UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

ENDOTHELIAL GLYCOCALIX SHEDDING AS A PROPOSED PATHOPHYSIOLOGICAL PATHWAY FOR PROTEINURIA IN CANINE HYPERCORTISOLISM

DAVID VIEIRA DA SILVA

ORIENTADOR(A):
Doutor Rodolfo Assis Oliveira Leal

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DESTRUIÇÃO DO GLICOCÁLICE ENDOTELIAL COMO POTENCIAL MECANISMO FISIOPTALÓGICO POR DETRÁS DA PROTEINÚRIA EM ANIMAIS COM HIPERCORTISOLISMO

Resumo:

A proteinúria é um dos sinais clinico-patológicos mais relevantes em pacientes com síndrome de Cushing, podendo mesmo afetar cerca de 75% dos cães. Embora alterações hemodinâmicas como a hipertensão arterial e consequente alteração da taxa de filtração glomerular possam contribuir para esta proteinúria, a sua etiologia é pouco compreendida nestes animais. A literatura em medicina humana sugere uma possível relação entre a destruição do glicocálice endotelial (GE) glomerular e a perda de proteína pelos glomérulos renais. No entanto, o seu impacto em cães com síndrome de Cushing ainda não foi alvo de estudo. O ácido hialurónico ou hialuronato sérico, é um biomarcador da destruição do GE, validado em modelos animais. Este trabalho teve como objetivo avaliar o hialuronato sérico em cães diagnosticados com síndrome de Cushing, clarificando sua potencial relação com a proteinúria. Foi efetuado um estudo multicêntrico transversal utilizando amostras de soro de cães hipercortisolémicos entre 2022 e 2024, antes do início da terapêutica com trilostano, no qual foi doseado o hialuronato com recurso a um kit de ELISA (Hyaluronan Quantikine ELISA kit). Os resultados foram respectivamente correlacionados com os parâmetros hematobioquímicos e urinários (nomeadamente o rácio proteína-creatinina urinário). Em relação ao RPCU, os casos foram agrupados de acordo com a gravidade da proteinuria, em não proteinúricos (<0.5), proteinúricos (0.5-2) e marcadamente proteinúricos (>2). Concomitantemente, os resultados do ácido hialurónico obtidos foram comparados com controlos históricos. As concentrações séricas de hialuronato (mediana [intervalo mínimo-máximo) obtidas em cães hipercortisolêmicos (45,94 [9,70–265,07] ng/mL) mostraram uma amplitude semelhante às descritas em cães com estados hipercoaguláveis (92,40 [16,9–247,6] ng/mL) e uma mediana que foi equivalente ou superior à de cães saudáveis, conforme o estudo considerado. (45,7 vs 17.4 vs 17.9 ng/mL). A diferença nas concentrações do ácido hialurónico sérico entre os diferentes graus de proteinúria não demonstrou significância estatística (p=0,062). Da mesma forma, os níveis de hialuronato sérico não se correlacionaram com o RPCU (p=0,119). Este estudo revela um aumento nos níveis de hialuronato sérico em cães com síndrome de Cushing. Embora elevados, esses valores aparentemente não explicam a magnitude da proteinúria. A semelhança dos valores nos estados de hipercoagulabilidade e nos animais hipercortisolémicos sugere uma potencial associação com a destruição do GE.

Palavras chave: síndrome de Cushing, proteinúria, glicocálice endotelial, hialuronato, ELISA

ENDOTHELIAL GLYCOCALIX SHEDDING AS A PROPOSED PATHOPHYSIOLOGICAL PATHWAY FOR PROTEINURIA IN CANINE HYPERCORTISOLISM

Abstract:

Proteinuria is a common finding in dogs with naturally occurring Cushing´s syndrome, with an estimated incidence as high as 75%. Although hemodynamic factors such as hypertension and consequent changes on glomerular filtration rate may contribute to proteinuria, its etiology is still poorly understood. Emerging studies have revealed a compelling correlation between endothelial glycocalyx (eGlx) breakdown and proteinuria in humans. Serum circulating hyaluronan has been considered a reliable biomarker of eGlx shedding not only in humans but in various animal models. Although growing evidence highlights the role of eGlx shedding in the pathophysiology of various diseases, its impact in canine naturally occurring Cushing`s syndrome remains poorly understood. This study aims to evaluate serum hyaluronan in dogs with Cushing`s syndrome and its possible relationship with proteinuria. A cross-sectional multicentric study was conducted, including leftover samples from dogs with Cushing´s syndrome diagnosed from 2022 to 2024 before initiating trilostane therapy. Stored and frozen at -20 or -80°C until processing, serum hyaluronan was measured using a sandwich ELISA Kit (Hyaluronan Quantikine ELISA kit) previously validated for dogs. Concurrent urinary protein-creatinine ratio (UPC) and hematobiochemical values for each animal were obtained from medical records. Gathered values were compared with historic controls from previous studies. Dogs were grouped regarding their degree of proteinuria: non-proteinuric (UPC<0.5), proteinuric (0,5<UPC<2) and overtly proteinuric (UPC>2). The serum hyaluronan concentrations (median [min-max range]) obtained for dogs with hypercortisolism (45.94 [9.70-265.07] ng/mL) showed a similar range to those previously described in dogs in hypercoagulable states (92.40 [16.9-247.6] ng/mL), and median value either higher or similar to those observed in healthy dogs, depending on the study (45.7 vs 17.4 vs 17,9 ng/mL). The difference in serum hyaluronan concentrations between different degrees of proteinuria did not reach statistical significance ($p = 0.062$). No correlation was found between serum hyaluronan and UPC ($p = 0.119$). This study documents an increase in serum hyaluronan concentrations in dogs with Cushing´s syndrome. Despite no apparent association with proteinuric status, the serum hyaluronan values obtained in this study are similar in range to those found on dogs with in a hypercoagulable status, pointing towards an association between eGlx breakdown and the hypercoagulability in patients with naturally occurring Cushing´s syndrome.

Keywords: Cushing´s syndrome, Proteinuria, Endothelial Glycocalyx, Hyaluronan, ELISA

DESTRUIÇÃO DO GLICOCÁLICE ENDOTELIAL COMO POTENCIAL MECANISMO FISIOPTALÓGICO POR DETRÁS DA PROTEINÚRIA EM ANIMAIS COM HIPERCORTISOLISMO

Resumo alargado:

A síndrome de Cushing (SC) em cães é uma doença endócrina com diferentes etiologias. Para facilitar a sua classificação, a origem desta síndrome é classificada em função da hormona adrenocorticotrópica (ACTH). A ACTH é sintetizada na porção anterior da hipófise e estimula a produção de glucocorticoides pelo córtex das glândulas adrenais. As causas da SC são então definidas em ACTH-dependente ou independente. Entre as condições associadas ao aumento da concentração de ACTH, destacam-se os tumores hipofisários produtores de ACTH (HHD), que são a causa mais comum de hipercortisolismo, bem como a secreção ectópica de ACTH. Quanto às causas de hipercortisolismo ACTH-independente, destacam-se os tumores funcionais produtores de cortisol do córtex adrenal (HAD), hiperplasia funcional do córtex da adrenal associado à alimentação e, por fim, a administração crónica de glicocorticoides exógenos. Apesar de ser relativamente rara, a SC é uma das endocrinopatias mais frequentes em cães. Na grande maioria dos casos resulta de um tumor funcional hipofisário (na maioria das vezes benigno), produtor de ACTH ou de uma neoplasia adrenal secretante de glucocorticoides, independente da estimulação da ACTH. Raças como Jack Russel terrier, Bichon Frise, Yorkshire terrier, Staffordshire Bull terrier e os West Highland White terrier são particularmente predispostos a desenvolver esta condição. Dadas as inúmeras funções que os glucocorticoides assumem no organismo, uma exposição crónica e em excesso resulta numa ampla gama de sinais clínicos associados à doença, sendo de esperar uma combinação de efeitos gluconeogénicos, imunossupressores, anti-inflamatórios e catabólicos. Os sinais clínicos mais frequentemente observados são a poliúria, polidipsia, polifagia, aumento do perímetro abdominal, hepatomegália, além de uma constelação de manifestações dermatológicas como alopécia, atrofia da pele, comedões, calcinose cutânea, entre outros. Na análise de urina, com uma prevalência que pode atingir os 75% dos casos, a proteinúria é um dos achados mais frequentes desta síndrome, e cuja origem nesta endocrinopatia ainda é pouco clara. Poucas são as publicações que investigam a origem da proteinúria nesta doença. No entanto, são apontadas como possíveis causas as alterações hemodinâmicas (incluindo a hipertensão arterial, que induz um aumento na taxa de filtração glomerular) e alterações lipídicas sistémicas que, mesmo com glomérulos funcionais, podem potenciar a filtração glomerular de albumina. Estudos recentes em medicina humana sugerem que a degradação do glicocálice endotelial (GE) pode desempenhar um papel importante na disfunção renal, nomeadamente na proteinúria. Apesar da escassez de informação publicada sobre o GE em medicina veterinária, sabe-se que este desempenha um papel fundamental na homeostasia vascular. Começando pela regulação da permeabilidade dos vasos capilares,

uma rede complexa de proteínas e polissacarídeos, associada a uma matriz carregada negativamente, promovem a formação de uma barreira física, eletrostática e oncótica, impedindo desta forma a passagem de fluidos e solutos para o espaço extra vascular. Também armazena cofactores enzimáticos como a antitrombina e a prostaciclina que regulam e equilibram os estados de hiper e hipocoagulabilidade. É também essencial na regulação do tónus vascular (devido ao efeito mecanotransdutor) e indutor endotelial na produção de óxido nítrico), entre muitas outras funções. A avaliação da sua destruição pode ser feita por métodos diretos e indiretos. Um dos biomarcadores de avaliação indireta é a medição do ácido hialurónico ou hialuronato séricos, o qual está validado para uso em cães.

