fibrosis and pathological cardiovascular remodeling (Reina-Couto et al., 2016).

In recent years, several studies have explored the potential utility of cardiopulmonary and inflammatory biomarkers (Carretón et al., 2017a). Biomarkers, defined as measurable indicators of physiological or pathological processes, can aid in monitoring disease progression, evaluating treatment efficacy, and potentially preventing further tissue damage. Although their clinical relevance is increasing, the application of biomarkers in cardiac disease remains a relatively recent and evolving field of research (Carretón et al., 2017a).

Accordingly, this thesis will begin with a concise overview of the acute inflammatory response, followed by a summary of annexin A1 (AnxA1). It will then address HWD and its pathophysiological complications, particularly PH and caval syndrome. Special emphasis will be placed on the inflammatory mechanisms underlying HWD, as well as the currently identified inflammatory biomarkers associated with this condition.

1.1. Acute inflammatory response

The acute inflammatory response is a protective physiological mechanism triggered by tissue injury or pathogen invasion. It consists of two distinct phases: initiation and resolution. The initiation phase is an active process characterized by the release of pro-inflammatory mediators, increased vascular permeability, hemodynamic changes, and recruitment of neutrophils, events driven by the inflammatory cascade. In contrast, the resolution phase, once thought to be passive, is now recognized as an active and tightly regulated process involving neutrophil apoptosis and the re-establishment of tissue homeostasis (Serhan, 2014).

The initiation phase of inflammation is triggered by a complex cascade of events, beginning with the release of arachidonic acid, a polyunsaturated fatty acid derived from the cleavage of membrane phospholipids by phospholipase A2 (Clark et al., 1991). Arachidonic acid is subsequently metabolized via the cyclooxygenase and lipoxygenase pathways, resulting in the production of pro-inflammatory eicosanoids. The cyclooxygenase pathway generates prostaglandins and thromboxanes, which mediate vasodilation, hemodynamic alterations, and increased vascular permeability. Concurrently, the lipoxygenase pathway leads to the synthesis of leukotrienes, which promote neutrophil recruitment and chemotaxis (Goetzl et al., 1995). Interestingly, certain lipoxygenase-derived metabolites, such as lipoxins, also play a role in inflammation resolution. These endogenous lipid mediators attenuate oxidative damage and modulate the inflammatory response, thereby exerting dual pro-resolving and anti-inflammatory functions (Reina-Couto et al., 2014). Beyond eicosanoids, several cytokines and chemokines, including tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), along with components of the complement system, contribute to the amplification of the inflammatory response by promoting leukocyte activation and recruitment. The coordinated action of these mediators defines the initiation phase, which aims to neutralize the offending agents and ultimately restore homeostasis (Serhan, 2014).

If the resolution mechanisms fail to repair the injury and eliminate the offending agents, a cycle of sustained activation ensues. This is characterized by excessive stimulation of pro-inflammatory pathways and impaired regulation of pro-resolving and anti-inflammatory mechanisms, resulting in persistent inflammation. Such dysregulation contributes to chronic inflammatory states, tissue fibrosis, and pathological remodeling (Reina-Couto et al., 2016). At this stage, the administration of anti-inflammatory drugs becomes essential to interrupt the fibrotic progression and facilitate the resolution of inflammation (Flower, 1988).

Steroidal drugs, such as glucocorticoids, exert potent anti-inflammatory effects through binding to cytosolic receptors. These compounds are synthetic analogs of endogenous steroid hormones, such as hydrocortisone, and mimic their physiological actions, making them more effective than many non-

steroidal anti-inflammatory drugs (Flower, 1988). In the classical acute inflammatory pathway, glucocorticoids inhibit phospholipase A2, thereby blocking the release of arachidonic acid and subsequently suppressing the synthesis of prostaglandins and leukotrienes. These eicosanoids are key mediators of neutrophil chemotaxis and overall immune activation, and their inhibition leads to a dose-dependent suppression of the inflammatory response. Additionally, glucocorticoids inhibit the transcription of genes encoding pro-inflammatory mediators. Notably, the anti-inflammatory effects of glucocorticoids can be reversed by exogenous administration of arachidonic acid, as their action occurs upstream of its release (Flower, 1988).

If the initiation phase is successfully completed, the second phase, resolution, is initiated. This phase is marked by the production of specialized pro-resolving mediators, including lipoxins, resolvins, protectins, and maresins. These mediators are biosynthesized from omega-3 and omega-6 fatty acids and function primarily by inhibiting further neutrophil recruitment and promoting neutrophil apoptosis. The ultimate goal of this phase is the restoration of tissue homeostasis (Reina-Couto et al., 2016).

1.2. Annexin A1 and its relevance

Annexin A1, also known as lipocortin-1, is a 37-kDa protein member of the annexin superfamily (Pepinsky et al., 1988; Raynal & Pollard, 1994). It is a known calcium-dependent protein whose expression is characteristically regulated by glucocorticoids (Buckingham & Flower, 1997; Flower, 1988). Glucocorticoids are central to inflammation resolution, partly by upregulating AnxA1 expression. Elevated glucocorticoid levels enhance AnxA1 synthesis, which activates its receptor to mediate anti-inflammatory effects (Buckingham & Flower, 1997).

Annexin A1 plays a pivotal role in immune modulation due to its anti-inflammatory properties. Structurally, this glycoprotein contains four to eight repeats of a highly conserved motif, each comprising 70 to 80 amino acids. Its unique N-terminal sequence confers biological variability and heterogenicity within the annexin family. Additionally, annexins are characterized by conserved phospholipid- and calcium- binding sites within these motifs (Buckingham & Flower, 1997; Flower, 1988).

Inflammatory stimuli or elevated glucocorticoid levels upregulate AnxA1 expression by increasing mRNA transcription in immune cells such as neutrophils and monocytes (Sheikh & Solito, 2018). Under inflammatory conditions, characterized by intracellular calcium concentrations ≥1 mM, AnxA1 undergoes a conformational change that exposes its phospholipid-binding sites. This structural rearrangement facilitates AnxA1 translocation to the plasma membrane and exposes its N-terminal domain (Rosengarth et al., 2001). AnxA1 mediates its anti-inflammatory effects primarily through

binding to formyl peptide receptors (FPRs), especially FPR2—also known as formyl peptide type 2/lipoxin A4 receptor (FPR2/ALX)—a G-protein-coupled receptor. FPR2 preferentially binds full-length AnxA1 but also interacts with the AnxA1-derived synthetic peptide Ac2-26, which additionally binds FPR1 (de Jong et al., 2017). Expression of AnxA1 receptors is highest on the plasma membranes of macrophages, monocytes, and neutrophils (Goulding & Guyre, 1992; Goulding et al., 1996; Perretti & Flower, 1996).

Anti-inflammatory pathways activated by AnxA1 or its synthetic peptides supress NF-κB activity (Yang et al., 2009; Zhang et al., 2010), resulting in decreased expression of pro-inflammatory mediators such as TNF-α, IL-1β and IL-6, alongside increased levels of anti-inflammatory mediators, like IL-10 and activation of pro-resolution pathways (Gobbetti & Cooray, 2016). Functionally, AnxA1 reduces neutrophil adhesion and transmigration, promotes neutrophil apoptosis, and inhibits platelet–neutrophil aggregation. It also suppresses leukocyte and monocyte recruitment while enhancing efferocytosis, the phagocytic clearance of apoptotic cells (de Jong et al., 2017; Sugimoto et al., 2016).

Beyond its receptor-mediated effects, AnxA1 directly exerts anti-inflammatory action by inhibiting phospholipase A2 during inflammatory cascade. This inhibition prevents the conversion of membrane phospholipids into arachidonic acid, thereby limiting the production of key inflammatory mediators via cyclooxygenase and lipoxygenase pathways (Sheikh & Solito, 2018).

Annexin A1 is widely distributed throughout the body, present in white blood cells, stromal cells, and biological fluids. Neutrophils and monocytes are the primary cellular sources, while lymphocytes contribute to a lesser extent. Conversely, B lymphocytes and platelets do not express AnxA1. Its expression typically increases with cell differentiation (Perretti & Dalli, 2009). Under physiological, non-inflammatory conditions, AnxA1 is detected in healthy cells, with highest levels in the thymus, spleen, lungs, and brain (Flower, 1988). Chronic inflammation is associated with a dysregulated balance between pro- and anti-inflammatory mediators, often due to AnxA1 degradation or insufficient expression (La et al., 2001).

In human medicine, AnxA1 has been studied as an inflammatory biomarker, particularly in cardiovascular diseases and cancer, where its expression correlates with disease severity (Sheikh & Solito, 2018). Additionally, AnxA1 is considered a potential therapeutic target for chronic inflammatory diseases such as atherosclerosis and myocardial infarction, given its cardioprotective properties. Its role in modulating neuroinflammation and providing neuroprotection in stroke models has also garnered significant interest in neurology (de Jong et al., 2017; Solito et al., 2008).

1.3. Heartworm diseases: a brief review

Heartworm disease is a severe, vector-borne, zoonotic, and potentially life-threatening parasitic condition caused by *Dirofilaria immitis* (*D. immitis*), a filarial nematode that primarily infects dogs and, less frequently, cats worldwide, including Portugal (Esteves-Guimarães et al., 2024; Esteves-Guimarães et al., 2025; Vieira et al., 2015). Domestic dogs and wild canids act as the definitive hosts, while felines are considered atypical hosts due to their lower susceptibility. Transmission occurs via culicid mosquitoes, primarily of the genera Culex, Aedes, and Anopheles. The pathogenicity of HWD is primarily due to the presence of adult worms in the pulmonary arteries and right heart, as well as the release of *Wolbachia*, an endosymbiotic bacterium implicated in inflammatory complications (Bussadori, 2023; Montoya-Alonso et al., 2022). Although the global distribution of *D. immitis* varies, climate change and increasing temperatures are expanding mosquito habitats, facilitating the emergence of HWD in previously non-endemic regions (Pietrzak et al., 2024; Ware et al., 2022).

Dirofilaria immitis has a relatively long-life cycle, typically lasting 7 to 9 months. Mosquitoes acquire first-stage larvae (L1) during a blood meal from an infected host. Within the mosquito, L1 develops into second-stage larvae (L2), and subsequently into the infective third-stage larva (L3), which migrates to the mosquito's proboscis. Transmission to the definitive host occurs during the mosquito´s subsequent blood meal (Bussadori, 2023; Nelson et al., 2024; Ware et al., 2022). The development of L3 is temperature-dependent and requires approximately 80% relative humidity. In suboptimal conditions, the development period extends beyond the typical 10-14 days (Taylor, 1960; Ware et al., 2022). Once inoculated into the definitive host, L3 converts to L4 within 3 to 12 days and migrates through subcutaneous tissue and muscle. By 50 to 70 days post-infection, L4 develops into immature adults, which enter the circulatory system and migrate to the pulmonary arteries, where they mature and elicit pathological changes (Kotani & Powers, 1982; Nelson et al., 2024; Ware et al., 2022).

The clinical manifestations of HWD depend on worm burden, duration of infection, and the host´s immune response. In most cases, dogs remain asymptomatic, particularly during the early stages of infection. Clinical signs range from mild to severe. Mild cases may present with no symptoms or a mild cough, whereas moderate cases are characterized by exercise intolerance and abnormal pulmonary auscultation. In severe cases, clinical signs may include dyspnoea, hepatomegaly, syncope, and ascites. In advanced stages, dogs may develop caval syndrome, a life-threatening complication (Bussadori, 2023; McCall et al., 2008a; Ware et al., 2022).

As outlined above, mosquitoes are essential vectors in the transmission of HWD. Although dogs exhibit inherently high susceptibility, the primary strategy for preventing transmission lies in interrupting the parasite´s life cycle through regular chemoprophylaxis and vector control, particularly in endemic regions. Macrocyclic lactones, such as ivermectin and milbemycin oxime, are the first-line

preventive agents and should be initiated by 8 weeks of age (Nelson et al., 2024). These agents act synergistically with the host´s immune system to eliminate susceptible larval stages, including microfilaria, L3 and L4, and, in some cases, juvenile and adult worms (McCall et al., 2008a). In highly endemic areas, the additional use of repellents and ectoparasiticides is recommended to further reduce mosquito exposure and transmission risk (Nelson et al., 2024).

Current diagnostic guideline advise against initiating heartworm preventive treatment when a dogs' infection status is unknown. Consequently, annual screening for HWD is recommended for all dogs over seven months of age. The two main diagnostic methods used are antigen testing and microfilariae detection (Nelson et al., 2024; Ware et al., 2022). Antigen tests detect a major glycoprotein secreted by adult female worms, using an enzyme-linked immunosorbent assay (ELISA) (Courtney & Cornell, 1990). These tests are highly specific and can yield positive results even with a single adult female worm, including in occult infections. Antigen tests typically become positive around five months post-infection (Atkins, 2003). Microfilariae testing, in contrast, usually detect infection approximately six months post-exposure. It involves microscopic examination of a fresh blood sample to identify circulating microfilariae (Rawlings, 1986), with sensitivity enhanced by concentration techniques such as the modified Knott test or filtration methods (Georgi & Georgi, 1992). Despite the overall reliability of these diagnostic tools, false-negative and false-positive results can occur. Therefore, follow-up testing one year after the initial screening is recommended to confirm infection status (Nelson et al., 2024; Ware et al., 2022).

Additional diagnostic tools can aid in assessing the severity of HWD. Thoracic radiography is valuable for evaluating cardiopulmonary involvement and often reveals characteristics findings such as pulmonary artery enlargement and tortuosity (Bowman & Atkins, 2009; Bussadori, 2023). Point of care ultrasound (POCUS) is particularly useful in dogs presenting with respiratory distress, ascites or abdominal distention, right-sided heart murmurs, abnormal pulmonary auscultation, or advanced clinical signs such as cyanosis or syncope. It can detect cardiac abnormalities, as well as distension of the caudal vena cava and hepatic veins. However, comprehensive echocardiography remains the gold standard for cardiac assessment. It enables direct visualization of adult heartworms and provides detailed evaluation of intracardiac and pulmonary changes associated with disease progression (Nelson et al., 2024; Ware et al., 2022).

Heartworm disease is more manageable in asymptomatic dogs compared to those with moderate or severe clinical signs. The main therapeutic objective is to eliminate all life stages of *D. immitis* while minimizing complications, thereby improving the patient's clinical condition and overall quality of life. A fundamental initial step in treatment is strict physical activity restriction, which reduces the risk of cardiopulmonary complications, particularly pulmonary thromboembolism (Nelson et al., 2024). Although often unavoidable, especially in advanced HWD, pulmonary thromboembolism results

from embolization of dead worm fragments, which activate the complement cascade and promote platelet aggregation and fibrin deposition. Clinical signs of thromboembolism may appear 7-10 days following the initiation of adulticide therapy and may include fever, coughing, and worsening signs of right-sided heart failure (Hirano et al., 1992; Ware et al., 2022).

Once HWD is confirmed, treatment should begin with stabilization of the patient. In symptomatic cases, prednisolone should be administered. Regardless of clinical presentation, all dogs should receive monthly macrocyclic-lactone-based heartworm prevention, doxycycline, strict activity restriction, and a monthly mosquito-repellent ectoparasiticide. If microfilariae are detected via microfilaria testing, treatment with an antihistamine or a glucocorticoid such as prednisolone should be started, if not already started. Macrocyclic lactones not only prevent new infections but also contribute to the reduction of circulating microfilariae (Nelson et al., 2024; Ware et al., 2022). Doxycycline plays a central role in HWD treatment due to its activity against *Wolbachia*, the endosymbiotic bacteria essential for microfilarial embryogenesis. Administered at 10 mg/Kg twice daily for 28 consecutive days, doxycycline significantly reduces *Wolbachia* loads, thereby suppressing microfilaremia and interrupting the development of microfilariae beyond the L3 stage (Rossi et al., 2010). Additionally, doxycycline exhibits immunomodulatory properties, reducing inflammation and contributing to the elimination of L3 and L4 larval stages after one month of therapy (McCall et al., 2011). In animals that cannot tolerate doxycycline, rifampicin can be used as an alternative. Glucocorticoids such as prednisone, administered at anti-inflammatory doses, may help control clinical signs and prevent anaphylactic reactions. Their use is recommended either in symptomatic patients during the initial stabilization phase or later during treatment in conjunction with melarsomine. Non-steroidal anti-inflammatory drugs, including aspirin, are not recommended due to their limited efficacy and potential risks (Nelson et al., 2024; Ware et al., 2022).

Following completion of doxycycline therapy, a one-month rest period is advised before initiating adulticide treatment with melarsomine. During this interval, monitoring for anaphylaxis and managing discomfort with analgesics such as tramadol or gabapentin is recommended. The adulticide protocol begins with a single 2.5 mg/Kg intramuscular injection of melarsomine administered deeply into the epaxial muscles between the third and fifth lumbar vertebrae (Nelson et al., 2024). Melarsomine is specifically effective against immature worms, particularly L2 and L4 stages, and retains its efficacy even when administered concurrently with prednisolone (Dzimianski et al., 1989, 2010).

The standard adulticide regimen continues with a second melarsomine injection administered one month after the first, followed by a third injection 24 hours later. Post-treatment exercise restriction should continue for an additional 6 to 8 weeks. One month after the final injection, a quantitative microfilariae test should be performed. If microfilariae persist, a microfilaricide should be administered and testing repeated after four weeks. Persistent positive results may suggest macrocyclic

lactone resistance and warrant further investigation. Once microfilaremia has been eliminated, lifelong prophylaxis with macrocyclic lactones should be instituted, especially in endemic areas. Gradual return to normal activity is allowed, and annual screening is recommended to ensure continued protection (Nelson et al., 2024).

In advanced stages of HWD, PH may develop as a consequence of mechanical obstruction of the pulmonary vasculature and chronic pulmonary parenchymal inflammation (Johnson, 1999). In severe cases, the disease may progress to caval syndrome, characterized by the migration of adult worms into the right atrium and vena cava. This condition is life-threatening and requires emergency surgical extraction of the worms via the jugular vein (Nelson et al., 2024; Ware et al., 2022).

1.4. Heartworm diseases: pathophysiological complications

1.4.1. Pulmonary hypertension

Pulmonary hypertension is a hemodynamic and physiological condition characterized by elevated pressure within the pulmonary vasculature (Reinero et al., 2020). In human medicine, PH is classically defined by a mean pulmonary arterial pressure (PAP) exceeding 25 mmHg at rest, as measured by right heart catheterization (Hoeper et al., 2013). However, recent evidence suggests that a mean PAP above 20 mmHg should be considered pathological, as the former threshold is now viewed as arbitrary and not representative of the upper physiological limit in the general population (Condon et al., 2019). In veterinary medicine, PH is typically diagnosed when systolic PAP exceeds 30 mmHg or mean PAP surpasses 20 mmHg (Bussadori, 2023; Kligfield, 1998).

The pathophysiology of PH is multifactorial and may result from increased pulmonary blood flow (e.g., congenital left-to-right shunts), elevated pulmonary vascular resistance (as observed in HWD), or increased pulmonary venous pressure (Reinero et al., 2020). Pulmonary hypertension is commonly classified as either precapillary or postcapillary (Kellihan & Stepien, 2010). Postcapillary PH is primarily associated with left-sided heart disease and is driven by increased pulmonary venous pressure or pulmonary blood flow. In contrast, precapillary PH is characterized by elevated PAP due to increased pulmonary vascular resistance (PVR), in the absence of elevated left atrial pressure, thereby maintaining a normal pulmonary arterial wedge pressure (PAWP) (Kellihan & Stepien, 2012).

Reinero et al. (2020) proposed a classification in dogs adapted from the human classification system (Simonneau et al., 2013). This system categorizes PH into six groups based on aetiology, with group 5 encompassing parasitic diseases such as *D. immitis* and *Angiostrongylus vasorum*. This classification integrates pathophysiological, haemodynamic, and clinical parameters, aiming to improve

diagnostic accuracy and facilitate more targeted therapeutic strategies, ultimately enhancing clinical outcomes (Kellihan & Stepien, 2010).

As previously mentioned, although right heart catheterization remains the gold standard for diagnosing PH, its invasive nature limits its use in veterinary medicine. Instead, transthoracic echocardiography with spectral Doppler is routinely employed to estimate the probability of PH. Systolic PAP can be estimated by measuring the peak of tricuspid valve regurgitation velocity (TRV) and applying the modified Bernoulli equation to determine the pressure gradient between the right ventricle and the right atrium, assuming there is no right ventricular outflow tract obstruction (Reinero et al., 2020; Ware et al., 2022). Tricuspid valve regurgitation velocity is a central parameter for estimating PH probability and is influenced by multiple factors, including right ventricular function and pericardial compliance (Reinero et al., 2020; Studley et al., 2003). Additionally, pulmonary regurgitation velocity can be used to estimate mean and diastolic PAP. The diastolic PAP can be derived from the peak diastolic pulmonary regurgitation velocity combined with the estimated right ventricular pressure (Ge et al., 1992), while the mean PAP can be estimated from the peak diastolic velocity alone (Masuyama et al., 1986). PH severity is often classified as mild, moderate, or severe based on pressure gradient estimates, although these cut-offs are not standardized. A TRV > 3.4 m/s, corresponding to a pressure gradient > 46 mmHg, is widely accepted as indicative of at least moderate PH (Reinero et al., 2020; Ware et al., 2022).

However, echocardiography alone is insufficient for definitive PH diagnosis. A comprehensive assessment incorporating echocardiographic findings, clinical signs, and ancillary diagnostic tests is essential. In regions endemic for HWD, PH is a frequent complication. Clinically, the most common sign is coughing, often accompanied by a heart murmur on auscultation. Other signs include exercise intolerance and syncope, while advanced cases may exhibit features of right-sided heart failure, such as ascites, cyanosis, and jugular venous distension or pulsation (Johnson et al., 1999; Ware et al., 2022).

Pulmonary hypertension classification is critical for guiding therapeutic decisions, as the underlying aetiology and chronicity influence treatment response and prognosis. Management has two primary goals: alleviating clinical signs and addressing the underlying cause to slow or halt disease progression and complications (Kellihan & Stepien, 2010). For PH secondary to HWD, treatment should follow established heartworm management protocols as previously outlined. Supportive strategies include exercise restriction, prevention of concurrent infections (respiratory or parasitic), and avoidance of unnecessary procedures (Reinero et al., 2020). Pharmacological treatment of PH focused on pulmonary vasodilation, with first-line therapy targeting the nitric oxide pathway via phosphodiesterase-5-inhibitor (PDE5i), such as sildenafil. These agents reduce PVR, limit PAP elevation, and mitigate disease progression. Sildenafil is generally well tolerated in dogs and has been associated with improved clinical outcomes, particularly in exercise tolerance and quality of life (Bach et al., 2006).

1.4.2. Caval syndrome

Caval syndrome is a severe and potentially fatal complication of chronic HWD, particularly affecting middle-aged male dogs with a high parasite burden (Atkins, 1987). The syndrome is characterized by the abnormal migration of *D. immitis* into the right ventricle, right atrium and vena cava (Jackson et al., 1966). As the disease progresses, mean PAP rises, while endothelial-dependent relaxation of the pulmonary artery diminishes, contributing to the development of PH (Strickland, 1998). Although primarily associated with advanced HWD, caval syndrome may also occur iatrogenically following the administration of dichlorvos (Eaton & Rosol, 1989) or, less commonly, after melarsomine therapy (Strickland, 1998).