Este trabalho teve como objetivo determinar a concentração deste biomarcador de destruição do GE em cães com SC e correlacionar estes valores com a gravidade da proteinúria. Pretendeu-se ainda realizar uma análise comparativa entre os valores de pressão arterial sistólica, triglicéridos e colesterol séricos em função das concentrações do ácido hialurónico. Para isso, foi efetuado um estudo multicêntrico transversal no qual o hialuronato sérico foi medido através de um kit ELISA (Hyaluronan Quantikine ELISA kit). Foram utilizadas amostras de soro de cães com hipercortisolismo, recolhidas entre 2022 e 2024 e congeladas a -20ºC e -80ºC em dois hospitais veterinários universitários, (Hospital Escolar Veterinário da Faculdade de Medicina Veterinária da Universidade de Lisboa e Hospital Escolar Veterinário da Universidade de Bolonha). Para a sua seleção, foram avaliados o historial clínico, resultados no teste de supressão com a dexametasona em dose baixa (LDDST), concentração da ACTH endógena (eACTH) e exames imagiológicos. Os cães incluídos no estudo foram divididos de acordo com a etiologia suspeita em HAD e HHD e hipercortisolémicos sem origem determinada. A divisão entre estas causas foi efetuada de acordo com a concentração de eACTH (sendo que valores ≤5 pg/mL foram considerados indicativos de HAD), em associação com os resultados do padrão do LDDST e as alterações observadas nos exames de imagem. Foram recolhidos simultaneamente dados relativos à pressão arterial sistólica, colesterol, triglicéridos e rácio proteína creatinina urinários (RPCU). No caso do RPCU, os cães foram classificados em três grupos com base na gravidade da proteinuria: não proteinúricos (<0.5), proteinúricos (0.5-2) e marcadamente proteinúricos (>2). Foi efectuada uma avaliação descritiva das variáveis. Uma vez que os valores do ácido hialurónico apresentavam uma distribuição não normal, os estudos comparativos foram feitos a partir da mediana e intervalo interquartis. De modo a aumentar a amostra da população de cães hipercortisolémicos e, respeitando a política dos 3 R's, as medianas e os intervalos interquartis do hialuronato sérico foram comparados com grupos de animais saudáveis e com outras comorbilidades (submetidos à medição deste parâmetro pela mesma metodologia). Estes grupos eram constituídos por cães saudáveis, cães em estados de hipercoagulabilidade

e cães diagnosticados com doença mixomatosa da válvula mitral, todos avaliados com a mesma metodologia. As concentrações séricas de hialuronato (mediana [intervalo mínimo-máximo) obtidas em cães hipercortisolémicos (45,94 [9,70–265,07] ng/mL) mostraram uma amplitude semelhante às descritas em cães com estados hipercoaguláveis (92,40 [16,9–247,6] ng/mL) e uma mediana que foi equivalente ou superior à de cães saudáveis, conforme o estudo considerado (45.7 vs 17.4 vs 17,9 ng/mL). No entanto, não foram realizadas correlações estatísticas mais rigorosas devido à ausência dos valores individuais do hialuronato nos cães saudáveis, nos cães em estados de hipercoagulabilidade e com doença mixomatosa da válvula mitral. No grupo de cães com hipercortisolismo, foi realizada uma análise de correlação de Spearman para avaliar possíveis associações entre o RPCU e a concentração sérica do ácido hialurónico. No entanto, não foi identificada qualquer correlação significativa entre o hialuronato sérico e o RPCU ($p = 0,119$). Adicionalmente, para investigar potenciais diferenças entre a gravidade da proteinúria e os níveis de hialuronato, foi realizado um teste de Kruskal-Wallis. Da mesma forma, as concentrações séricas de ácido hialurónico nos diferentes graus de proteinúria (< 0,5 RPCU, 0,5 < RPCU < 2 e RPCU > 2) não apresentaram diferenças estatisticamente significativas ($p = 0,062$). Por último, foram também testadas hipóteses de associação entre os níveis de ácido hialurónico e as variáveis pressão arterial sistólica, colesterol e triglicéridos. No entanto, não foi identificada uma correlação que explique a magnitude da destruição do glicocálice e as variáveis consideradas ($p > 0,05$ em todas a variáveis).

Este estudo demonstrou que os níveis de hialuronato estão aumentados em cães com SC, sugerindo uma possível destruição do glicocálice endotelial. No entanto, a perda da integridade do GE não parece estar diretamente relacionada com a proteinúria em cães com SC. Embora seja reconhecida uma associação entre a hipercolesterolemia e a degradação do GE em humanos, esta causa não se verificou nos cães deste estudo. Foram também realizadas avaliações da pressão arterial sistólica e dos níveis de triglicerídeos, mas aparentemente, também não explicam a magnitude dos valores do hialuronato associados ao hipercortisolismo. A semelhança dos níveis do hialuronato observados em estados de hipercoagulabilidade e em cães com hipercortisolismo sugere uma possível relação com a destruição do GE, reforçando a evidência prévia sobre o papel do GE na manutenção da homeostasia vascular e no controlo dos estados de hipo e hipercoagulabilidade

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List of abbreviations and symbols:

<- Less than

>- Higher than

ACTH- Adrenocorticotropic hormone

ACTHst- ACTH stimulation test

ADHC- Adrenal-dependent Hypercortisolism

ALP-Alkaline phosphatase

Bp- Blood pressure

Cm- Centimetres

CRH- Corticotrophin releasing hormone

CS- Cushing´s syndrome

CT- Computed tomography

DM- *Diabetes Mellitus*

eGlx- Endothelial Glycocalyx

ELISA- Enzyme-linked immunosorbent assay

FAT- Functional adrenal tumour

GAG- Glycosaminoglycan

GLX- Glycocalyx

GFB- Glomerular filtration barrier

HAD- Hipercortisolismo Adrenal Dependente

HC- Hypercortisolism

HHD- Hipercortisolismo Hipofisário Dependente

HDDST- High-dose dexamethasone suppression test

Kg- Kilograms

LDDST- Low-dose dexamethasone suppression test

MMVD-Mixomatous mitral valve disease

mg/Kg- Milligram per kilogram

mL- Millilitre

mmHg- Millimetres of mercury

ng/mL- nanograms per Millilitre

nmol/L- nanomole per liter

MRI- Magnetic Resonance Imaging

NPV- Negative predictive value

PBR- Perfused boundary region

P/B ratio- Pituitary Height/ Brain ratio

PDH- Pituitary dependent Hypercortisolism

PPV- Positive predictive value

PTH-Parathormone

PU- Polyuria

rh- Recombinant human

SSTR- Somatostatin type receptor

UCCR- Urine cortisol to creatinine Ratio

UPC- Urine protein to creatinine ratio

US- Ultrasonography

µg/Kg- Picogram per kilogram

µg/Dog- Picogram per dog

%- Percentage

e.g.- *exempli gratia*

ºC- Degree Celsius

SECTION 1- Traineeship Report:

My externship took place at the Veterinary Teaching Hospital of The Faculty of Veterinary Medicine - University of Lisbon, from September 4th of 2023 to March 8th of 2024, spanning a total of 27 weeks. In addition to the standard 7-to-8-hour shifts, I had the opportunity to assist Veterinary Doctors and nurses through 12 hours night and inpatient care shifts. The first half of my Traineeship was spent in the department of Internal Medicine under the supervision of Prof. Dr. Rodolfo Oliveira Leal. In the second trimester, I was scheduled on a rotation basis across the different hospital services along with the other trainees. During this time period I was able to gain a variety of skills in small animal veterinary medicine and also participate in the laboratory activities related to the technical aspects of the upcoming research project.

1.1 Internal Medicine

Approximately 500 hours were spent in this department. Under the mentorship of Prof Dr. Rodolfo Assis Leal Oliveira Dipl.ECVIM-CA, his residents Dra. Joana Dias and Dra. Beatriz Mendoza and the PhD-candidate Patrícia Marques, I had the opportunity to assist and learn from these professionals. Over the past five years of veterinary medical school, my increasing interest in this field led me to apply for this particular externship. During this period, I spent the majority of my time as an assistant internist. My duties started at 8:30 am during the morning rounds. I was fortunate to listen and participate in the discussion on the better management of the inpatient care patients. At around 9am my duty was to begin the consultations, under the professor's supervision. A thorough physical examination and a complete anamnesis helped me formulating my problem list and further reason my differential diagnosis. Afterwards, a fruitful exchange of ideas with the supervisor helped me consolidate my knowledge and rationalise complementary diagnostic tests. Blood samples via venipuncture, venous catheterization, urine sampling via cystocentesis and bladder catheterization were taught by the nurse team, led by Élia Cosme. During this time period, as a last year student, I was also able to interact with students from the previous years in a hospital environment. Conferencing with the owners and students gave me communicating skills, crucial for any veterinary practitioner. Later in the day, following all the consultations, the discussion of the daily cases between internal medicine members enhanced shared knowledge. This leads to a more accurate and effective approach to the most challenging cases. After debating on complex clinical cases, the doctors in this department write and answer emails to the owners

communicating complementary test results and actualize further steps on the patient's treatment. Also in this department, I had the opportunity to assist practical procedures such as endoscopies (rhinoscopy, tracheoscopy, bronchoscopy with bronchoalveolar lavage, esophagoscopy, gastroenterostomy, colonoscopy, cystoscopy), fecal transplants, bone marrow biopsies, lymph nodes fine-needle aspiration, and joint taps. In the context of residency supervision and program, Professor Rodolfo Leal conducted a weekly oral exam simulation with Dra Joana Dias and Dra Beatriz Mendoza. Additionally, each team member presented an article weekly from top-tier veterinary medicine journals, namely the Journal of Veterinary Internal Medicine.

This 500-hour experience provided valuable insights into managing and treating patients with endocrinopathies, respiratory, digestive, haematological, and urinary tract conditions, as well as chronic infectious diseases like leishmaniasis and ehrlichiosis.

1.2 Surgery

Approximately 80 hours (two weeks) were spent in this department. The day began at 8am where we meet animal owners and perform an admission questionnaire focusing on the animal's body weight, hours of fasting, any known medical conditions or ongoing medication prior to a surgery. Following this, we would confirm the animal's body weight, carry out a thorough physical exam, place a venous catheter and administer premedication. Under the supervision and the help of the nurses, anaesthesiologists and surgeons, we prepared the animal by performing the trichotomy, clean and disinfect the interventional sites and induce the anaesthesia. According to plan, the trainees embraced different rules such as assistant anaesthesiologist, assistant surgeon and circulating assistant. I got the opportunity to participate in endocrine, orthopaedic, gastrointestinal, neurologic, maxillofacial, reproductive and emergency surgeries. Following the procedure, a critical care operation on the recovery of the animal included a second physical exam, rectal temperature, heart rate, respiratory rate measurements, hydration assessment and reflexes evaluation until the animal is fully awake and stabilised. Later in the day a productive discussion on the daily surgeries among trainees, nurses and the surgeons helped us clarify our questions on the clinical cases.

During this time period I have also had the chance to learn how to place nasogastric and oesophageal tubes.

1.3 General practice

Similarly to surgery, two weeks in total were spent in this department. During these 8-hour shifts, the trainee was able to assist doctors and nurses during first and second opinion consultations, or specific procedures performed by the nurse team. In most of the cases, the doctors allowed us to have the first interaction with the owners. Our job was to collect all the necessary information to write a complete anamnesis, identify the primary and secondary problems and formulate a differential diagnosis list. Afterwards, the doctor would join the consultation and read the trainees report, complete the anamnesis and perform the physical exam. To gather additional details about the patient's condition, we help the medical staff in collecting blood through venipuncture or urine typically via cystocentesis or bladder catheterization. Frequently, the animals required immediate evaluation for internal changes that necessitate imaging tools. As trainees, we would prepare the animals in the right positions for the X-ray, or restrain the patients for quick ultrasound evaluations.

In this department, I learnt and optimized a range of practical procedures such as drawing blood, placing venous catheters, collecting urine samples via cystocentesis and bladder catheterization, performing abdominocentesis and thoracocentesis, evaluating ear and skin cytology, performing quick ultrasound assessments, and even participating in cardio-respiratory resuscitation. Emergency experiences enhanced my critical thinking and quick-decision making and the constant interaction between the staff members and the patients owners improved my communication skills.

1.4 Exotic animals

One week in total was spent in this department. Unfortunately, many owners cancelled consultations over the week. Nevertheless, I was able to assist and participate in a few interesting consultations and procedures. As trainees, we were taught how to restrain and approach these animals and learn to collect biological samples such as blood and urine. I took part in consultations involving parrots, rabbits, domesticated rats, turtles, and ferrets. Two notable procedures I assisted were the dental filling of a rabbit and the sterilisation of a female domesticated rat. Additionally, in this week, we have had the chance to deal with an emergency of a bunny with a total urine obstruction flow due to a significant urolith located in the bladder.

1.5 Ophthalmology

The week I spent in the ophthalmology department provided me with a deeper knowledge of the major ophthalmological diseases. Besides conducting general physical

exams, I was able to perform an ophthalmological examination which included evaluating palpebral and pupillary reflexes and menace response, performing Schirmer and fluorescein test, measuring intraocular pressure, and examining the anterior and posterior segments of the eyes. I also took part in a minor surgery to remove a palpebral nodule from a dog's eyelid. Oftentimes, blood was collected for additional tests such as retrovirus testing (serology) and toxoplasma screening in cats, as well as leishmania and ehrlichia serology in dogs. Through this experience, I became familiar with the most common medications used in ophthalmological treatments, attended both first opinion and follow up consultations and learnt how to manage frequent ophthalmologic conditions like corneal ulcers.

1.6 Dermatology

The rotation in dermatology lasted one week with 8-hour shifts. Trainees assisted and participated in referral consultations and follow-ups. Under the doctor's guidance we performed dermatological examinations and complementary diagnostic tests such as skin cytology, ear cytology followed by the coloration with a commercial Romanowsky stain (Diffquick), trichogram, video otoscopy and skin biopsies. After each consultation, a fruitful conversation among trainees and the doctors helped us clarify our differential diagnosis list and treatment protocols.

1.7 Oncology

One week in total was spent in this rotation. As trainees, we had the opportunity to participate in referral, first opinion appointments and chemotherapy sessions. During consultations, a careful and thorough approach to the patient is essential for making an accurate diagnosis. This information is critical for the patient's prognosis and a possible treatment approach. Furthermore, hospital chemotherapy procedures began with the reception of the patient, weighing of the animal, placing a venous catheter and collecting blood for a complete blood count (CBC) analysis. Subsequent to a normal blood work, the responsible nurse or the doctor would prepare the chemotherapy and give it to the animal. We monitored the patient during the procedures and afterwards delivered the patient back with the owner. Throughout the week, I was fortunate to learn how to approach these animals and, most importantly, how to communicate such delicate information to their owners.

1.8 Cardiology and Neurology

Over the course of one week, I rotated among these two services. Starting with neurology, I had the opportunity to learn how to gather patients past medical history in a

neurology perspective and practise a full neurological exam. Most of the patients required post imaging evaluation to characterize the lesion. Occasionally, blood samples were collected if meningitis or a genetic disorder was suspected. Assisting and participating in referral, first and second opinion neurology appointments, gave me valuable insights and a deeper understanding on how to approach a neurological patient and their conditions.

In the cardiology department as trainees, we would hear the doctor collecting the anamnesis while we, at turns, would auscultate the patient and look for sound abnormalities in the cardio-respiratory system. After this, a brief discussion with the doctor helped us refine our auscultation skills. During the echocardiography exam, the doctor guided us through the process, explaining each cardiac alteration when a disease was detected. Through this experience I have acquired a deeper understanding on the most frequent cardiac diseases such as myxomatous mitral valve disease (MMVD) and dilated cardiomyopathy in dogs, as well as hypertrophic cardiomyopathy in cats.

1.9 Diagnostic Imaging

Two weeks in total were spent in this department. Starting with X-rays, I have participated in the admission, contention, sedation and positioning of the animals and had the opportunity to interpret the images with the doctor in charge. In computed tomography and magnetic resonance booths, I helped placing intravenous catheters, administering medication, endotracheal intubation, positioning and anaesthesia monitorization. Similarly to the X-ray, we had the opportunity to interpret the images and gained a deeper knowledge on the differences between these two models of imaging. Regarding Ultrasonography, my duties included trichotomy, positioning and containment of the patients. I gained experience in identifying various abdominal organs on ultrasound and recognizing the most prevalent abnormal findings. Additionally, I assisted in conducting ultrasound-guided percutaneous needle cytology and biopsies.

1.10 Reproduction

During the week spent in the reproduction department the trainee had the opportunity to assist the doctors in their daily practice. Over the course of this week, I acquired a deeper understanding on how to perform a physical reproductive tract examination. This included visualisation, palpation and even swab collection of genital tract fluids for a further cytology examination. Furthermore, I was granted the chance to visualise ultrasonography exams for the foetal development evaluation and to complement the diagnosis of diseases such as pyometras in bitches and queens, as well as benign prostate hypertrophy in dogs. This

experience gave me tools on how to approach, diagnose and treat some of the most common diseases regarding the reproductive system.

SECTION 2- LITERATURE REVIEW:

2.1 : Naturally occurring Cushing´s Syndrome

Naturally occurring Cushing´s Syndrome (CS) is the consequence of an excessive production of endogenous cortisol (Hypercortisolism (HC)), commonly arising from either a pituitary adenoma/adenocarcinoma (PDH) or a cortisol secreting functional adrenal tumour (FAT). CS classification is based upon the concentration of adrenocorticotropic hormone (ACTH) which directly stimulates the adrenal gland cortex to produce glucocorticoids. ACTH-dependent Cushing´s syndrome comprises PDH and ectopic ACTH syndrome(Galac et al. 2005), while ACTH-independent Cushing´s syndrome includes cortisol secreting FAT, primary ACTH-independent adrenocortical hyperplasia, such as food-induced hypercortisolism, and iatrogenic form of hypercortisolism, given the chronic use of external glucocorticoid therapy. While PDH and cortisol secreting FAT are well-recognized, reports of primary ACTH-independent adrenocortical hyperplasia and ectopic ACTH syndrome are limited to scarce case reports (Galac et al. 2005; Galac et al. 2008).

2.2 : Epidemiology of naturally occurring Cushing´s Syndrome

From two large studies of over 230,000 dogs, the overall prevalence of CS is 0.17% to 0.28% (O'Neill et al. 2016; Carotenuto et al. 2019). The majority (80% to 85%) of these are due to a functional tumour of the *pars distalis* or *pars intermedia* of the pituitary gland, known as PDH. In most cases, excessive ACTH secretion leads to bilateral adrenocortical hypertrophy of the adrenal glands (Bennaim et al. 2019).

Autonomous cortisol-secreting tumours (FAT), causes adrenal dependent hypercortisolism (ADHC) and occurs in approximately 10-15% of cases (Bennaim et al. 2019). The elevated concentration of glucocorticoids triggers a negative feedback mechanism in the hypothalamus and pituitary gland, reducing the release of corticotropin-releasing hormone (CRH) and ACTH, respectively.

The most recent studies have reported a median age at the first suspicion of CS to be approximately 10,2 years (Schofield et al. 2020), which aligns with previous predictions ranging from 9 to 11 years (Carotenuto et al. 2019). Dogs aged 12 years or older have 5 to 7 times the odds to be diagnosed with CS compared to those aged between 6 to 8,9 years (O'Neill et al. 2016). Some studies suggest that females are more prone to be diagnosed with CS (Carotenuto et al. 2019). However previous studies found no evidence of sex predisposition (O'Neill et al. 2016). Furthermore, historically, the neutered status of an animal was associated

with increased odds of developing CS (Carotenuto et al. 2019). Recently, there has been an hypothesis suggesting that in younger spayed female dogs, CS originates primarily from PDH. However, if they are neutered at an older age, the probability of being of adrenal origin increases (Espiñeira et al. 2021). This is attributed to the elevated concentration of luteinizing hormone observed in later stages, which is associated with a concurrent increase in luteinizing hormone receptors on the adrenal glands (Espiñeira et al. 2021).

The breeds most represented across different studies include Jack Russel terrier, Bichon Frise, Yorkshire terrier, Staffordshire Bull terrier and West Highland White terrier (O'Neill et al. 2016; Schofield et al. 2020). Additionally, other breeds such as Dachshunds, Boxers and Standard Schnauzer have also been noted (Carotenuto et al. 2019). Among the overall population, crossbreds have a lower probability of developing the disease. Interestingly, the Border Collie and the Labrador retriever showed an even lower odds of manifesting CS compared to crossbreds (O'Neill et al. 2016). The size of the dog is also a discriminating factor associated with the origin of the HC. Dogs weighing less than 20 Kilograms (Kg) were more prone to have PDH, whereas larger dogs had an increased probability of having ADHC (Reusch and Feldman 1991).

2.3 : Clinical and clinicopathologic findings

Given the myriads of actions that glucocorticoids can partake in across the various systems of the dog, it is logical to expect a lengthy list of clinical signs associated with the chronic overexposure to them. Consequently, a combination of gluconeogenic, immune-suppressive, anti-inflammatory, protein catabolic and lipolytic effects will be expected (Behrend 2015). A proper anamnesis and a good physical examination are the best indicators to test for CS (Behrend et al. 2013).

Clinical signs consistently reported as the most frequent are polyuria (PU) and polydipsia (82-91%)(Reusch and Feldman 1991; Bennaim et al. 2019). This is most likely associated with secondary nephrogenic *diabetes insipidus*, owing to cortisol-induced inhibition of vasopressin at the renal tubular level, an increased threshold for neurohypophysis vasopressin release, or even central *diabetes insipidus* if a large tumour grows on the pituitary and compresses or invades the neurohypophysis. Other clinical signs include polyphagia (46-57%), abdominal enlargement (67-73%), hepatomegaly (50-67%) and excessive panting (61%). Polyphagia is associated with the direct effect of glucocorticoids (Bennaim et al. 2019), and might even be exacerbated if a concurrent DM is present (Miceli et al. 2017). Abdominal enlargement is believed to be a direct consequence of hepatomegaly secondary to glycogen deposition

(Bennaim et al. 2019), fat accumulation within the abdomen, an enlarged bladder due to polyuria, and decreased muscle tone caused by cortisol-induced protein catabolism (Reusch and Feldman 1991; Bennaim et al. 2019). Excessive panting, for instance, is thought to be caused by pulmonary thromboembolism, pulmonary mineralization and other causes of hypoxemia such as respiratory muscle weakness in combination with the obesity associated with CS, or chronic interstitial lung changes (Berry et al. 2000).

Within dermatological practice, the constellation of clinical signs most commonly reported include, endocrine alopecia, thin skin and poor healing. Alopecia is believed to result from follicular atrophy induced by chronic glucocorticoid exposure. Thin skin and poor healing may result from inhibition of the collagen synthesis by fibroblasts (Behrend 2015) . Pyoderma, for instance, results from a combination of local immunosuppression and the structural cutaneous changes (Bennaim et al. 2019). Calcinosis Cutis is thought to be associated with the rearrangement of the molecular structure of the collagen, forming an organic matrix that attracts and binds calcium (Doerr et al. 2013). Hyperpigmentation of the skin and comedones are also dermatological signs associated with CS.

Other less common clinical manifestations include: insulin resistance, thromboembolism, ligament rupture, facial nerve palsy, pseudomyotonia, testicular atrophy and persistent anestrus (Behrend et al. 2013).

Despite the diversity of signs associated with this syndrome, clinicopathological changes often provide practitioners with clues to either reinforce suspicion of CS and proceed to the endocrine testing, or to access possible comorbidities related to this syndrome (Behrend et al. 2013).

When analysing a complete blood count, cushingoid dogs may demonstrate an erythrocytosis and thrombocytosis, due to direct bone marrow stimulation. Erythrocytosis can also be associated with an hypoxemic state secondary to respiratory muscle weakness or even pulmonary thromboembolism (Berry et al. 2000). Neutrophilia, monocytosis, lymphopenia and eosinopenia display a leucogram pattern often referred to as “Stress Leukogram”, which may also be present. Neutrophilia and monocytosis are related to steroid-enhanced capillary demargination, lymphopenia is due to steroid-induced lympholysis, and eosinopenia is attributed to bone marrow sequestration (Bennaim et al. 2019).