On physical examination, a new heart murmur, typically involving the tricuspid valve, may be auscultated. Additional clinical signs reflecting low cardiac output include weak pulse, tachycardia, tachypnoea and other manifestations of right-sided heart failure (Strickland, 1998). Caval syndrome is also associated with intravascular haemolysis, resulting in hemoglobinemia, haemoglobinuria, and a regenerative haemolytic anaemia. These haemolytic processes are attributed to mechanical trauma from the intravascular worms. Concurrent renal and hepatic dysfunction is common, often evidenced by elevated serum biochemical markers (Ishihara et al., 1978; Ware et al., 2022).

Echocardiography is the primary diagnostic modality, typically revealing right ventricle dilatation and hypertrophy. It can also demonstrate a visible worm mass traversing the tricuspid valve, along with moderate to severe tricuspid insufficiency and tricuspid regurgitation, detectable by spectral and colour Doppler imaging (Atkins et al., 1988; Strickland, 1998).

Caval syndrome is a multisystemic condition with a guarded to poor prognosis, even with appropriate treatment. Management requires prompt surgical extraction of the worms, usually via the jugular vein (Jackson et al., 1977), in conjunction with supportive care such as fluid therapy and oxygen supplementation, to reduce PAP and right ventricular afterload. Additional supportive interventions may be warranted on a case-by-case basis (Rawlings & Tackett, 1990). Once the patient is stabilized, adulticide therapy with melarsomine is recommended to eliminate any remaining parasites not removed during surgery (Strickland, 1998).

1.5. Inflammatory mechanisms in heartworm disease

The pathophysiology of HWD is mainly due to the presence of worms in the heart. These worms damage the pulmonary arteries when they reach their final location, although the right heart chambers can also be damaged (Ames & Atkins, 2020; Falcón-Cordón et al., 2022; McCall et al., 2008b). In fact, heartworms cause vascular inflammation and endothelial damage, resulting in the proliferation of villi in the intima. This activates an inflammatory response involving leukocyte and platelet chemotaxis,

with releasing factors that promote smooth muscle proliferation and collagen accumulation, resulting in fibrosis. This culminates in proliferative endarteritis, resulting in occlusion of the vascular lumen and remodelling of the arteries (Bowman & Atkins, 2009; Carretón et al., 2017a).

During HWD treatment, the death of parasites can lead to complications such as pulmonary thromboembolism (McCall et al., 2008a). Prednisolone, which is sometimes used in treatment, increases the thrombotic risk (Johannesdottir et al., 2013; Stuijver et al., 2013).

Pulmonary thromboembolism is a frequent complication, often triggered by the death of adult worms, leading to granulomatous inflammation and arterial obstruction. Hypoxia, caused by ventilation-perfusion mismatch and endothelial-derived vasoactive substances, results in sustained vasoconstriction. All these pathological alterations had a negative impact, increasing the afterload of the right ventricle and leading to a hypertensive stage. This justifies the fact that HWD can lead to PH, already referred previously and, in more severe cases, right-sided heart failure (Bowman & Atkins, 2009; Carretón et al., 2017a).

Microfilariae further contribute to inflammation, particularly in the lungs. Eosinophilic pneumonitis is an immune-mediated reaction to the clearance of antibody-microfilaria complexes from the pulmonary microcirculation. This mainly occurs in occult heartworm infections. These complexes are surrounded by neutrophils and eosinophils, which can lead to the formation of granulomas. Focal lesions are more common than generalized ones and are more closely linked to the death of the worms. Dead parasites obstruct microcirculation, exacerbate endothelial damage, increase coagulation, and may cause consolidation of lung lobes (Bowman & Atkins, 2009).

The endosymbiotic bacteria, *Wolbachia*, commonly associated with heartworms, also intensifies the release of pro-inflammatory mediators and consequently amplifying the inflammatory response (Falcón-Cordón et al., 2022; McCall et al., 2008b; Méndez et al., 2014). This bacteria also contributes to immune-mediated kidney disease due to alterations in the glomerular basement membrane, which leads to proteinuria, a condition commonly associated with *D. immitis* and exacerbated by PH (Carretón et al., 2020; Ludders et al., 1988). However, kidney values (creatinine and urea) are normal, meaning there is no azotaemia. Additionally, microfilariae can deposit in the kidney and cause damage (Carretón et al., 2020), and their sudden death can result in the release of large amounts of antigenic particles (Carretón et al., 2014a).

1.6. Biomarkers in heartworm disease

In canine HWD, vascular and inflammatory alterations can cause changes in cardiac, haemostatic and inflammatory biomarkers (Yoon et al., 2017). Among the most relevant cardiac biomarkers are cardiac troponin T, cardiac troponin I (cTnI), myoglobin, and N-terminal pro-B-type

natriuretic peptide (NT-proBNP). Cardiac troponin T, cTnI and myoglobin are markers of myocardial injury, released into circulation in response to ischemia or necrosis (Carretón et al., 2014a, 2017a). Troponin T levels are generally undetectable in both healthy and heartworm-infected dogs (Carretón et al., 2011), whereas cTnI levels increase in *D. immitis* infections, particularly in dogs with high worm burdens (Carretón et al., 2013). Myoglobin levels also tend to be elevated; however, their diagnostic specificity is limited in veterinary medicine, as myoglobin can originate from both cardiac and skeletal muscle. Furthermore, myoglobin is rapidly degraded and, given the low incidence of primary cardiac ischemic in dogs, its clinically utility in HWD is restricted (Carretón et al., 2011).

The production of NT-proBNP in stimulated by chronic ventricular pressure or volume overload, and serves as a well-established biomarker of cardiac function (Boswood et al., 2008; Oyama et al., 2013). It is widely used for the diagnosis and monitoring of congestive heart failure. In veterinary medicine, NT-proBNP levels are elevated in both post-capillary, and, to a lesser extent, pre-capillary PH. However, in dogs with HWD and PH, NT-proBNP concentrations do not appear to differ significantly between microfilaremic and amicrofilaremic individuals (Costa-Rodríguez et al., 2023). Notably, elevated NT-proBNP levels are primarily observed in dogs with severe HWD, whereas normal dogs and dogs with moderate clinical signs typically exhibit values below the reference range. This suggests that significant increases in NT-proBNP are associated with advanced cardiopulmonary damage, including PH and pulmonary thromboembolism, which are more common in severe stages of the disease (Carretón et al., 2014b).

Among haemostatic biomarkers, D-dimer reflects fibrin degradation and is a sensitive indicator of thromboembolic activity. Elevated D-dimer concentrations are commonly observed in dogs with a high worm burden, particularly during treatment, unlike dogs with milder infections (Carretón et al., 2011, 2017a). Additionally, D-dimer levels may increase in dogs treated with prednisolone, as glucocorticoids exhibit procoagulant effects and may exacerbate vascular inflammation (Bowman & Atkins, 2009; Rawlings, 1986).

Inflammatory biomarkers include acute phase proteins (APPs), which are nonspecific indicators of systemic inflammation and tissue injury (Carretón et al., 2017a). These comprise positive APPs, such as C-reactive protein (CRP), ceruloplasmin, and haptoglobin (Hp) (Ceron et al., 2005), as well as negative APPs like albumin and paraoxonase-1 (PON-1). In canine HWD, a marked decreased in negative APPs is observed, particularly in dogs with PH (Carretón et al., 2017b; Méndez et al., 2014).

Positive APPs, such as CRP, rise significantly in HWD and play an important role in chronic vascular remodelling (Carretón et al., 2017b), while Hp levels decline, especially in microfilaremic dogs (Méndez et al., 2014). However, severe tissue damage occurs in other parasitic diseases, such as ehrlichiosis (Mylonakis et al., 2011) and babesiosis (Matijatko et al., 2007). C-reactive protein is

synthesized by hepatocytes in response to inflammatory cytokines, such as TNF-α and interleukins, and may serve as a prognostic marker for PH, independently of parasite burden (Venco et al., 2014).

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide produced by endothelial and other cell types, contributing to vascular remodelling, cell proliferation, and cardiopulmonary damage in PH. ET-1 levels correlate strongly with the severity and progression of PH in HWD (Carretón et al., 2017a; Falcón-Cordón et al. 2022). Conversely, oxidative stress markers have shown limited diagnostic utility in this context (Carretón et al., 2017b). Adiponectin, an adipokine with anti-inflammatory and cardioprotective properties, also plays a role in modulating the inflammatory and remodelling processes associated with PH (Ouchi & Walsh, 2007; Villarreal-Molina & Antuna-Puente, 2012; Weng et al., 2011).

During HWD treatment, levels of CRP and IL-6 often rise, suggesting ongoing inflammatory responses (Yoon et al., 2017). Following appropriate adulticide therapy, CRP levels tend to decrease, reflecting reduced vascular inflammation due to the clearance of microfilaria and Wolbachia endosymbionts (Carretón et al., 2013). Greater reductions in CRP are observed in dogs with higher worm burdens (Méndez et al., 2015). Although parasite death may cause arterial lesions, pre-treatment with anti-inflammatory strategies can help mitigate this effect and reduce vascular injury during adulticide administration (Kramer et al., 2011).

2. STUDY: ANNEXIN A1, AS A POTENTIAL BIOMARKER IN CANINE HEARTWORM DISEASE

2.1. Objectives

This study aimed to evaluate the potential of AnxA1 as a biomarker in HWD and its complication, PH. Serum AnxA1 concentrations were measured in three groups: healthy dogs, dogs diagnosed with HWD, and dogs with HWD-associated PH. The study also sought to investigate associations between AnxA1 levels and various clinical and haematological parameters, including complete blood count, sex, reproductive status, and age, both within and across groups. In the diseased cohorts, additional variables, such as clinical signs, NT-proBNP, CRP, cortisol concentrations, and estimated parasite burden, were analysed to explore potential correlations with serum AnxA1 levels.

2.2. Material and methods

A total of 135 dogs were recruited between 2020 and 2024 from the Veterinary Teaching Hospital at the University of Las Palmas de Gran Canaria, located in the Canary Islands (Spain), a hyperendemic region for HWD caused by *D. immitis* (Montoya-Alonso et al., 2024; Montoya-Alonso et al., 2022). All animals underwent a thorough clinical evaluation, including anamnesis, physical examination, complete blood count, and screening for HWD using a commercial immunochromatographic test (Uranotest Dirofilaria®, Urano Vet SL, Barcelona, Spain), which reports 94% sensitivity and 100% specificity for *D. immitis*. Testing was performed according to the manufacturer's instructions. Dogs testing positive were further evaluated for microfilaremia using a direct smear and the modified Knott technique.

Detailed data were recorded for each dog, including age, breed, sex, reproductive status, weight, and body condition score, assessed using a nine-point scale (Laflamme, 1997). Based on HWD test results, dogs were categorized into two groups: a control (CTRL) group of healthy, uninfected dogs (Uranotest Dirofilaria®-negative) and an HWD group (Uranotest Dirofilaria®-positive). Healthy dogs, recruited between 2023 and 2024, also underwent biochemical analysis. Infected dogs were evaluated for clinical signs and classified as symptomatic if they exhibited at least one sign consistent with HWD, such as coughing, exercise intolerance, abnormal lung sounds, dyspnoea, hepatomegaly, syncope, or ascites. In all infected dogs, serum concentrations of NT-proBNP, CRP, and cortisol were measured at the time of diagnosis.

Inclusion criteria required written informed consent from all dog owners, no prior diagnosis of HWD, and the absence of known concurrent diseases. Dogs with suspected concomitant cardiopulmonary conditions, based on clinical history, anamnesis, physical examination, or clinical signs, were excluded. Additionally, animals previously treated with glucocorticoids, such as prednisolone, were not eligible for inclusion.

Echocardiographic assessments were performed on all infected dogs using an ultrasound machine with spectral and colour Doppler and multifrequency probes (2.5-10 MHz, Viviq Iq®, General Electric, Boston, MA, USA). Examinations were conducted in conscious, unsedated animals positioned in right and left lateral recumbency, with the transducer placed between the third and fifth intercostal spaces. An electrocardiogram was monitored throughout the procedure, and all evaluations were performed by the same cardiologist. A complete echocardiographic study was conducted, recording the following parameters: pulmonary artery flow velocity (vel. PA, m/s); pressure gradient across the pulmonary valve (ΔP PV, mmHg); right pulmonary artery diameter in systole (RPAs); right pulmonary artery diameter in diastole (RPAd); right pulmonary artery distensibility index (RPADi); pulmonary valve regurgitation velocity (vel. PR, m/s); TRV (m/s); and pressure gradient across the tricuspid valve (ΔP TV, mmHg). For each variable, three complete and consecutive cardiac cycles were acquired. The presence and severity of PH were determined based on echocardiographic findings, following the ACVIM guidelines, which stratify PH as mild, moderate or severe (Reinero et al., 2020).

Parasite burden was assessed via echocardiography using a four-point semi-quantitative scoring system. Scores 1 and 2 indicated a low parasite burden: score 1 denoted no visible worms, while score 2 represented a small number of worms localized in the distal pulmonary artery. Scores 3 and 4 reflected a high parasite burden: score 3 indicated worms occupying the right and main pulmonary arteries, and score 4 represented extensive infestation involving the entire right pulmonary artery, the main pulmonary artery, and the pulmonary valve (Venco et al., 2003).

Based on the presence or absence of PH, the infected dogs were categorized into two subgroups. Initially, the study included three groups of 45 dogs each. However, six dogs were excluded due to prior administration of prednisolone at the referring veterinary clinic in response to life-threatening pulmonary thromboembolism, two from the HWD without PH group and four from the HWD with PH group, per the study's exclusion criteria. The final distribution consisted of 45 healthy dogs (CTRL group), 43 dogs diagnosed with HWD without PH (HWD group), and 41 dogs diagnosed with HWD and concurrent PH (HWD+PH group).

Blood samples were collected from the cephalic vein into serum tubes and refrigerated until processing. Centrifugation was performed within two hours of collection, at 1432 g for 10 minutes. The resulting serum was aliquoted into Eppendorf tubes and stored at −80°C until analysis. NT-proBNP, CRP and cortisol concentrations were measured using the VCHECK immunochromatographic analyser

(Bionote®, Big Lake, MN, USA), according to the manufacturer's instructions. However, the VCHECK system does not quantify NT-proBNP values below 499 pmol/L, despite the reference range for healthy dogs being <900 pmol/L (Harr et al., 2022). Similarly, the detection limit for CRP is 10 mg/L, with values below this threshold considered normal. The reference range for cortisol was 1-5.5 µg/dL. Serum AnxA1 levels were determined using a Canine Annexin A1 ELISA Kit (MyBiosource®, Inc., CA, USA). All analyses were conducted at the Pharmacology and Therapeutics Unit, Department of Biomedicine, Faculty of Medicine, University of Porto, according to the manufacturer's protocol.

Data normality was assessed using the D'Agostino & Pearson test, and all variables failed to meet the assumption of normality ($p > 0.05$). Continuous variables were reported as medians with interquartile ranges (25^{th}-75^{th} percentiles). When testing a hypothesis about continuous variables, the Kruskal-Wallis test was used for comparisons across more than two groups, while the Mann-Whitney U test was employed for pairwise comparisons. A p-value < 0.05 was considered statistically significant, and 95% confidence intervals were used throughout. Statistical analysis was conducted using GraphPad Prism 10.5.0 (GraphPad Prism Software®, Inc., CA, USA).

All the owners gave their consent to participate in this study. The study was approved by the ethical committee of the Veterinary Medicine Service of the University of Las Palmas de Gran Canaria and was carried out in accordance with the current European legislation on animal protection.

2.3. Results

A total of 129 dogs were divided into three groups as previously described. The CTRL group included 45 healthy dogs: 20 mixed breeds (44.4%), 8 Canarian Warren Hounds (17.8%), and 2 dogs (4.4% each) from Belgian Malinois, Miniature Pinscher, and American Staffordshire Terrier breeds. Additionally, one dog (2.2%) each represented the following breeds: Andalusian Terrier, French Bulldog, Chihuahua, American Cocker Spaniel, Argentinian Mastiff, Siberian Husky, German Shepherd, Canary Dog, English Setter, Shih Tzu, and Staffordshire Bull Terrier. Sex and reproductive status distribution are detailed in Table 3, while age and body weight data are summarized in Table 4. The HWD group, comprising 43 dogs without PH, included 27 mixed breeds (62.8%), 3 Canary Dogs (7.0%), 2 Canarian Warren Hounds (4.7%), and one dog (2.3%) from each of the following breeds: Alaskan Malamute, Doberman, Majorero Dog, English Bulldog, Chihuahua, Siberian Husky, Labrador Retriever, German Shepherd, Belgian Malinois, American Pit Bull Terrier, and Rottweiler. The HWD+PH group consisted of 41 dogs, including 23 mixed breeds (56.1%), 2 dogs (4.9% each) from Andalusian Terrier, French Bulldog, Labrador Retriever, American Pit Bull Terrier, Canarian Warren Hound, and Canary Dog breeds, and one dog (2.4%) each of Majorero Dog, Pug, Chihuahua, Fox Terrier, Garafian Shepherd, and Miniature Pinscher. For the HWD and HWD+PH groups, sex, reproductive status, clinical signs, and

parasite burden scores are presented in Table 3. NT-proBNP, CRP, and cortisol levels measured at diagnosis, along with age and body weight, are summarized in Table 4.

Table 3: Distribution of dogs by sex, reproductive status, clinical signs and parasite burden across the different study groups (with percentages).

		Number	**Percentage (%)**		**Number**	**Percentage (%)**
CTRL Group (n=45)	Male	26 dogs	57.8%	Intact	24 dogs	92.3%
				Neutered	2 dogs	7.7%
	Female	19 dogs	42.2%	Intact	17 dogs	89.5%
				Spayed	2 dogs	10.5%
HWD Group (n=43)	Male	21 dogs	48.8%	Intact	11 dogs	52.4%
				Neutered	10 dogs	47.6%
	Female	22 dogs	51.2%	Intact	17 dogs	77.3%
				Spayed	5 dogs	22.7%
	Symptoms			Yes	5 dogs	11.6%
				No	38 dogs	88.4%
	Parasite burden[1]			Score 1	1 dog	2.3%
				Score 2	25 dogs	58.1%
				Score 3	17 dogs	39.5%
HWD+PH Group (n=41)	Male	24 dogs	58.5%	Intact	17 dogs	70.8%
				Neutered	7 dogs	29.2%
	Female	17 dogs	41.5%	Intact	12 dogs	70.6%
				Spayed	5 dogs	29.4%
	Symptoms			Yes	32 dogs	78.0%
				No	9 dogs	22.0%
	Parasite burden[2]			Score 2	13 dogs	31.7%
				Score 3	15 dogs	36.6%
				Score 4	13 dogs	31.7%

[1]26 dogs (60.4%) with a low parasite burden and 17 dogs (39.5%) with a high parasite burden.

[2]13 dogs (31.7%) with a low parasite burden and 28 dogs (68.3%) with a high parasite burden.

Table 4: Demographic and biomarker data of dogs across study groups: age, body weight, NT-proBNP, CRP, and cortisol levels.

		Age (years)	Body weight (Kg)	NT-proBNP (pmol/L)	CRP (mg/L)	Cortisol (mcg/dL)
CTRL Group (n=45)	Median (25th - 75th percentile)	5 (3-9)	18 (9.25-23)			
	Minimum - Maximum	1 - 14	4.5 - 53			
HWD Group (n=43)	Median (25th - 75th percentile)	5 (3-7)	15.7 (9.1-26.0)	505.7 (499-662.9)	15.0 (9.99-24.0)	2.24 (1.32-2.98)
	Minimum - Maximum	1 -12	4.5 - 43	499 - 1061.8	9.99 - 201	0,99 - 9.35
HWD+PH Group (n=41)	Median (25th - 75th percentile)	6 (5-8)	10.65 (6.0-25.0)	1287 (499-2605)	21.27 (9.99-32.85)	2.26 (1.26-3.25)
	Minimum - Maximum	1 - 14	3.29 - 43.3	499 - 5632.5	9.99 - 112.00	0.99 - 9.96

In specific cases, a subset of dogs from the HWD and HWD+PH groups received one or more medications prior to blood collection, as outlined in Table 5. These drugs were administered as initial doses during transport to the referral hospital, due to life-threatening pulmonary thromboembolism emergencies. Additionally, Table 5 highlights (underlined) the first three antiparasitic drugs, which were given monthly as prophylaxis against parasitic diseases, including HWD.

Table 5: Medications administered prior to blood collection in dogs from HWD and HWD+PH groups.

	HWD Group	HWD+PH Group
Ivermectin and pyrantel pamoate monthly	3 dogs	3 dogs
Milbemycin oxime monthly	3 dogs	
Afoxolaner	1 dog	
Doxycycline	3 dogs	1 dog
Metronidazole		1 dog
Furosemide		2 dog
Sildenafil		1 dog
Diazepam and vitamins		1 dog

Annexin A1 levels could not be quantified in two dogs from the HWD group using the ELISA kit, as their absorbance values exceeded the upper limit of the standard curve, even at the maximum recommended dilution of 1:10. According to the kit manufacturer, dilutions beyond this ratio fall outside the validated range, rendering accurate quantification impossible for these samples. Additionally, AnxA1 concentrations in three dogs from the CTRL group and two from the HWD group were located at the extremes of the standard curve, a region associated with reduced measurement reliability. Nevertheless, these values were retained in the statistical analysis, as further dilution would have resulted in concentrations beyond the assay's detectable range.

2.3.1. Annexin A1 serum levels

The median AnxA1 serum concentration was 546.4 pg/mL (253.2-1695) in the CTRL group, 831.5 pg/mL (384.2-2766) in the HWD group, and 606.2 pg/mL (314.8-1440) in the HWD+PH group. No statistically significant differences were found among the groups (p = 0.2012) (Figure 2). Further analysis comparing the HWD and HWD+PH groups also revealed no significant difference in AnxA1 levels (p = 0.1913).

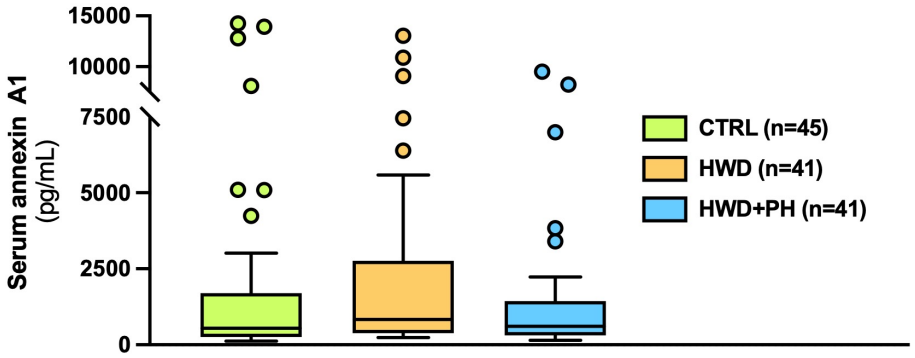

Figure 2 : Serum AnxA1 concentration distribution across study groups.