Biochemical profile alterations illustrate the various metabolic pathways in which glucocorticoids partake. About 76-100% of cushingoid dogs may show an elevated alkaline phosphatase (ALP) due to an overexpression of an ALP isoenzyme unique to dogs (Behrend et al. 2013). Vacuolar hepatopathy, associated with glycogen storage, may not only increase

ALP but alanine aminotransferase in 80 to 85% of cases (Bennaim et al. 2019). Hypercholesterolemia (90%) is the consequence of steroid induced lipolysis (Bennaim et al. 2019) and decreased blood urea nitrogen (34-56%) is due to PU (Behrend et al. 2013). Hyperglycemia (20-57%) is explained by the increased hepatic gluconeogenesis and decreased peripheral glucose utilization, given the glucocorticoid interference on insulin action at the cellular level (Miceli et al. 2017). Hyperphosphatemia is most likely related to a decrease in urinary phosphate excretion. This results in direct stimulation of parathormone (PTH) secretion by the parathyroid gland, leading to secondary hyperparathyroidism. Interestingly, calcium levels, which were expected to be high due to calcium retention induced by increased (PTH), tend to remain within the normal range due to the calciurese induced by glucocorticoids (Corsini et al. 2021).

The clinical workup of a patient experiencing Hypercortisolaemia can often give misleading indications of other possible systemic diseases. Starting with acute pancreatitis, increased lipase synthesis or cellular permeability to lipases can be induced directly or indirectly by steroids. Consequently, when measuring parameters like 1,2-o-dialuryl-rac-glycero-3-glutaric acid-(6`-methylresorufin) ester (DGGR Lipase) or 1,2-diaglyceride in dogs with CS, values tend to be elevated, reducing the specificity on acute pancreatitis diagnosis. Canine pancreatic lipase immunoreactivity assessment remains uncertain due to conflicting results from previous studies (Linari et al. 2021).

Increased pre and post prandial serum bile acid concentrations have been reported in 11,8% and 37,5% of the cases respectively (Bennaim et al. 2018). Additionally, abnormalities in various coagulation parameters appear to be common in these patients (Pace et al. 2013; Park et al. 2013). More than 80% exhibit at least one altered parameter from the proposed hypercoagulability panel. Increased maximum amplitude in tromboelastography measurement, elevated thrombin-anti-thrombin complexes, shorter prothrombin time and higher fibrinogen concentrations are among the most recurrent findings (Pace et al. 2013).

Given the chronic nature of this disease, serum concentration of other hormones such as triiodothyronine and thyroxine can decrease, potentially leading to a misdiagnosis of Hypothyroidism (Ferguson and Peterson 1992).

Urinalysis (US) and urine specific gravity are two important and commonly performed tests, yet very intriguing when concerning CS. Urine specific gravity tends to be lower than 1.020 due to the PU/PD associated with this syndrome. Bacteriuria or positive urine bacterial cultures are often present in these patients due to local immunosuppression and glycosuria, increasing the likelihood of urine tract infections (Forrester et al. 1999). With an incidence as

high as 75% among patients, pathological proteinuria is a common finding in dogs with CS. Albumin appears to be the protein most frequently excreted in these cases, but the mechanisms and physiopathology supporting this observation are yet to be elucidated (Smets et al. 2010).

2.4 : Diagnosis of naturally occurring Cushing´s Syndrome

Despite the extensive list of clinical signs highlighted previously, it is important to bear in mind that the definitive diagnosis of CS involves, not only the clinical presentation, but also endocrine testing as well as an imaging evaluation (Behrend et al. 2013).

2.4.1 : Endocrine testing

Endocrine testing involves a two-step process that firstly screens for a potential hypercortisolemia and then, if possible, discerning its origin. Screening methods include the ACTH-stimulation test, the low-dose-dexamethasone suppression test (LDDST), and the urine cortisol to creatinine ratio (UCCR) (Behrend 2015). Understanding the source of the hypercortisolemia is mandatory for an accurate and specific treatment. Some of the tests, such as LDDST, can tackle those two challenges with a single strategy. The result of UCCR when combined with the oral high-dose-dexamethasone suppression test (HDDST) can also screen and distinguish etiology of CS. However, this test is seldomly performed nowadays. It is vital to bear in mind that the reason for such a diversity of tests, is that none of them is entirely reliable (Bennaim et al. 2019).

In order to appraise the reliability of a test, predicting tools such as sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), must be evaluated. The sensitivity of a test is defined as the proportion of patients with a disease who will have a positive test result, and specificity is the proportion of patients without the disease who will have a negative test result. One significant limitation associated with these predictive tools, is their lack of practical utility in helping clinicians estimate the probability of the disease in individual patients. This limitation is due to the fact that these parameters are defined based on populations with or without the disease. Since patients typically present with a set of signs rather than a diagnosis, clinicians cannot directly apply these parameters to them as they would not know at the time whether the patient has the disease or not.

The central focus of endocrine testing is to determine the diagnosis, therefore, understanding the likelihood that the test result will accurately reflect the correct diagnosis.

With this, a predictive value offers the clinician a probability of the disease based on the tests result (Akobeng 2007).

2.4.1.1 : Low-Dose-Dexamethasone Suppression Test (LDDST)

In healthy dogs, dexamethasone administration induces a rapid and prolonged inhibition of cortisol secretion (Meijer et al. 1978). This is due to direct negative feedback triggered in the hypothalamus and the anterior lobe of the pituitary gland. (Behrend 2015) resulting in suppression of CRH production. Consequently, ACTH secretion by the anterior lobe of the hypophysis is also suppressed, thereby restraining cortisol production by the adrenal cortex.

When performing a LDDST, the sensibility and specificity for the diagnosis of CS are reported as being 85%-100% and 44%-73%, respectively. A High NPV reinforces the test effectiveness in ruling out a diagnosis of CS in unaffected dogs. However, the diagnostic performance of the LDDST must be approached with caution (Bennaim et al. 2019).

The protocol consists of three different serum or plasma collections and cortisol measurement, before (T0) 4 (T4) and 8 (T8) hours after a low dose of dexamethasone (0,01mg/Kg) injection. A cortisol concentration higher than 39 nmol/L (1.41 microg/dl), 8 hours after dexamethasone administration is considered abnormal and, therefore, associated with CS (Lim et al. 2023)

As mentioned earlier, this test can confirm the diagnosis and potentially distinguish its origin. Various cortisol concentrations can be observed at T4 an T8, and with this, different patterns can be extrapolated. Some of these patterns can affirm the diagnosis and potentially identify its source, while others can help rule out the suspicion of CS.

2.4.1.2 : Urinary Cortisol to Creatinine Ratio (UCCR) and Oral High Dose Dexamethasone Suppression Test (HDDST)

This test has also been used to screen and possibly differentiate the origin of the HC in dogs (Behrend 2015). The UCCR refines the normal cortisol concentration variations by accessing its production over a specified period, typically overnight. When combined with HDDST synchronously, it has the potential to demonstrate both increased production and decreased sensitivity to glucocorticoid feedback. After dexamethasone administration, a decrease in blood cortisol concentration is typically reflected in a lowered UCCR, which may

suggest the presence of PDH (Galac et al. 1997). However, this test relies heavily on owner´s compliance, and a lack of a lowered UCCR does not confirm an adrenal tumour diagnosis (Behrend 2015).

2.4.1.3 : Adrenocorticotropic hormone stimulation test (ACTHst)

To assess the adrenocortical reserve, the ACTHst is conducted by measuring serum cortisol concentrations before and 60-90 minutes after the administration of synthetic ACTH, also known as tetracosactide or cosyntropin (Behrend et al. 2013). Various formulations of synthetic ACTH are available (Cohen and Feldman 2012). The sensitivity of the test varies widely, reported as 0-95%, depending on the population and the presumed HC origin. Sensitivity for PDH ranges between 80-92% while for ADHC it ranges from 0-63%. However, some studies may have overestimated sensitivity by including more advanced or severely affected cases. The low sensitivity observed for ADHC is believed to be linked to a different tumour response to exogenous ACTH. These neoplasms sometimes produce other steroid-derived hormones besides cortisol, which can lead to absent or weak stimulation. In such cases, consideration should be given to cortisol-precursor-secreting adrenal tumours or exogenous steroid administration (Bennaim et al. 2018). When accessing specificity, in contrast to what previous studies referred to, the value ranges from 59-61% if the cut off is set for 550 nmol/L. However, using an optimal higher cutoff value of 683 nmol/L (24.8 µg/dL) yields a significantly greater specificity at 94%, but moderately compromising the sensitivity of the test to 84%.(Nivy et al. 2018). The ACTHst is considered to be the gold standard for the diagnosis of iatrogenic HC in which a cortisol concentration before and after the stimulation is below the reference range (Bennaim et al. 2019).

2.4.2 : Diagnostic Imaging

Diagnostic imaging is relevant to understand the origin of the HC. Although endocrine testing may clarify the etiology of CS, imaging evaluation should be undertaken to complement functional assessment and understand the localization, characteristics, and potential compression or involvement of nearby structures. Also, with this knowledge, it is possible for the clinician to target the tumour with a better and specific treatment (Sanders et al. 2018).

2.4.2.1 : Diagnostic imaging of the pituitary gland

To visualize the pituitary gland accurately, it is essential to conduct either a computed tomography (CT) or magnetic resonance imaging (MRI) scan (Behrend et al. 2013). CT scans of healthy dogs have demonstrated that the pituitary gland undergoes physiological changes related to cranium size. (Vlugt-Meijer et al. 2003). To differentiate between a normal and enlarged pituitary, the Pituitary height/brain (P/B) ratio allows to recognize pathological differences regardless of the physiological cranium changes among the different breeds. A P/B ratio > 0.31 mm/mm2 suggests an increased pituitary gland (Vlugt-Meijer et al. 2003).

When contrasting CT scans with MRI, the latter offers superior image quality of the pituitary gland. However, when assessing a potential adenoma, the thickness of the slice becomes pivotal for microadenoma detection. Indeed, a 3 mm slice thickness has been designated as the maximal tumour detection limit for dogs with PDH. The supplementary data provided by dynamic contrast-enhanced CT, owing to the "pituitary flush," can also be observed in MRI, although with a distinct physiological explanation. The concentration of vasopressin in the posterior lobe manifests as a bright spot in MRI images. Consequently, the absence or a decrease of signal may be linked to possible compression by a hypophysial tumour (Kurokawa et al. 1998). In summary, dynamic CT and MRI of the pituitary offer equally diagnostic capabilities in PDH diagnosis (Vlugt-Meijer et al. 2003; Behrend et al. 2013).

2.4.2.2 : Diagnostic Imaging of the Adrenal gland

Abdominal ultrasound and CT scanning are both excellent imaging tools for gaining insights into the size, thickness, symmetry, shape, and possible vascular implications associated with tumours of the adrenal gland (Gregori et al. 2015; Soulsby et al. 2015). Changes in the adrenal gland can stem from various causes, such as chronic illness, aging and tumours occurring either within the gland or externally, where specific trophic hormones are produced, thus altering its dimensions (Pagani et al. 2016). When there's a suspicion of CS, various presentations of the adrenal gland are anticipated. Due to the trophic effect of ACTH, PDH typically manifests as a bilateral and symmetrical enlargement in the size and shape of the adrenal glands. Alternatively, if an adrenal functional tumour is suspected, asymmetrical and heterogeneous glands are expected. However, in clinical practice, this logic

differentiation is not always possible. Dogs with PDH may exhibit nodular hyperplasia while ADHC may not always cause atrophy of the contralateral gland. Additionally, different tumours can affect both glands. In a study involving 91 animals, only 56% of hypercortisolaemic patients could have their origin distinguished using ultrasonography (Melián et al. 2001; Pagani et al. 2017). Ultrasonography can also provide essential information regarding the potential malignancy of adrenal neoplasms. Large masses or nodules larger than 2 centimetres (cm), with or without vascular invasion, are considered indicative parameters for predicting malignant adrenal gland neoplasia. However, while vascular invasion is a specific parameter, it is not always sensitive for predicting malignancy (Pagani et al. 2016).

For cases in which a malignant neoplasm is suspected, CT scans can help the clinicians having a better inside on potential vascular invasion or metastasis (Gregori et al. 2015). Therefore, preoperative evaluation becomes crucial in order to gain insights into potential surgical challenges and guarantee optimal anaesthesia planning. Triple-phase helical CT is recommended for aiding in the preoperative differential diagnosis of adrenal gland tumours (Yoshida et al. 2016). Hypercortisolaemic dogs should undergo CT scans of the pituitary and adrenal glands (Bokhorst et al. 2019). One specific parameter, the adrenal diameter ratio (calculated as the maximal diameter of the larger gland divided by the minimal diameter of the smaller gland), appears to be potentially useful in distinguishing between PDH and ADHC, or in suspecting the possibility of simultaneous PDH and ADHC. If this value is higher than 2.08 ADHC is highly suspected (Piñeiro et al. 2011).

2.5 : Treatment of naturally occurring Cushing´s syndrome

Treating a dog with CS, aims to reduce cortisol production, whether it stems from an autonomous cortisol-producing neoplasm or excessive ACTH production. This treatment seeks to alleviate clinical signs, mitigate long-term complications, lower mortality rates, and improve overall quality of life (Sanders et al. 2018). Untreated dogs with PDH have a mean survival expectancy of 359 days (95% confidence interval, 48 to 916 days) to 506 days (Kent et al. 2007; Nagata et al. 2017). For ADHC, the only survival data currently available pertains to post-surgical outcomes (Mayhew et al. 2014).

2.5.1 : Treatment at the pituitary level

2.5.1.1 : Hypophysectomy

Surgical removal of the pituitary lesion through transsphenoidal hypophysectomy has proven successful in treating PDH in dogs (Meij et al. 2002). In a study of 306 animals, 91%

survived 4 weeks post-hypophysectomy, and 92% of these had a confirmed remission of the disease with a mean survival time of 781 days (Rijn et al. 2016). Post-surgery complications include perioperative death, transient mild postoperative hypernatremia, prolonged or permanent central *diabetes insipidus*, recurrence of HC, and transient reduction or cessation of tear production (Rijn et al. 2016). Hormone replacement therapy is necessary due to the removal of active endocrine tissue from the pituitary gland. Lifelong treatments with oral cortisone and levothyroxine are required. In only 10% of cases, synthetic vasopressin (desmopressin) is not discontinued over time (Meij 2001). An early diagnosis of the pituitary lesion, combined with a non-enlarged or moderately enlarged pituitary gland, is expected to result in the best outcome when hypophysectomy is the initial treatment choice (Fracassi et al. 2014; Rijn et al. 2016). Factors that negatively influence the prognosis include a high P/B value, advanced age, elevated preoperative endogenous ACTH and UCCR levels, and dexamethasone resistance in endocrine testing (Hanson et al. 2007; Rijn et al. 2016).

2.5.1.2 : Radiation therapy

Pituitary radiotherapy can also be performed to reduce tumour size, but it does not alleviate the signs associated with CS. Its best use is in combination with medical treatment or following hypophysectomy to reduce severe neurological signs or address potential post-surgery recurrence of HC (Kent et al. 2007; Sawada et al. 2018).

2.5.1.3 : Other therapeutic approaches

Novel but poorly described techniques, such as stereotactic radiotherapy and stereotactic radiosurgery, are potential treatment options for intracranial tumours like PDH. These new methods can precisely target well-defined neoplasms, with this, the potential side effects are diminished, due to lower radiation exposure of the patients and a decreased number of anesthetic protocols required (Mariani et al. 2015; Hansen et al. 2019; Nolan and Gieger 2019). Researchers have also evaluated the expression of certain receptors in post-hypophysectomy pituitaries to identify potential drugs that could specifically bind to these receptors. In canine corticotroph adenomas, the receptor subtype predominantly expressed is Somatostatin receptor type 2 (SSTR2) while Dopamine receptor type 2 and especially Somatostatin receptor type 5 (SSTR5) are expressed at much lower levels (Bruin et al. 2008). Given the inhibitory action of Dopamine receptors in the anterior lobe of the pituitary (ANTAKLY et al. 1987) drugs such as cabergoline have been assessed in dogs with PDH. One study showed a 42.5% favourable response to cabergoline in dogs with PDH, particularly if the tumour was located in the *pars intermedia* or if a <5 mm intrasellar neoplasm was present

(Castillo et al. 2008). Targeting Somatostatin type receptors (SSTR), drugs such as pasireotide and octreotide have garnered interest. Pasireotide binds to SSTR type 1, 2, 3, and 5 (Weckbecker et al. 2002), while octreotide binds with high affinity to SSTR2 and also to SSTR3 and SSTR5 (Cuevas-Ramos and Fleseriu 2014). Given the prior knowledge that corticotrope adenomas predominantly express SSTR2 and SSTR5 (Bruin et al. 2008), octreotide emerges as a potential treatment option for PDH (Sanders et al. 2018). Previous reports on the use of octreotide in treating pancreatic insulin-producing tumours, demonstrated that its effect were limited to 3 to 4 hours following percutaneous injection (Robben et al. 2006) therefore, the reliability of treatment would increase if an oral form were developed (Sanders et al. 2018). Dopamine/somatostatin chimeras such as BIM-23A760, represent novel treatment options currently in development, which have shown promising results in *in vitro* studies (Ibáñez-Costa et al. 2017).

2.5.2 : Treatment at the adrenal level

2.5.2.1 : Adrenalectomy

When it comes to treating adrenal tumours, adrenalectomy is recommended in most cases (Gilson et al. 1994). Historically, the recession of the tumorous gland can be performed through an open ventral celiotomy or a paracostal approach (Mayhew et al. 2014). However, the laparoscopic approach has been increasingly adopted (Naan et al. 2013). Regarding perioperative mortality, survival rates one-year post-surgery can reach 83% in cases without vascular involvement (Cavalcanti et al. 2020). This contrasts with a 76% survival rate in cases with vascular involvement, where the mean survival expectancy is 547 days post-surgery. Perioperative complications can include haemorrhage, tachycardia, bradycardia, hypotension, and hypertension (Massari et al. 2011). Postoperative complications may involve pancreatitis and thromboembolism. Median survival times for dogs with adrenal-dependent CS undergoing adrenalectomy can be as high as 953 days (Mayhew et al. 2014). Several important factors must be considered before surgery. Adrenal tumours greater than 3 cm in diameter are associated with lower postoperative survival expectancy (Sanders et al. 2019). Surgery is not recommended if metastasis is observed in previous imaging studies (Massari et al. 2011), and a poor prognosis is expected if a thrombus extends beyond the diaphragm (Mayhew et al. 2019).

2.5.2.2 : Radiation therapy

As previously described for pituitary tumours, radiotherapy can also be performed for adrenal tumours. A protocol involving a dose ranging from 30 to 45 Gray over three to five

days was able to reduce tumour diameter by up to 30% and resulted in a median overall survival time of 1,030 days in an experimental study involving 9 dogs (Dolera et al. 2016)

2.5.2.3 : Medical treatment

A diverse array of medications can be considered when evaluating a case of CS. Trilostane is a steroid analogue that inhibits the enzyme 3-beta-hydroxysteroid dehydrogenase, essential for the synthesis of all steroid hormones in the adrenal gland (Potts et al. 1978) Since its suppression of cortisol production lasts less than 12 hours, a twice-daily administration combined with a low dose seems to improve clinical response, keep the total daily dose relatively low, and significantly reduce the incidence of adverse effects. The recommended dose is 0,5-1mg/Kg Per Os q12h for dogs with PDH and 0,2-0,5 mg/Kg for dogs with adrenal dependent CS. Interestingly, larger dogs need a lower dose per Kg when compared to small dogs (Vaughan et al. 2008; Bermejo et al. 2020). Due to the inhibition of adrenal gland hormone production, hypoadrenocorticism is a possible side effect of this medication. Up to a quarter of dogs treated with Trilostane may experience an hypoadrenocorticism crisis (vomiting, diarrhoea, weight loss, inappetence, lethargy, weakness, and/or collapse) after four years of treatment (King and Morton 2017). An underlying concomitant disease might contribute to the onset of these clinical signs (Lamoureux et al. 2023). In most cases, discontinuing the medication is sufficient to mitigate these side effects. However, some reports have indicated possible persistent hypoadrenocorticism and even death (Ramsey et al. 2008; King and Morton 2017). Close monitoring of the patient is mandatory. It is the clinician's job to anticipate and accurately assess adrenal function to prevent possible crises. The ACTH stimulation test, previously described for the diagnosis of CS, can also be used to assess adrenocortical reserve and predict possible hypocortisolaemia (Neiger et al. 2002). The optimal time to perform this test is 2-3h after trilostane administration (Griebsch et al. 2014). However, other research has shown that in cases where hypocortisolaemia was present during the test, repeating it after 9- 12 hours revealed much higher cortisol concentrations. Therefore, decreasing the dosage seems more appropriate than completely discontinuing the medication in patients with no evident clinical signs (Midence et al. 2015). Other studies on this topic evaluated cortisol concentrations before and after administering the trilostane pill, as well as after ACTH stimulation, and compared these results to the clinical signs reported by the owners. The findings showed that pre-trilostane and post-trilostane concentrations more effectively differentiated between dogs with excellent control and those that were inadequately controlled (Macfarlane et al. 2016). In addition, a further study evaluated the possible differences between two pre-trilostane cortisol concentrations and concluded that stress during the re-

evaluation could potentially alter the values. Therefore, any significant events during this measurement should be considered when adjusting the trilostane dose (Boretti et al. 2018). Recent literature emphasizes that thorough history taking and meticulous physical examination are irreplaceable by changes in laboratory findings. Serum haptoglobin concentration, along with serum alanine aminotransferase (ALT) and gamma-glutamyl transferase, combined with pre-pill cortisol levels, can provide further clarification when assessing poorly controlled animals undergoing trilostane therapy (Golinelli et al. 2021). In terms of prognosis, dogs with PDH treated with trilostane have a survival expectancy of 852 days (Fracassi et al. 2015). For dogs with adrenal tumours, the median survival time is approximately 14 months (Arenas et al. 2014).