Some animals in the HWD and HWD+PH groups received medications prior to blood collection, as detailed in Table 5. Given that certain administered drugs, particularly antiparasitic agents (underlined in Table 5) and other listed medications, may influence serum AnxA1 levels, a secondary analysis was performed excluding these animals to minimize potential confounding effects. To ensure a more accurate evaluation of AnxA1 concentrations within the disease groups, all dogs recorded in Table 5 were excluded from this analysis, including those that had received only antiparasitic treatment, due to its possible impact on AnxA1 expression. The results of this refined analysis are shown in Figure 3. The Mann-Whitney U test yielded a p-value of 0.6502, indicating no statistically significant differences in serum AnxA1 concentrations between the HWD and HWD+PH groups. These findings are consistent with those observed in the initial analysis.

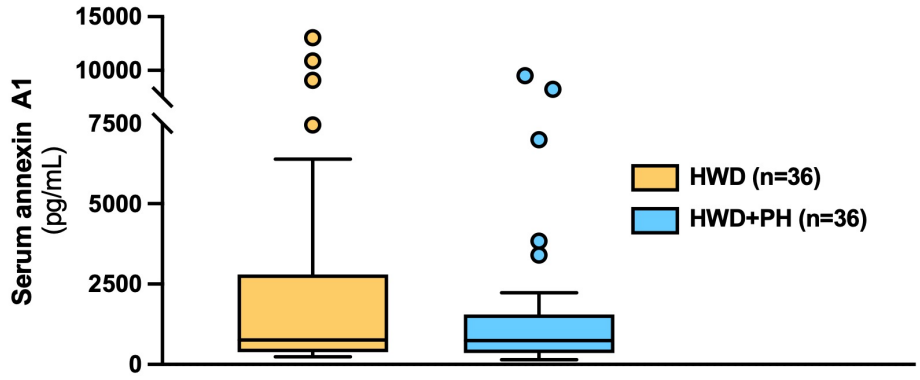

Figure 3: Distribution of AnxA1 serum levels in the diseased groups (HWD and HWD+PH), without prior medication.

In the HWD+PH group, PH severity was classified as mild (n=15), moderate (n=8), or severe (n=18). Serum AnxA1 levels were compared among these categories, with no significant differences observed (p = 0.5567). These findings suggest that AnxA1 concentrations are not associated with the severity of PH (Figure 4).

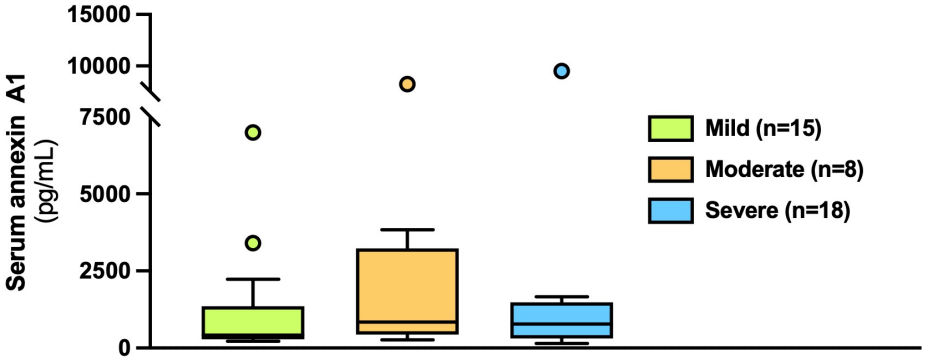

Figure 4 : Distribution of AnxA1 serum levels in the HWD+PH group, stratified by PH severity.

2.3.2. Annexin A1 serum levels and haematological parameters

A complete blood count was conducted for all three study groups. Given that HWD is an inflammatory condition, differences in leukocyte counts were anticipated. Comparative analysis revealed a statistically significant difference among the groups (p = 0.0002). Notably, the HWD+PH group exhibited a significantly elevated leukocyte count compared to both the CTRL and HWD groups, aligning with the expected inflammatory response. These findings are illustrated in Figure 5.

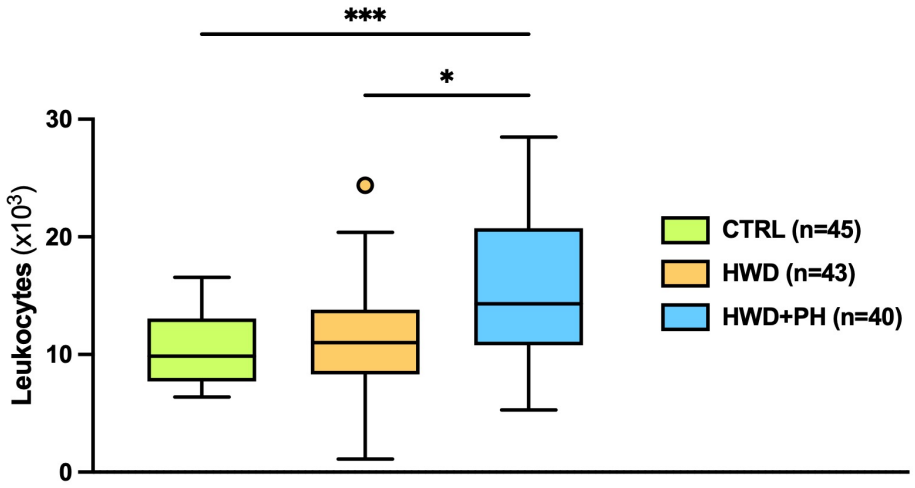

Figure 5: Distribution of leukocytes count in the study groups. * p< 0.05.***p=0.0002.

Leukocytes are key mediators of pro-inflammatory responses, whereas AnxA1 is associated with anti-inflammatory activity. To further explore the relationship between these parameters, a Spearman correlation analysis was conducted. In the CTRL group, a moderate negative correlation was identified between leukocyte count and serum AnxA1 levels ($p = 0.0252$; $r = -0.3333$, Figure 6), suggesting that elevated leukocyte levels are associated with reduced AnxA1 expression. However, no significant correlations were observed in the HWD ($r = 0.05647$; $p > 0.05$) or HWD+PH ($r = 0.04785$; $p > 0.05$) groups.

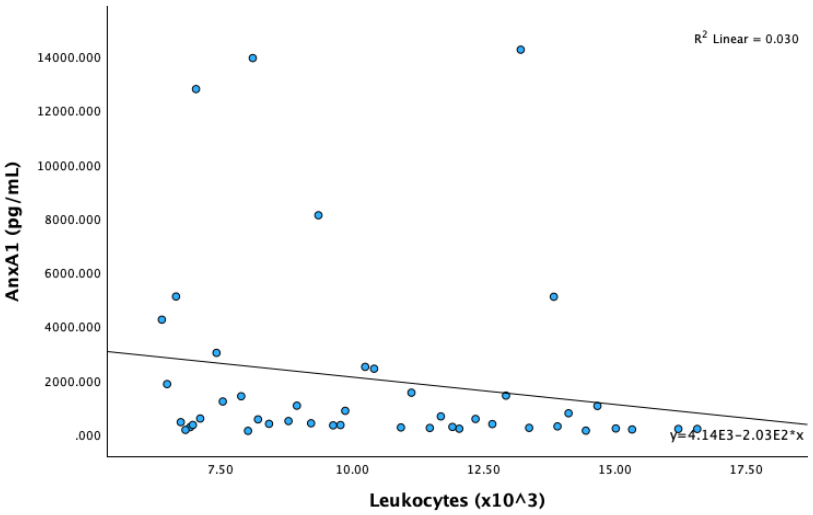

Figure 6: Correlation between AnxA1 serum levels and leukocytes counts in CTRL group.

To explore additional haematological alterations, further analyses were conducted to identify complete blood count parameters with statistically significant variation among the groups. A significant

reduction in red blood cell (RBC) count was observed in the HWD+PH group compared to the HWD group (p = 0.0326), as illustrated in Figure 7.

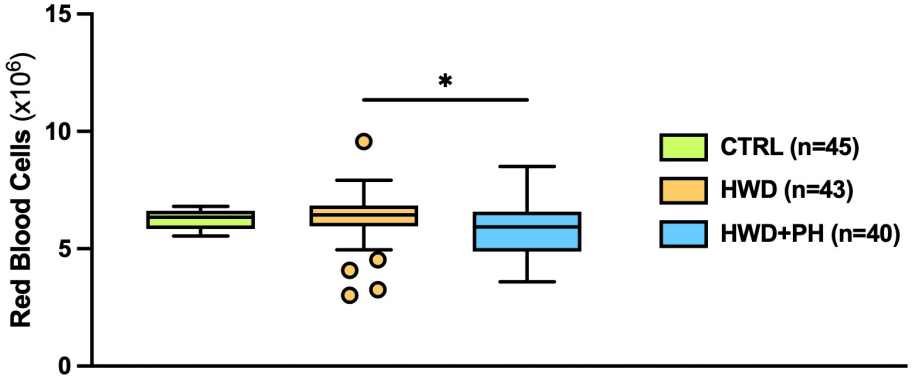

Figure 7: Distribution of RBC counts in the study groups. * p< 0.05

Given the previously observed differences, the correlation between AnxA1 and RBC was assessed using Spearman's method. In the HWD group, a moderate negative correlation was identified (r = –0.4439; p = 0.0036), indicating that higher RBC counts were associated with lower serum AnxA1 levels (Figure 8). In contrast, no significant correlations were found in the CTRL (r = 0.1236; p > 0.05) or HWD+PH (r = –0.05535; p > 0.05) groups. Additionally, a moderate negative correlation between AnxA1 and haematocrit was observed within the HWD group (r = –0.358; p = 0.021), further supporting the association between red cell parameters and AnxA1 expression in dogs with HWD.

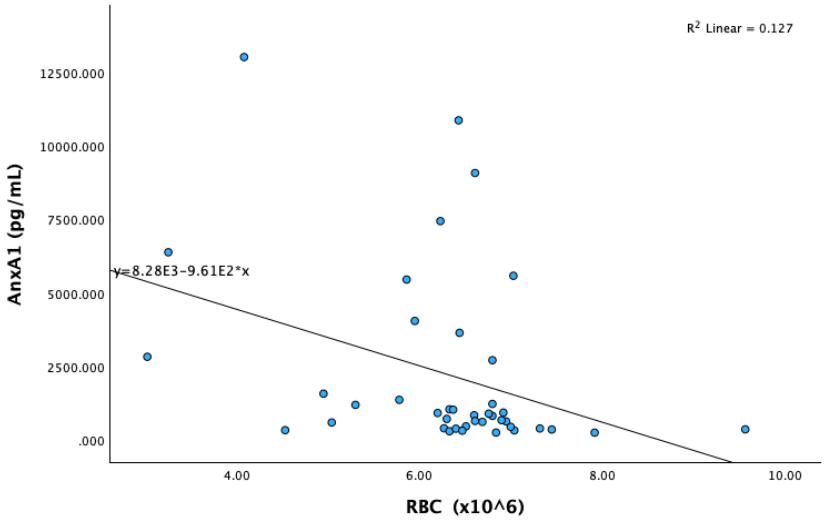

Figure 8: Correlation between AnxA1 serum levels and RBC counts in HWD group.

2.3.3. Sex-related variations in annexin A1 serum levels

Although AnxA1 serum levels did not differ significantly between groups, this anti-inflammatory protein may be influenced by factors beyond inflammation, including sex-related biological and hormonal differences. All groups included both sexes, as detailed in Table 3. To evaluate the potential influence of sex on AnxA1 levels, a three-step analysis was conducted. First, comparisons among male subgroups (CTRL, HWD, HWD+PH) revealed no significant differences ($p = 0.1962$). The same was observed among female subgroups ($p = 0.1952$). Second, sex-based comparisons within each group showed significantly higher AnxA1 levels in females than males in CTRL (864.5 pg/mL (402.1; 5101) vs. 338 pg/mL (231.3; 1148), $p = 0.0304$) and HWD (1217 pg/mL (420.1; 5215) vs. 630.3 pg/mL (330.5; 919.7), $p = 0.0264$) groups. No difference was found in HWD+PH ($p = 0.7485$). Third, comparisons between intact males and females within each group revealed no significant differences ($p > 0.05$), although a trend toward higher AnxA1 levels in intact females was noted in CTRL (864.5 pg/mL (391.9; 6607) vs 356.4 pg/mL (236.0; 1334), $p = 0.0880$). Similarly, no significant differences were observed between neutered males and spayed females ($p > 0.05$), though small sample sizes may have limited statistical power. Results are summarized in Figure 9.