Mitotane, an historical treatment option for adrenal tumours, targets the adrenal gland directly. By inhibiting the enzyme sterol-O-acyl-transferase1 in the mitochondria of adrenocortical cells, it causes an accumulation of free cholesterol and fatty acids. Consequently, an Endoplasmic Reticulum stress is induced and the apoptosis cascade is activated (Sbiera et al. 2015). Mitotane also inhibits the steroidogenic enzyme cytochrome P450 involved in cortisol synthesis and activates other cytochrome P450 enzymes such as 3A4 that enhances the metabolism of glucocorticoids and other drugs (Hermsen et al. 2011; Kroiss et al. 2011). Despite the statistical similarities in efficacy and life expectancy compared to trilostane therapy (BRADDOCK et al. 2003; Alenza et al. 2006; Arenas et al. 2014), reports indicate that mitotane treatment is associated with more side effects and is less safe than trilostane (Ramsey 2010).

2.6 : Complications and comorbidities

With a prevalence reaching as high as 80%, systolic hypertension is a well-recognized and frequent consequence of CS in dogs (José et al. 2020) described as systolic blood pressure (Bp) greater than 160 mmHg (Acierno et al. 2018). Different pathophysiology pathways have been proposed, such as the cortisol-induced erythropoietin and thromboxane A2 synthesis. Both contribute to vasoconstriction, but also stimulate the bone marrow to produce and release platelets, a common finding in dogs with CS. (Kelly et al. 2000; Nakahata 2008; José et al. 2020).

Diabetes Mellitus, occurs due to glucocorticoid induced peripheral insulin resistance (Tappy et al. 1994) and a decreased insulin secretion by the pancreatic Beta-cells (Gilor et al. 2016). Unfortunately, CS was associated with shorter survival time in dogs with concomitant DM (Tardo et al. 2019).

Up to a quarter of the population of dogs with CS may develop biliary mucocele (Mesich et al. 2009). The proposed pathophysiology is associated with a reversible change in the bile salt composition, which induces inflammation in the gallbladder epithelium. This inflammation leads to reactive mucinous hyperplasia and increased sludge deposition in its lumen (Klinkspoor et al. 1995). Other hypothesis suggests that the immunosuppressive state of CS and alterations on the gallbladder motility might increase the risk of bacterial cholecystitis (Mesich et al. 2009).

Other less common conditions associated with CS include calcium-containing uroliths in about 4% of patients (Hoffman et al. 2018) and pulmonary thromboembolism given the hypercoagulability state in CS as previously mentioned (Pace et al. 2013).

2.7 : Proteinuria

The term proteinuria relates to an abnormal increase in protein content in the urine. Several tests can be performed to detect and measure proteinuria, including conventional urinalysis, urine albumin concentration assays and the urine protein to creatinine ratio (UPC) determination (Lees et al. 2005).

Normal urine production results from an orchestrated and diverse set of interaction between cells, enzymes and hormones on the healthy kidney tissue. Focusing on the glomeruli, a sieve-like structure known as the glomerular filtration barrier (GFB) separates the glomeruli from the beginning of the tubular nephron system. This barrier prevents the excretion of essential blood components, such as proteins and other macromolecules. The microscopy architecture of the filtration barrier is formed by three distinct layers, the glomerular endothelial cell, the glomerular basement membrane and the visceral epithelial cell or podocyte (Toblli et al. 2012). These structures together form pores with a size of around 60 000 to 70 000 Daltons. Additionally, they are covered by negatively charged glycoproteins, creating an ionic charge barrier that impedes the excretion of negatively charged molecules (Vaden and Elliott 2016). Despite this complex sieve-like structure, the glomerulus leaks small quantities of Albumin, a negatively charged protein with a molecular weight of 69 000 Daltons. However, rapid endocytosis by the proximal tubular cells prevents significant quantities of albumin from entering the urine (Maack 2013; Vaden and Elliott 2016). Low molecular weight proteins that comprise hormones, enzymes, immunoprotein fragments (Ex: Bence johns' proteins) and host specific antigens are extensively filtered, but absorbed and catabolized by the proximal renal tubules. Therefore, a functional and healthy kidney should be able to either block or reabsorb circulating proteins from the bloodstream, preventing them from entering the urinary system

(Maack 2013). Any inflammation or infection present in the urinary tract can cause proteinuria due to exfoliation of the epithelium, or the presence of RBC or leukocytes (Lees et al. 2005).

If a patient is suspected to have persistent proteinuria, a proactive approach by the clinician is mandatory due to the potential negative outcomes associated with this condition. Persistent proteinuria has been shown to be linked with a threefold increased risk of developing a uremic crises and death in dogs with chronic kidney disease when the UPC is greater than 1 (Jacob et al. 2005). Further studies correlated the proteinuric state with a subsequent renal impairment by measuring the glomerular filtration rate (GFR). Dogs with a UPC lower than 1 had a significant increase in survival expectancy. Conversely, if associated with hypertension, dogs with a chronic kidney condition, a significant increase in mortality is expected (Wehner et al. 2008).

2.7.1 : Causes of proteinuria

Precise assessment of proteinuria demands the evaluation on the localization, persistence and magnitude. Persistent proteinuria is defined as having three separate positive measurements taken at two-week intervals (Lees et al. 2005). To effectively approach and treat persistent proteinuric dogs, localizing and identifying the underlying cause is mandatory. Pre-renal causes occur due to an increased delivery of low molecular weight proteins to nephrons, that exceeds the absorption capability of the proximal tubular cells. Examples include lymphoma or multiple myeloma(Palgrave et al. 2010) , which secrete immunoglobulin light chains, haemoglobinuria secondary to intravascular haemolysis or myoglobinuria from rhabdomyolysis (M. Syme and Rosanne Jepson 2024). Post-renal conditions like urinary tract infections, urolithiasis, vaginitis or even transitional cell carcinomas promote the entry of protein in the urine in association with an exudation of blood, serum or neoplastic cells into the lower urinary or genital tracts (Vaden and Elliott 2016). Renal causes include altered permselectivity of the GFB in conditions such as increased glomerular pressure, amyloidosis or membranoproliferative glomerulonephritis. Impaired tubular reabsorption of plasma proteins can occur due to any form of renal tubular dysfunction (Lees et al. 2005). In these cases, normoglycemia may be present alongside glycosuria, highlighting the essential role of the proximal tubules in glucose reabsorption, as observed in Fanconi Syndrome (Lees et al. 2005; Vaden and Elliott 2016).

2.7.2 : Proteinuria in Cushing Syndrome- What do we know?

With a considerable high incidence, seven out of ten patients with CS will suffer from pathological proteinuria (Mazzi et al. 2008). Interestingly, urinary albumin is increased in

animals treated with glucocorticoids, as well as canine Cushing´s patients (Waters et al. 1997; Mazzi et al. 2008). Research on humans indicates that around 80% of those with Cushing´s disease have increased albuminuria, which largely resolves following effective treatment (Koh et al. 2000). As previously mentioned, renal proteinuria is the consequence of an increased glomerular filtration from either damage to the GFB, higher intraglomerular pressure or even an impaired proximal tubular reabsorption (Lees et al. 2005; Vaden and Elliott 2016). Proposed physiopathological mechanisms for this condition include disturbances on lipid metabolism and glomerular hemodynamic changes caused by glucocorticoids. Even with a normal permselectivity of the GFB, an excessive binding of free fatty acids to serum albumin can possibly lead to an increased urinary albumin excretion. Factors contributing to increased glomerular proteinuria may include heightened intraglomerular pressure and damage to the glomerular basement membrane (Hayashi et al. 1990; Koh et al. 2000; Schellenberg et al. 2008). Some reports state that glucocorticoid-induced hypertension is the primary reason for proteinuria. Even though systemic hypertension does not directly correlate to proteinuria, glucocorticoids induce an increase in renal plasma flow and GFR (Hall et al. 1980; Kubota et al. 2001; Schellenberg et al. 2008). A 42-day treatment with exogenous glucocorticoid in dogs, was sufficient to produce microscopical changes in the kidney, such as hypercellular glomerular tuffs, glomerular adhesions and moderately thickened Bowman's capsule (Waters et al. 1997). Therefore, increased intraglomerular pressure might lead to reversible or irreversible histomorphology changes in the glomeruli depending on the degree or chronicity of disease (Schellenberg et al. 2008). As a result of this potential irreversible changes caused by glucocorticoids, optimal control of hypertension and proteinuria might require angiotensin receptor blockers in addition to prior proposed medical treatment for CS (Fowler et al. 2021; José et al. 2021).

2.8 : Endothelial Glycocalyx

Overlaying the extracellular membrane of many living cells, a well-organised meshwork of glycosaminoglycans (GAG), sugar chains, proteins, and enzymes form a negatively charged gel-like matrix named Glycocalyx (Glx) (Lawrence-Mills et al. 2022). Numerous studies linked its critical role in maintaining vascular health and homeostasis with a myriad of different pathophysiological processes in different species (Salmon et al. 2012; Alphonsus and Rodseth 2014; Kolářová et al. 2014). Emerging evidence suggests that compromised integrity of the glomerular endothelial glycocalyx (eGlx) may significantly contribute to the onset of proteinuria, particularly albuminuria, in cases of glomerular injury (Salmon et al. 2012; Salmon and Satchell 2012; Rabelink and Zeeuw 2015).

2.8.1 : Endothelial Glycocalyx structure

With a similar overarching structure in both prokaryotic and eukaryotic cells, including epithelial and endothelial cells, the eGlx is composed of a rich variety of molecules. Proteoglycans (syndecans, glypicans), which make up the eGlx backbone, consist of negatively charged and covalently bonded core proteins and unbranched glycosaminoglycans (GAG) side chains. These proteoglycans vary in the number and type of side chains, size of core proteins, and their extent to membrane binding. GAG side chains are linear polymers of disaccharides that differ in length and molecular structure modifications like acetylation and sulfation (Lawrence-Mills et al. 2022). GAG hyaluronan binds directly to the endothelial cell membrane via surface receptors (Shaw et al. 2022).The eGlx contains many glycoproteins with small-branched carbohydrate chains, including endothelial cell adhesion molecules and parts of the coagulation and fibrinolytic systems. This layered structure also has soluble components like receptors (e.g., fibroblast growth factor receptor, enzymes, and growth factors) and other plasma-derived molecules, like albumin that temporarily bind to eGlx components (Jacob et al. 2007). The diverse composition of the eGlx is constantly balanced with the blood, and its structure changes in response to the surrounding environment (Shaw et al. 2021).

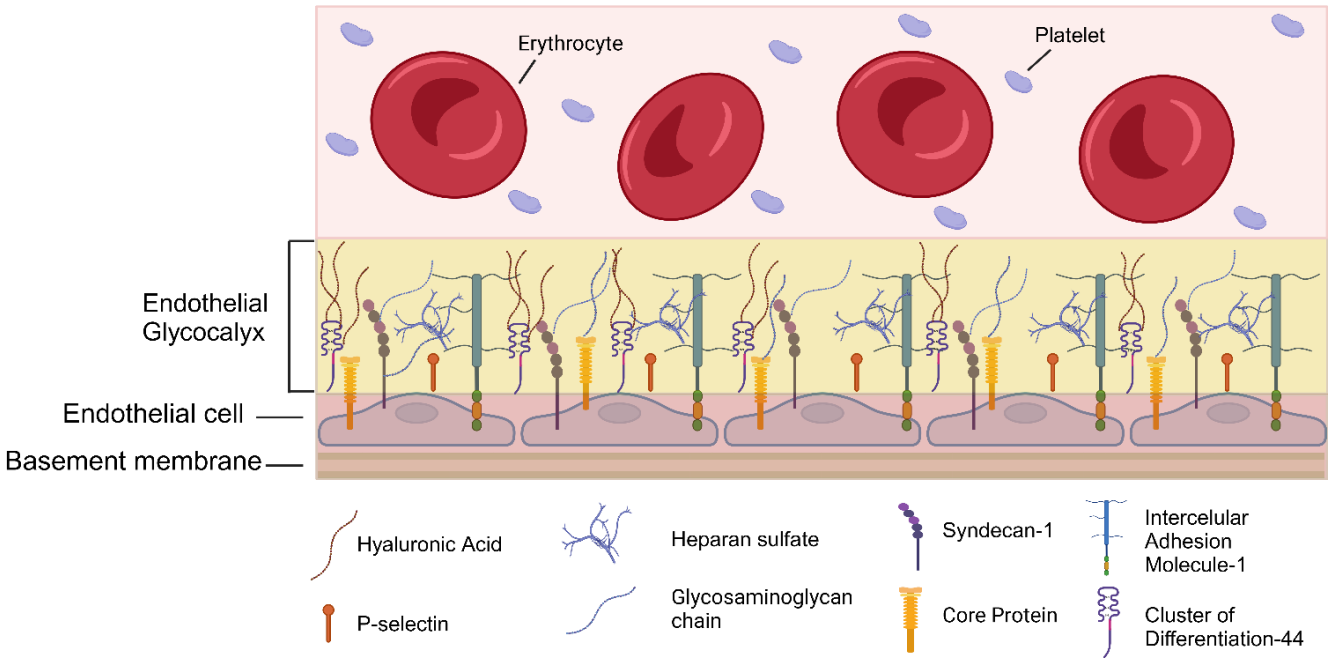


Figure 1: Illustration of some key structural components of the endothelial Glycocalyx.

2.8.2 : Endothelial Glycocalyx function

Despite the paucity of research published on eGlx in veterinary medicine, it is known that it contributes to vascular health through various mechanisms (Lawrence-Mills et al. 2022). Starting with the regulation of the vascular permeability, a complex meshwork of proteins and disaccharides combined with a densely negative charged matrix created by the sulphated GAGs, promotes the formation of a physical and electrostatic sieve-like barrier. Ultimately, molecules larger than 70 000 Da cannot reach the endothelial cell membranes within the vascular lumen (Vink and Duling 1996). Furthermore, the attachment of circulating albumin to the eGlx generates an oncotic pressure within the Glx itself, thereby increasing local oncotic pressure. Consequently, the regulation of fluid exchange between the blood vessels and the interstitial space is due to differential oncotic pressures of the vessel lumen, the eGlx and the interstitial fluid (Jacob et al. 2007; Salmon et al. 2009). By covering the luminal surface of the endothelial cell, the eGlx, also regulates and articulates the interaction between the cells within the vascular space. Degradation of the eGlx exposes the intracellular adhesion molecule-1 resulting in leukocyte adherence and activation (Mulivor and Lipowsky 2004). Storing enzymatic co-factors such as antithrombin and tissue-type plasminogen activator, the eGlx integrity also balances the hyper or hypo-coagulable state (Johansson et al. 2011).

Additionally, the ability to bind the enzyme superoxide dismutase protects the endothelium from oxidative stress by scavenging free oxygen radicals (Li et al. 1998). The eGlx can also act as mechanotransducer, enabling endothelial cells to respond through changes in the hemodynamic regulation of the blood vessels (Lawrence-Mills et al. 2022). Wall-shear-stress is transmitted through de eGlx via transmembrane proteins that bind to endothelial cell cytoskeleton (Pries et al. 2000). Chronic shear stress exposure is thought to increase GAG synthesis (ARISAKA et al. 1994). Furthermore, increases in blood flow can induce conformational changes within the Glycocalyx structure triggering the release of nitric oxide (Mochizuki et al. 2003), mediating local vasodilation and increased vascular permeability (Santos-Parker et al. 2017).

2.8.3 : Indirect Endothelial Glycocalyx measurement

After highlighting the pivotal role of the eGlx in maintaining vascular homeostasis, it is reasonable to infer that certain diseases through hemodynamic alterations, can lead to its deterioration and breakdown. Regarding the veterinary world, evidence of its breakdown and shedding have been reported in patients with septic peritonitis (Shaw et al. 2021), myxomatous mitral valve disease and hypercoagulable state (Lawrence-Mills et al. 2022), canine parvoviral infection (Naseri et al. 2020) and a model for septic shock (YINI et al. 2015). In order to visualize and measure eGlx breakdown, direct visualization methods such as perfusion fixation and staining with heavy metal ions or immersion fixation and lectin staining are challenging to perform and analyse in a clinical perspective. Therefore, the indirect measurement with enzyme-linked immunosorbent assays (ELISA), or with sidestream dark field imaging are more clinically applicable and minimally to non-invasive (Lawrence-Mills et al. 2022). Sydecan and heparan sulphate ELISAs kits have been used to measure and quantify eGlx breakdown in a model for septic shock (YINI et al. 2015), however it was unclear if these kits were validated for use in dogs (Lawrence-Mills et al. 2022). On the other hand, hyaluronan assays, validated in dogs, have been used to assess breakdown and shedding in many diseases (Naseri et al. 2020; Shaw et al. 2021; Lawrence-Mills et al. 2022). Hyaluronic acid has also been measured as a biomarker for vascular health in conditions such as portosystemic shunts, both before and after surgical intervention (Seki et al. 2010; Devriendt et al. 2021). Sidestream darkfield imaging is another non-invasive technique to evaluate eGlx integrity. This imaging method, already utilized in human medicine, estimates eGlx depth by measuring the perfused boundary region (PBR)(Pouska et al. 2018). The PBR indicates the area within the eGlx where RBCs can flow through. Thus, eGlx damage leads to an increased PBR (Lawrence-Mills et al. 2022).

Sidestream darkfield imaging has already been used to measure the eGlx of sublingual microvasculature in animals (Yozova et al. 2022). However, accurately assessing the sublingual microvasculature and obtaining clear images requires several minutes, necessitating that the patients be under anaesthesia (Lawrence-Mills et al. 2022).

SECTION 3- ENDOTHELIAL GLYCOCALIX SHEDDING AS A PROPOSED PATHOPHYSIOLOGICAL PATHWAY FOR PROTEINURIA IN CANINE HYPERCORTISOLISM

3.1 : Background

Naturally occurring hypercortisolism most commonly results from an excessive cortisol production due to either a PDH or a FAT (Rijnberk 1996). Among clinicopathological findings, dogs with hypercortisolism often present with proteinuria (which may be present in about 75% of these cases (Smets et al. 2010)). Proteinuria may be due to pre-renal causes (e.g. hyperproteinaemia status), post-renal (urinary infection) or of renal origin (tubular or glomerular) (Lees et al. 2005; Vaden and Elliott 2016). Given the myriad of actions they can have on multiple organs and systems, hemodynamic changes in hypercortisolism, such as hypertension, and the consequent changes on the GFR in dogs, may eventually result in proteinuria and glomerulosclerosis (Smets et al. 2010; Smets et al. 2012). Covering the endothelial surface, a loosely adherent matrix of glycosaminoglycans, proteoglycans and other adsorbed plasma components, forms the eGlx. As the primary interface between blood and the vessel wall, the endothelial surface layer regulates vital endothelial cell functions under physiological and pathophysiologic conditions (Salmon and Satchell 2012; Lawrence-Mills et al. 2022). In veterinary medicine, there is growing evidence that supports the role of eGlx shedding in various animal models. However, only a few studies have examined its role in naturally occurring diseases such as septic peritonitis, hypercoagulability, myxomatous mitral valve disease, and canine parvoviral enteritis (Smets et al. 2010; Lawrence-Mills et al. 2022). Emerging studies have revealed a compelling correlation between eGlx shedding and proteinuria in human patients. Since the regular arrangement (Squire et al. 2001) and negative charge (Stace and Damiano 2001), eGlx fibers allows it to act as a sieve for macromolecules, such as albumin. The loss of integrity in the glomerular eGlx can lead to increased permeability to albumin and other macromolecules. Consequently, the compromised glomerular eGlx integrity may significantly contribute to the development of proteinuria, specifically albuminuria, in cases of glomerular injury (Salmon et al. 2012; Salmon and Satchell 2012; Rabelink and Zeeuw 2015). To date, literature examining the relationship between hypercortisolism and its effect on canine eGlx shedding is scarce (Lawrence-Mills et al. 2022). Therefore, the main objective of this project is to investigate a potential correlation between the breakdown of eGlx and the occurrence of proteinuria in dogs suffering from hypercortisolism.

3.2 : Objectives

The eGlx has several key functions, some of which are tightly related to changes in blood pressure and glomerular function (CURRY 1999; Weinbaum et al. 2007).

In humans, increased glycocalyx shedding, measured through serum hyaluronan concentrations, has been linked mostly to ischemic and inflammatory states, but it has also been shown that hypercholesterolemia (Meuwese et al. 2009), chronic kidney disease and cardiac disease increase eGlx shedding (Padberg et al. 2014; Liew et al. 2021). In veterinary medicine, increased glycocalyx shedding has been documented in patients in hypercoagulable states, including four dogs with hypercortisolism (Lawrence-Mills et al. 2022).

By linking proteinuria to increased eGlx shedding, this project aims to explore the role of glycocalyx breakdown as a contributing factor for proteinuria in dogs with hypercortisolism. The hypothesis is that hyaluronan levels will be significantly higher in dogs with hypercortisolism when compared to healthy dogs, showing a positive correlation with UPC values.

3.3 : Materials and methods

An observational cross-sectional multicentric study was conducted with the following tasks and rearrangement:

3.3.1 : Sample selection

Serum samples of untreated hypercortisolaemic dogs diagnosed from 2022 to 2024 were obtained and selected from a biobank of leftover samples, stored and frozen at -20ºC or -80ºC after a maximum of 24h post collection. These samples have been collected from two veterinary universities: Veterinary Teaching Hospital, Faculty of Veterinary Medicine - University of Lisbon (HEV-FMV) and the Veterinary Teaching Hospital of the University of Bologna. Samples from dogs with a final diagnosis of hypercortisolism and proteinuria assessment (by UPC measurement) without any concurrent illnesses that might affect serum hyaluronan concentrations or UPC were selected.

The control groups (composed of healthy, hypercoagulable state and Myxomatous mitral valve disease dogs) were obtained from previous studies that utilized the same hyaluronan measurement kit validated for dogs (Beiseigel et al. 2021; Lawrence-Mills et al. 2022). By

adhering to the three “Rs” policy, a larger pool of hypercortisolaemic dogs were analysed, enabling a more robust statistical analysis.

3.3.2 : Measurement of serum hyaluronan concentration

Serum hyaluronan concentrations were assessed using a sandwich ELISA- Hyaluronan Quantikine ELISA kit (R&D Syems, Inc), previously validated for use in dogs (Beiseigel et al. 2021; Lawrence-Mills et al. 2022; Shaw et al. 2022).

3.3.2.1 : Assay procedure

A total of 40 serum samples collected from dogs diagnosed with hypercortisolism, previously collected and frozen at -20ºC to -80ºC, were used to measure its concentration in Hyaluronan.