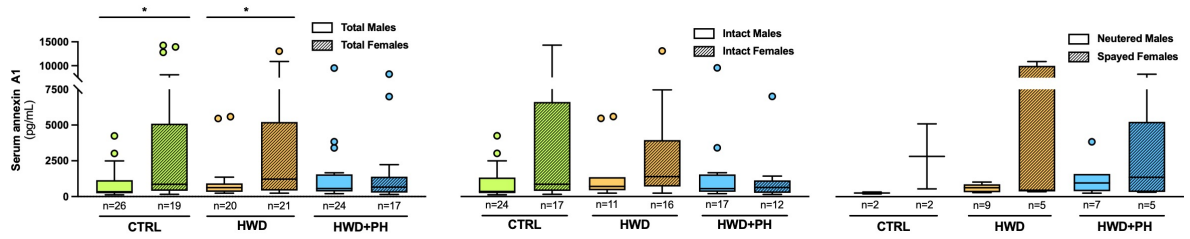

Figure 9: Distribution of AnxA1 serum levels by sex and reproductive status across the three study groups. **Left:** Total male and total female dogs from the three study groups. **Middle:** Intact males and intact females from the three study groups. **Right:** Neutered males and spayed females from the three study groups. * $p < 0.05$.

2.3.4. Age-related variations in annexin A1 serum levels

Age-related changes in cytokine expression, which regulate inflammatory pathways, may also affect anti-inflammatory proteins like AnxA1. To explore whether AnxA1 serum levels vary with age as a potential adaptive response, animals were classified into age groups (young, adult, senior, and geriatric) (Table 6), based on Idexx Laboratories charts, considering both age and body weight (Jiménez, 2023). For statistical analysis, young and adult groups were combined, as were senior and geriatric groups, collectively termed older dogs, due to the limited number of senior and geriatric animals (Table 6).

Table 6: Number of animals from each study group distributed by age categories.

	CTRL Group	HWD Group	HWD+PH Group
Young	8 dogs	10 dogs	4 dogs
Adult	24 dogs	28 dogs	28 dogs
Senior	11 dogs	2 dogs	7 dogs
Geriatric	2 dogs	1dog	2 dogs

Comparisons of AnxA1 levels between age groups revealed that, among young and adult dogs, the HWD group exhibited significantly higher AnxA1 levels than the CTRL group (758.4 pg/mL (379.3; 2737) vs. 360.9 pg/mL (213.5; 1168), p = 0.0352) (Figure 10). No significant differences were found in older dogs (Figure 10). Further analysis comparing young/adult dogs to older dogs across all groups (Figure 11) showed a significant increase in AnxA1 levels only in the CTRL group, with older dogs presenting higher levels (360.9 pg/mL (213.5;1168) vs. 1404 pg/mL (609.3; 4667), p = 0.0077). No significant age-related differences were observed in the HWD or HWD+PH groups.

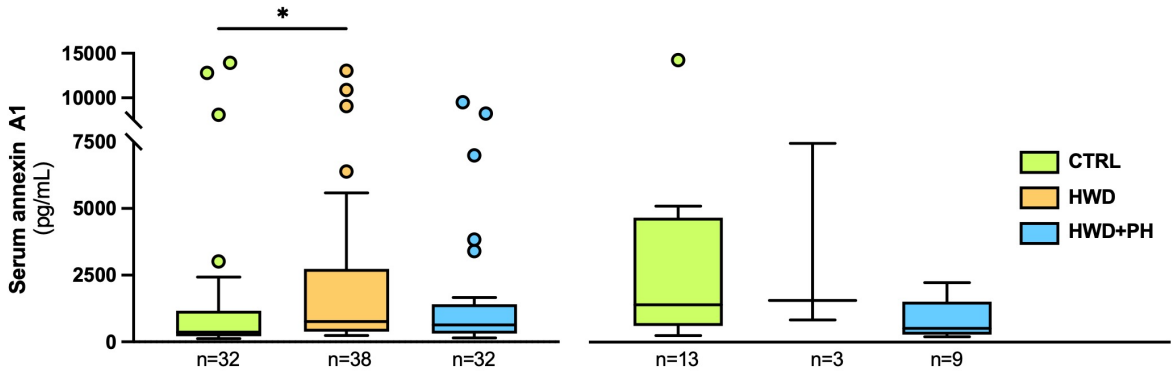

Figure 10: Distribution of AnxA1 serum levels in the three study groups in young and adult dogs (left) and older dogs (right). * $p < 0.05$.

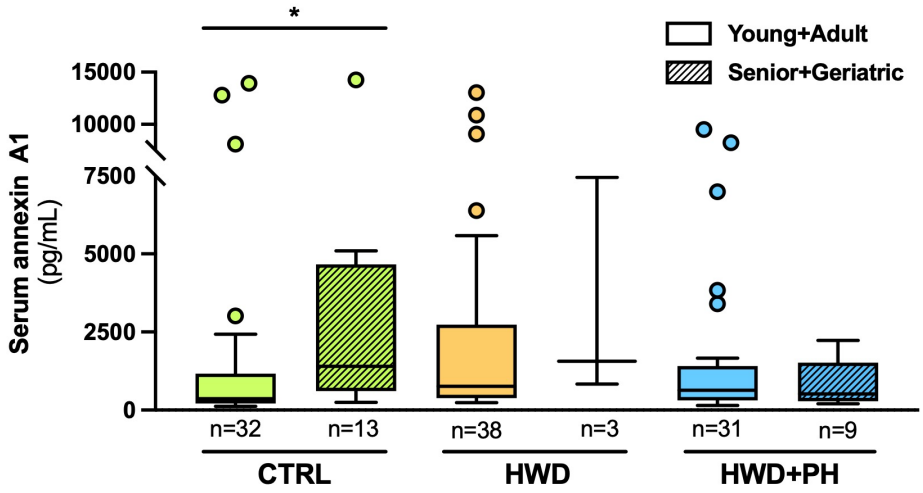

Figure 11: Distribution of AnxA1 serum levels in young and adult dogs compared to older dogs (senior + geriatric) in study groups. * $p < 0.05$.

To further assess the relationship between AnxA1 and age, a Spearman correlation analysis was performed. A moderate positive correlation was observed in the CTRL group ($p = 0.002$; $r = 0.4484$, Figure 12), indicating that AnxA1 serum levels tend to increase with age. In contrast, no significant correlations were found in the disease groups, with p-values > 0.05 and r-values of -0.04665 for HWD and -0.02292 for HWD+PH.

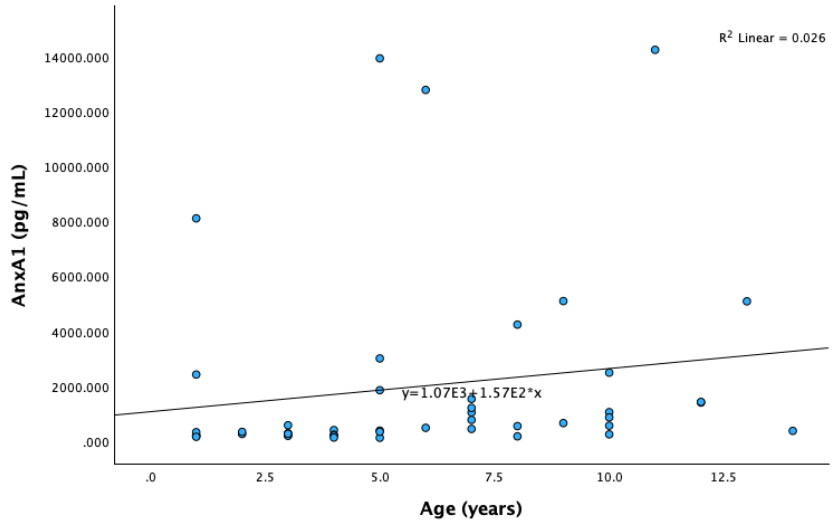

Figure 12: Correlation between AnxA1 serum levels and age in the CTRL group.

2.3.5. Impact of clinical signs in heartworm disease on annexin A1

A preliminary statistical analysis was performed to investigate the association between serum AnxA1 levels and the presence of clinical signs, aiming to determine if more severe disease, indicated

by clinical signs, is linked to elevated anti-inflammatory protein levels. Although dogs in the HWD+PH group exhibited significantly more clinical signs than those in the HWD group, no significant differences in AnxA1 serum levels were observed when comparing symptomatic dogs across disease groups. Similarly, comparisons of asymptomatic dogs between these groups also showed no significant variation in AnxA1 levels.

Annexin A1 serum levels were compared between symptomatic and asymptomatic dogs in the HWD and HWD+PH groups to assess potential associations with clinical signs. No significant differences were detected, indicating that AnxA1 levels do not correlate with the presence of clinical signs (Figure 13).

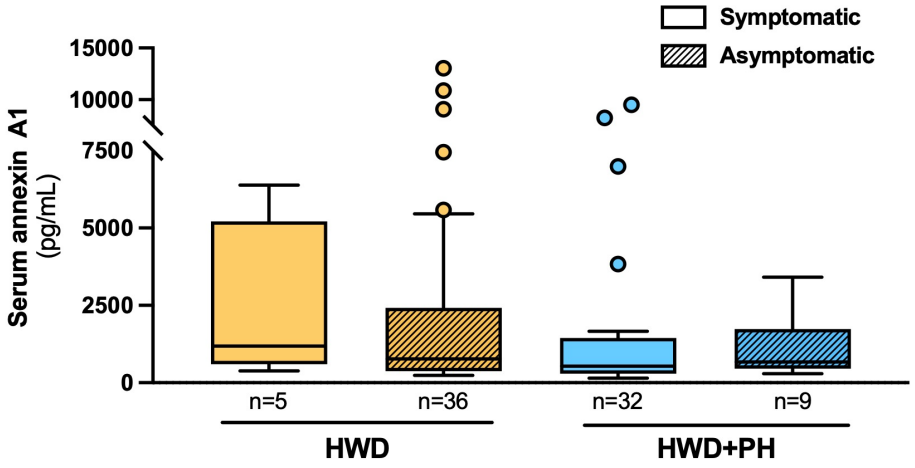

Figure 13: Distribution of AnxA1 serum levels in the symptomatic and asymptomatic dogs from HWD Group (left) and HWD+PH Group (right).

2.3.6. Blood biomarkers in heartworm disease and its association with annexin A1

Blood samples were collected on the day of HWD diagnosis, and serum levels of NT-proBNP, CRP, and cortisol were measured in both HWD and HWD+PH groups (Figure 14). NT-proBNP levels were significantly higher in the HWD+PH group compared to the HWD group ($p < 0.0001$), indicating a strong association between elevated NT-proBNP and the presence of PH. CRP levels showed no statistically significant difference between the two groups, although the p-value of 0.0912 suggests a trend toward higher CRP levels in the HWD+PH group. Given that AnxA1 is a glucocorticoid-regulated protein, serum cortisol levels were also measured to explore a potential relationship. No significant differences were observed between groups ($p = 0.9520$), indicating that cortisol concentrations were not associated with the presence of PH in dogs with HWD.

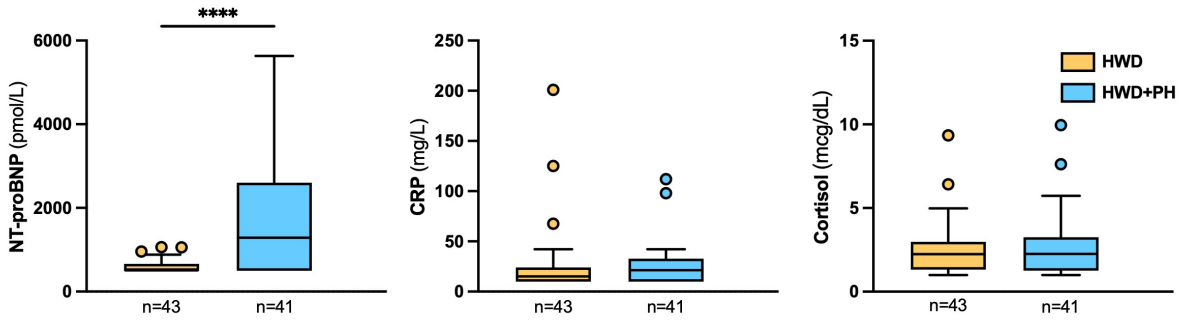

Figure 14: Distribution of NT-proBNP, CRP and cortisol serum levels in the diseased groups. **** p< 0.0001.

Correlations between AnxA1 and the measured blood biomarkers (NT-proBNP, CRP, and cortisol) were evaluated in both HWD and HWD+PH groups. No significant correlations were found between AnxA1 and NT-proBNP in either group (HWD: r = –0.00355, p > 0.05; HWD+PH: r = –0.1303, p > 0.05), indicating no relationship between these markers. Similarly, correlations between AnxA1 and CRP were not statistically significant (HWD: r = –0.06094, p > 0.05; HWD+PH: r = 0.06500, p > 0.05), supporting the absence of association between AnxA1 and CRP levels. In contrast, a statistically significant moderate negative correlation was observed between AnxA1 and cortisol in the HWD+PH group (r = –0.3165, p = 0.0438, Figure 15) suggesting that higher cortisol levels are associated with lower AnxA1 serum levels. No such correlation was found in the HWD group (r = 0.02180, p > 0.05).

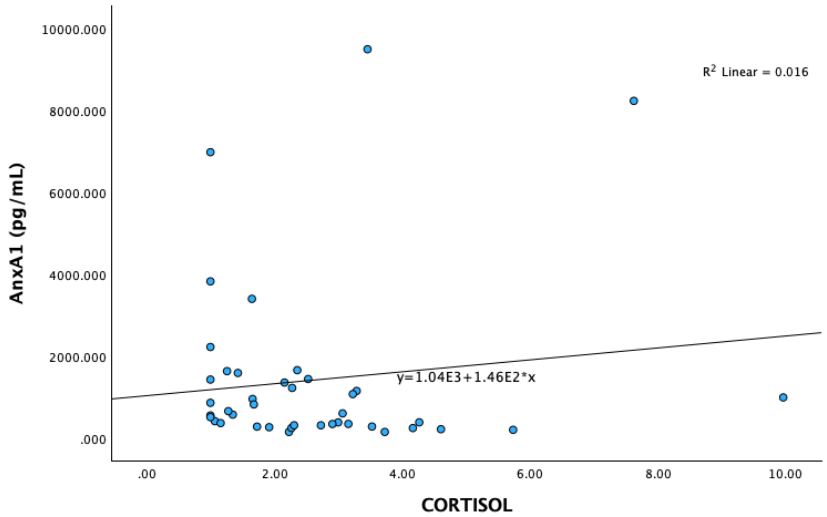

Figure 15: Correlation between AnxA1 and Cortisol in HWD+PH group.

2.3.7. Impact of parasite burden in heartworm disease on annexin A1

Parasite burden was significantly higher in HWD+PH dogs compared to HWD dogs (p = 0.0003), as shown in Figure 16. This suggests that the presence of PH in dogs with HWD is associated with a greater parasite load.

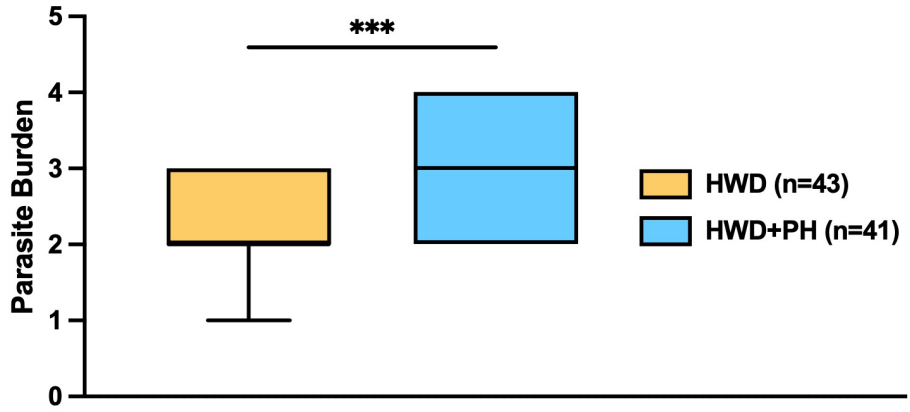

Figure 16: Distribution of Parasite Burden in the diseased groups (HWD and HWD+PH).***p=0.0003

To explore potential associations between AnxA1 serum levels and parasite burden, dogs were grouped by parasite scores (1–4). The HWD group included scores 1 to 3, while the HWD+PH group included scores 2 to 4. No statistically significant differences in AnxA1 levels were found across parasite scores in either group (p > 0.05). Further analysis was conducted by categorizing parasite burden as low (scores 1–2) or high (scores 3–4). Comparisons of AnxA1 levels between low and high burden groups yielded non-significant results (HWD: p = 0.2167; HWD+PH: p = 0.6232), indicating no association between parasite load and AnxA1 levels. These findings are summarized in Figure 17.

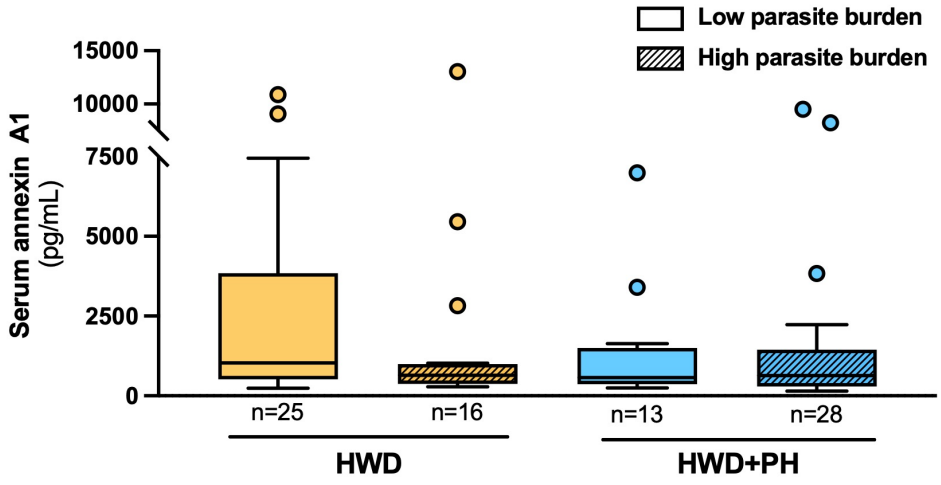

Figure 17: Distribution of AnxA1 serum levels in the low and high parasite burden in dogs from HWD Group (left) and HWD+PH Group (right).

2.4. Discussion

This study aimed to assess AnxA1 as a potential biomarker in canine HWD and secondary PH, given its established role in the modulation and resolution of inflammation. It was hypothesized that AnxA1 serum levels would increase with disease severity. In human medicine, elevated AnxA1 expression has been associated with poorer clinical outcomes in PH, suggesting prognostic potential; however, its diagnostic utility has not been established (Arvidsson et al., 2023). Contrary to expectations, no statistically significant differences in serum AnxA1 concentrations were observed between the study groups. This finding indicates that AnxA1 may not be exclusively responsive to inflammatory status and could be influenced by a broader range of physiological factors. Previous studies have reported that AnxA1 expression varies with sex and reproductive status (Hughes et al., 2013), as well as with age (Singh et al., 2024). Such variables may contribute to interindividual variability in AnxA1 levels, potentially masking associations with disease severity in this context.

Of the 135 dogs initially enrolled, six were excluded due to prior treatment with prednisolone, a glucocorticoid known to elevate serum AnxA1 levels (Buckingham & Flower, 1997), which could introduce bias in AnxA1 assessment. Group distribution was comparable regarding sex and body weight; however, the HWD+PH group had a significantly higher median age. As AnxA1 levels tend to increase with age (Singh et al., 2024), age may have acted as a confounding factor in this group, where higher AnxA1 levels were also expected due to increased inflammatory severity. Although no statistically significant differences in sex distribution were found between groups, the low number of neutered males and spayed females, particularly in the control group, may have limited the ability to detect associations related to sex and reproductive status, which are known to influence AnxA1 expression (Hughes et al., 2013). Future studies with larger and more balanced subgroup sizes are needed to clarify these potential effects.

Annexin A1 (AnxA1) serum levels were also assessed in dogs from the disease groups that were not receiving medication at the time of blood sampling, as pharmacological treatment may influence AnxA1 expression. Certain drugs, such as glucocorticoids (Flower, 1988) and estrogens (Hughes et al., 2013), are known to modulate AnxA1 levels. Among the treatments administered to dogs in the HWD and HWD+PH groups, doxycycline and sildenafil are of particular interest due to their potential direct effects on AnxA1 expression. Doxycycline, commonly used in HWD to suppress microfilaremia (Rossi et al., 2010), possesses immunomodulatory properties, reducing inflammation by inhibiting cytokines such as IL-6, TNF-α, and IL-1β (Cazalis et al., 2008; McCall et al., 2011). This could result in decreased AnxA1 levels. Sildenafil, a phosphodiesterase type 5 inhibitor (PDE5i) used in PH management, enhances the nitric oxide pathway, which exerts anti-inflammatory effects by reducing leukocyte

adhesion and platelet aggregation (Kapil et al., 2020). This mechanism may increase AnxA1 expression as inflammation is controlled.

Conversely, antiparasitic treatments used to eliminate heartworms may exacerbate inflammation through mechanisms such as granulomatous reactions and pulmonary thromboembolism following worm death (Carretón et al., 2013), potentially leading to elevated AnxA1 levels. Other administered drugs (e.g., metronidazole, furosemide, diazepam, and vitamin supplements) may also influence AnxA1, although their specific effects remain unclear and require further investigation.

To minimize potential confounding effects, a subgroup analysis was conducted excluding medicated animals. Contrary to expectations, no significant differences in AnxA1 levels were observed between the HWD and HWD+PH groups. This suggests that factors beyond inflammatory burden, such as sex, reproductive status (Hughes et al., 2013), and age (Singh et al., 2024), as previously referred, may contribute to interindividual variability in AnxA1 expression.

Annexin A1 serum levels were evaluated across different categories of PH severity to investigate a potential association between increasing disease severity and elevated expression of this anti-inflammatory protein. It was hypothesized that more advanced PH would correspond to higher AnxA1 concentrations, reflecting enhanced activation of inflammatory pathways. This assumption was based on previous findings in canine models showing that CRP levels correlate with disease severity and duration (Carretón et al., 2017a), and on evidence from human studies suggesting a prognostic role for AnxA1 in PH (Arvidsson et al., 2023). However, no statistically significant differences in AnxA1 serum levels were observed between PH severity groups. These findings suggest that AnxA1 expression may not be directly influenced by the degree of inflammation associated with PH severity or that its regulatory role may occur through mechanisms not linearly related to PH progression.

The study also explored correlations between AnxA1 and complete blood count parameters. Leukocyte levels were significantly elevated in the HWD+PH group compared to the CTRL and HWD groups, reflecting the advanced disease stage and enhanced inflammatory response. This increase aligns with the known pathophysiology of inflammation, which involves neutrophil recruitment as a hallmark feature (Goetzl et al., 1995). In HWD, chronic vascular remodelling leads to persistent activation of pro-inflammatory mediators (Simón et al., 2012), resulting in elevated levels of cytokines such as TNF-a, IL-6 and IL-1β (Liu et al., 2022). These cytokines promote neutrophil chemotaxis and recruitment (Serhan, 2014), contributing to leucocytosis. Furthermore, severe cases of HWD are often associated with eosinophilia (Ettinger et al., 2017; Kim et al., 2020), which may also contribute to the elevated leukocytes counts observed in the HWD+PH group. These findings support the association between increased leukocytes levels and diseases severity.

A moderate negative correlation between leukocyte count and AnxA1 levels was observed in the CTRL group, suggesting that higher AnxA1 concentrations are associated with reduced leukocyte numbers. This may reflect the anti-inflammatory role of AnxA1, which binds to FPR receptors to inhibit neutrophil extravasation, leukocyte transmigration, and recruitment (Gavins & Hickey, 2012; Sugimoto et al., 2016). Additionally, AnxA1 promotes neutrophil apoptosis (de Jong et al., 2017; Sugimoto et al., 2016), further supporting the inverse relationship with leukocyte levels. However, this correlation was not evident in the HWD and HWD+PH groups. In these disease states, parasite-induced tissue damage in the pulmonary arteries and lungs (Carretón et al., 2017a) triggers a sustained pro-inflammatory response, characterized by elevated leukocyte counts. The absence of correlation in these groups may be attributed to the chronic nature of the disease and the extensive inflammatory burden, which may override the regulatory effects of AnxA1. Moreover, other factors, such as individual variability, disease duration, and physiological modulators, may also influence AnxA1 expression in these contexts.

A significant reduction in RBC count was observed in the HWD+PH group compared to the HWD group, consistent with anaemia commonly associated with advanced HWD and PH (Ettinger et al., 2017; Kim et al., 2020). This may result from mechanical destruction of RBCs within pulmonary arteries due to parasite presence, a process worsened by PH-related vascular damage. In the HWD group, a moderate negative correlation was found between AnxA1 and RBC count, indicating that higher AnxA1 levels are associated with lower RBC levels. This contrasts with the expected anti-inflammatory effect of AnxA1, which limits neutrophil activity and vascular injury (Gavins & Hickey, 2012; Sugimoto et al., 2016). The observed correlation may instead reflect inflammation-induced haemolysis, as AnxA1 is upregulated during neutrophil activation and degranulation (Torres et al., 2016). Similarly, a significant negative correlation between AnxA1 and haematocrit was found in the HWD group, consistent with its dependence on RBC levels (Klinken, 2002). No correlation was observed in HWD+PH, suggesting that chronic disease mechanisms may obscure this relationship. These findings underscore the complex interaction between inflammation, erythrocyte dynamics, and AnxA1 in HWD pathophysiology.

Sex-related differences in AnxA1 serum levels were observed, with female dogs in the CTRL and HWD groups showing significantly higher concentrations than males. Although no overall statistical difference was found between sexes within the broader study groups, these findings are consistent with the known regulatory influence of oestrogens, which upregulate AnxA1 expression and enhance its anti-inflammatory effects (Hughes et al., 2013). Oestrogens also modulate glucocorticoid responses and contribute to immune protection (Chamekh & Casimir, 2019), further supporting elevated AnxA1 in females. Additionally, FSH and LH have been positively associated with AnxA1 levels (Mulla et al., 2005). Conversely, androgens may suppress AnxA1 expression by promoting pro-inflammatory states (Chamekh & Casimir, 2019). In the HWD+PH group, this sex-related pattern was not evident, likely due

to the heightened inflammatory burden associated with advanced disease. Chronic inflammation may override hormonal modulation of AnxA1, thereby obscuring sex-related differences.

Further comparisons between intact females and males across all groups revealed no statistically significant differences in AnxA1 levels. Although a higher expression was anticipated in intact females due to endogenous oestrogens, this was not observed. Similarly, no differences were found between spayed females and neutered males. Considering that sterilization reduces sex hormone levels and may enhance pro-inflammatory responses (Hughes et al., 2013), its influence on AnxA1 expression cannot be excluded. Importantly, these subgroup analyses involved small sample sizes, particularly in the CTRL group, limiting statistical power. Therefore, the absence of significance may reflect insufficient representation rather than a true lack of biological difference. Further targeted studies with larger cohorts are warranted to clarify these observations.

Age-related variations in AnxA1 serum levels were assessed across the CTRL, HWD, and HWD+PH groups. An increased AnxA1 levels was observed in young and adult dogs with HWD compared to healthy controls, reflecting the anti-inflammatory response to the disease's inflammatory nature. This aligns with the known role of AnxA1 in modulating inflammation and correlates with disease severity. Conversely, a decreasing trend in AnxA1 levels was noted in older dogs within the HWD+PH group compared to the other groups, though not statistically significant.

For clinical relevance, age groups were categorized as young/adult versus older dogs. In the CTRL group, older dogs showed significantly higher AnxA1 levels compared to younger counterparts. This supports prior evidence that aging is associated with chronic low-grade inflammation, during which AnxA1 exerts a protective anti-inflammatory role (Singh et al., 2024). Younger dogs, by contrast, exhibit lower levels of pro-inflammatory cytokines such as IL-6 and TNF-α (Jimenez, 2023; Schmid et al., 2024), which may result in lower AnxA1 expression, thereby explaining the observed age-related differences in the CTRL group. In HWD and HWD+PH groups, no statistically significant differences in AnxA1 levels were observed across age groups. Correlation analysis revealed a moderate positive association between AnxA1 levels and age in the CTRL group, consistent with the elevated AnxA1 in older dogs. In contrast, no significant correlations were found in the disease groups, likely due to the overriding influence of pathological inflammation on AnxA1 regulation, potentially masking age-related effects.

Dogs in the HWD+PH group exhibited a significantly higher prevalence of clinical signs compared to those with HWD alone. Previous studies have linked symptomatic dogs to increased parasite burden and elevated pulmonary pressure (Serrano-Parreño et al., 2017), reflecting the greater severity of PH and the associated amplified inflammatory response. However, no significant correlation was found between AnxA1 levels and symptom presence within the disease groups. This unexpected

result suggests that despite the chronic pro-inflammatory state in symptomatic dogs, AnxA1 expression may be influenced by additional factors beyond disease severity and parasite load.

NT-proBNP levels were significantly elevated in dogs with PH secondary to HWD compared to those with HWD alone. This aligns with prior studies showing increased NT-proBNP in pre-capillary PH, including HWD-related PH, reflecting greater cardiac damage and thromboembolism risk (Costa-Rodríguez et al., 2023; Kellihan et al., 2011). NT-proBNP also correlates with pulmonary arterial pressure in dogs (Hori et al., 2012). Although NT-proBNP may be influenced by glomerular filtration rate and sex (Misbach et al., 2013), these factors did not confound results here.

No significant differences in CRP levels were observed between disease groups, despite expectations of increased CRP in HWD+PH due to amplified tissue damage, inflammation, vascular changes, and haemolysis (Méndez et al., 2014, 2015). Prior studies reported elevated CRP in older dogs and those with PH, correlating with disease severity and parasite load (Carretón et al., 2017b). This discrepancy may relate to the study sample, as older, more severely affected dogs in HWD+PH did not show increased CRP. While CRP has been proposed as a prognostic and monitoring biomarker for PH (Carretón et al., 2017a), this was not supported here. Cortisol levels did not differ significantly between HWD and HWD+PH groups, despite expectations of elevated cortisol in acute-phase inflammatory responses (Ceron et al., 2005). The chronic nature of PH may explain the absence of cortisol elevation.

Correlation analyses revealed no significant relationships between NT-proBNP and AnxA1 or between CRP and AnxA1, suggesting these biomarkers reflect distinct pathophysiological processes. The lack of correlation may be influenced by variations in sex, age, reproductive status, or differences in assay sensitivities. Conversely, a significant moderate negative correlation was observed between cortisol and AnxA1 in the HWD+PH group. Although glucocorticoids like cortisol typically upregulate AnxA1 synthesis (D'Acquisto et al., 2008; Perry & Medbak, 2013), the inverse relationship here may reflect AnxA1-mediated suppression of ACTH release, reducing cortisol production via a negative feedback loop (Buckingham et al., 2006). This suggests that in severe disease, increased AnxA1 expression dampens cortisol levels. No such correlation was found in the HWD group, likely due to milder disease severity.

Regarding parasite burden, dogs in the HWD+PH group exhibited a significant increase compared to the HWD group, consistent with disease severity. Higher parasite loads are typically linked to more severe clinical manifestations (Bussadori, 2023; McCall et al., 2008a; Ware et al., 2022). However, no significant differences in AnxA1 levels were found between low and high parasite burdens within groups. This contrasts with the initial hypothesis that AnxA1 serum levels would rise with disease severity and parasite load. These findings align with the lack of significant differences in AnxA1 levels observed between disease groups of varying severity.

This study had several limitations. First, animal enrolment criteria for the HWD and HWD+PH groups did not exclude dogs receiving medication, including annual preventive treatment for HWD, which may influence AnxA1 expression. Additionally, other medications potentially affecting AnxA1 levels were not controlled for, warranting stricter criteria in future studies. Second, the canine ELISA kit used employed a polyclonal antibody against AnxA1, raising the possibility of cross-reactivity with related annexin family proteins. Although the manufacturer reported no significant cross-reactivity, this remains a limitation. The kit was chosen as it is the only one validated for canine serum; monoclonal antibody kits are validated only for human samples. Third, small sample sizes in certain subgroups (notably neutered males and spayed females) limited statistical power in subgroup analyses. Finally, biomarkers (NT-proBNP, CRP, cortisol) were measured only at diagnosis, without longitudinal follow-up. This precluded assessment of temporal changes and comparison of biomarker progression with AnxA1 throughout disease course or treatment.

2.5. Conclusion

Annexin A1 is a key anti-inflammatory protein implicated in various diseases (Sheikh & Solito, 2018). Despite its regulatory role in inflammation, this study found no significant differences in serum AnxA1 levels among healthy dogs and those with HWD, with or without PH, indicating that AnxA1 alone may not be a reliable biomarker for these conditions.

Annexin A1 expression was influenced by individual factors: females exhibited higher levels, likely due to hormonal modulation by oestrogens (Hughes et al., 2013), and older dogs showed increased AnxA1, possibly reflecting chronic low-grade inflammation associated with aging (Singh et al., 2024). While leukocyte and red blood cell counts reflected inflammatory activity in HWD and PH, they did not correlate directly with AnxA1 levels, underscoring the complexity of inflammatory regulation in these diseases.

Established biomarkers such as NT-proBNP and CRP, previously linked to HWD and PH (Carretón et al. 2017b; Costa-Rodrígues et al., 2023; Hori et al., 2012; Méndez et al., 2015), showed no correlation with AnxA1, suggesting distinct regulatory pathways. Cortisol exhibited a negative correlation with AnxA1 in HWD+PH, potentially reflecting feedback suppression of ACTH (Buckingham et al., 2006). Although parasite burden influenced disease severity and PH, it was not associated with AnxA1 levels.

In conclusion, AnxA1 is not a suitable standalone biomarker for HWD or PH but is modulated by physiological variables. Study limitations included small subgroup sizes, prior medication exposure, and the use of a polyclonal ELISA kit. Future research should employ longitudinal designs, larger cohorts, and combined biomarker panels to further elucidate AnxA1's role in canine cardiopulmonary diseases.

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Annexin A1, as a Potential Biomarker in Canine Heartworm Disease

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