For the sample preparation, every canine serum required a five-fold dilution of 50µL of serum in 200µL of Calibrator Diluent RD5-18. For the reagent preparation, 20 mL of Wash Buff er Concentrate was added to 480 mL of distilled water to prepare 500 mL of Wash Buff er. The subtract solution was obtained by mixing in equal volumes (12ml) of the colour reagents A and B. Finaly, the standard was reconstituted with the Hyaluronan Standard and the Calibrator Diluent RD5-18. This reconstitution produced a stock solution of 40 ng/ml. To produce a series of dilutions, 200 µL of Calibrator Diluent RD5-18 was pipetted into each of 6 tubes. The undiluted Hyaluronan Standard (40ng/mL) served as the high standard while the Calibrator Diluent RD5-18 served as the zero standard (0 ng/mL). After the preparation of the reagents, the assay diluent RD1-14 was added to each of the 96 pools as well as the standard and the serum samples in a specific order.

Following the pipetting of samples and standards, a two-hour incubation was then conducted at room temperature on a horizontal orbital microplate shaker. The aspiration and washing process was then executed to remove any unbounded substances. A total of 5 washes with a wash buffer were performed prior to the Hyaluronan conjugate pipetting. Following the addition of 100µL of Hyaluronan conjugate to each well, a second two-hour incubation was performed. The washing process was repeated before adding 100µL of

Substrate Solution to every poll. A final 30-minute incubation was executed on the benchtop at room temperature, protected from light.

The last step consisted on the optical density determination of each well within 30 minutes after the last incubation, using a spectrophotometer analyser set to 450 nanometres.

3.3.3 : Data processing and statistical analysis

All the relevant data associated with each animal were collected and imported into a Microsoft Excel® 2016 database sheet. Statistical analyses were performed using commercially available software (IBM SPSS® Statistics for Windows, version 28.0.1.0). To characterize the population, a descriptive statistical analysis was conducted. The normality of variables was assessed using the Shapiro-Wilk test. If the data were normally distributed, serum hyaluronan concentrations between specified groups (e.g., gender, hypertensive status) were compared using an unpaired t-test. If the data were non-normally distributed, the Mann-Whitney U test was used as an alternative. When compared to the historic control groups, as hyaluronan concentrations were not normally distributed, the values for these groups were reported as (median [min-max range]).

In the subset of dogs with hypercortisolism, a Spearman rank correlation analysis was conducted to examine potential associations between urine UPC, blood pressure, cholesterol, triglycerides, and serum hyaluronan concentration. To investigate differences in serum hyaluronan concentration across varying degrees of proteinuria severity, a Kruskal-Wallis test was applied to three UPC-defined groups: non-proteinuric (<0.5), proteinuric (0.5–2.0), and overly proteinuric (>2.0). An exact Fisher's test was employed to assess associations between the categorical variables of blood pressure and cholesterol levels. Hypertensive status was defined as systolic blood pressure greater than 160 mmHg(Acierno et al. 2018) and hypercholesterolemia was defined as serum cholesterol greater than 300 mg/dL(Cardoso et al. 2016). All statistical tests were conducted with a 95% confidence interval. A significance level of $p<0.05$ was considered, meaning that when the p-value was ≤0.05, the results were deemed statistically significant.

A power analysis was performed and calculated using the mean values of the healthy and the hypercoagulable state dogs(Lawrence-Mills et al. 2022). Statistical relevance was extrapolated if the studied population had $n > 6$.

3.4 Results

3.4.1 : Characterization of the population

Forty dogs previously diagnosed with hypercortisolism were included in this study. Of these, twenty-nine were confirmed to have PDH based on the following criteria: lack of suppression, escape pattern, or partial suppression pattern in the LDDST, combined with an endogenous ACTH concentration > 6 picograms per millilitre. One dog was diagnosed with ADHC, characterized by a lack of suppression in the LDDST, an endogenous ACTH concentration < 6 picograms per millilitre, and adrenal gland abnormalities observed on ultrasonography. The remaining ten dogs diagnosed with hypercortisolism had an undetermined origin. A total of 21/40 (52,5%) females were included, of which 16/21 (76,1%) were neutered. The male population accounted for 19/40 (47,5%) animals and 11/19 (57,9%) were neutered. The age distribution of the population followed a normal curve, ranging from 7 to 16 years, with a mean age of 11.3 years (SD = 2.11). In terms of breed composition, 27/40 (67,5%) were pure-bred dogs, while 13/40 (32,5%) were mixed breeds. Among the pure breeds, 8/27 (29,6%) were Teckel, 3/27 (11,1%) French Bulldogs, 2/27 (7,4%) Beagles, 2/27 (7,4%) Italian Bloodhound. The remaining purebred dogs (12/27, 44.4%) represented various other breeds, each represented by one individual (Table 1).

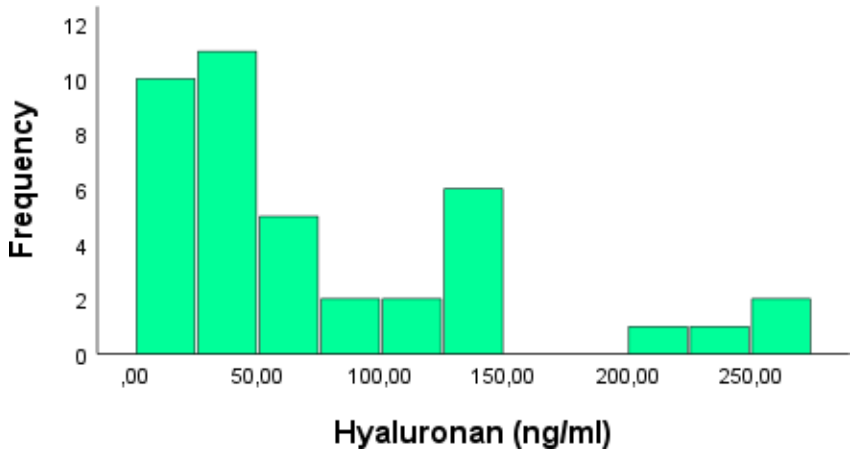
Table 1: Comparison of population characteristics

Population	Hypercortisolaemic dogs
Breed	Pedigree *n* = 27
	Cross Breeds *n* = 13
	Teckel *n* = 8
	French Bulldog *n* = 3
	Beagle *n* = 2
	Italian Bloodhound *n* = 2
	Boxer *n* = 1
	Belgian Malinois *n* = 1
	Chihuahua *n* = 1
	Italian Lagotto Romanoglo *n* = 1
	Labrador *n* = 1
	Maltese Bichon *n* = 1
	Miniature Dobermann
	Pinscher *n* =1
	Miniature Schnauzer Dog *n* = 1
	Poodle *n* = 1
	Portuguese Podengo *n* = 1
	Portuguese Water Dog *n* = 1
	Shih-Tzu *n* = 1
	Yorkshire Terrier *n* = 1
Sex	Female entire *n* = 5
	Female neutered *n* = 16
	Male entire *n* = 8
	Male neutered *n* = 11
Age in years Mean (range)	11,3 (7-16)
Hypercortisolaemic	PDH *n* = 29
	ADHC *n* = 1
	Undetermined *n* = 10

3.4.2 : Hyaluronan concentration descriptive analysis

In a sample of forty animals (*n* = 40), the serum hyaluronan concentrations ranged from 9,70 to 265,07 ng/mL with a median value of 45,94 ng/mL and an interquartile range of 99,45. As shown in (Graph 1), the distribution of hyaluronan concentration does not follow a normal distribution.

Graphs 1: Distribution of the population serum Hyaluronan concentration.



3.4.3 : Gender variation of Hyaluronan concentrations

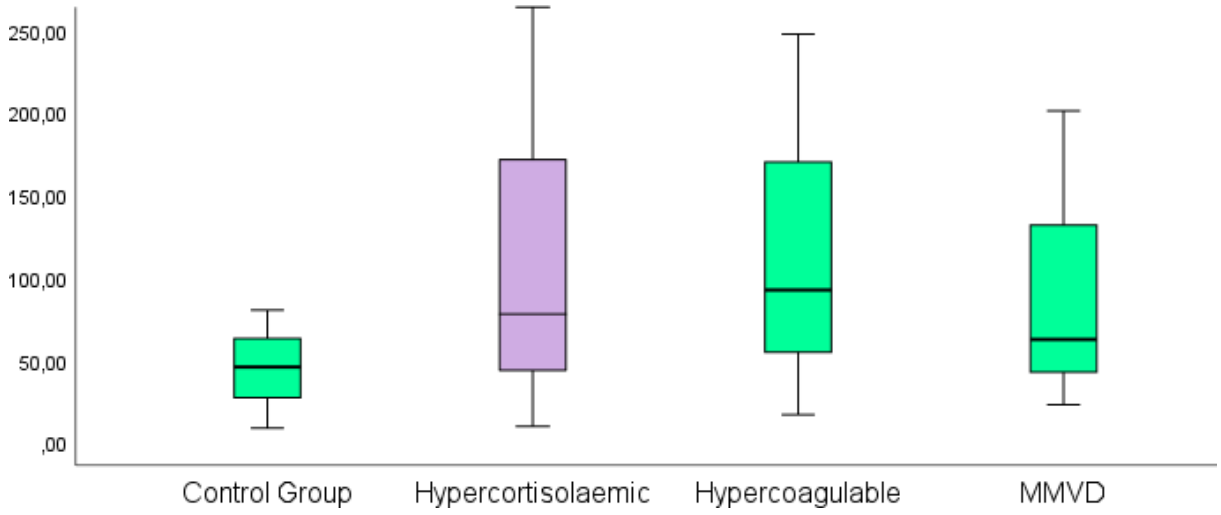
No statistical differences were observed between males and females (p = 0.708).

3.4.4 : Hyaluronan concentration: Hypercortisolaemic dogs and historic controls.

Current data was compared with historic controls which used the same kit. Serum hyaluronan concentrations median [min-max range]) ng/mL of the healthy dogs (*n* = 18); 45.7[8.70-80.20] ng/mL (Lawrence-Mills et al. 2022), (*n* =19); 17,4 ng/ml and (*n* =19) 17,8 ng/ml (Beiseigel et al. 2021) the hypercoagulable state group (*n* = 21) 92.40[16.90-247.60] ng/mL and the myxomatous mitral valve disease (*n* = 27) 62.40[22.80-201.00] ng/mL group were obtained from previous studies (Lawrence-Mills et al. 2022).

When comparing the hypercortisolemic population with the healthy dogs, a similar median value was observed in the Lawrence-Mills study (45.94 vs. 45.7), whereas the median value in the hypercortisolemic population was notably higher than those reported in the Beiseigel study (45.7 vs. 17.4 and 17.8). Graphically (Graph 2) a significant apparent difference in the minimum and maximal hyaluronan concentrations can be observed. Unfortunately, due to the missing individual hyaluronan concentration data for each healthy animal, no analytical comparisons (Mann-Whitney U test) could be performed in this study. Nevertheless, an analytical evaluation between the control group and the Hypercoagulable state group did reveal a significant difference between these two groups (Lawrence-Mills et al. 2022). With this, given the proximity between the minimum (9,70 vs 16,90 ng/mL), and the maximum values (265,07 vs 247,60 ng/mL), along with the graphical appearance, an apparent similarity between groups can be considered. Therefore, the hypercortisolaemic group appears to have higher hyaluronan concentrations compared to the controls. No information regarding the comparison between the Hypercortisolaemic group and MMVD group could be extracted, due to the missing individual hyaluronan concentration of the MMVD group.

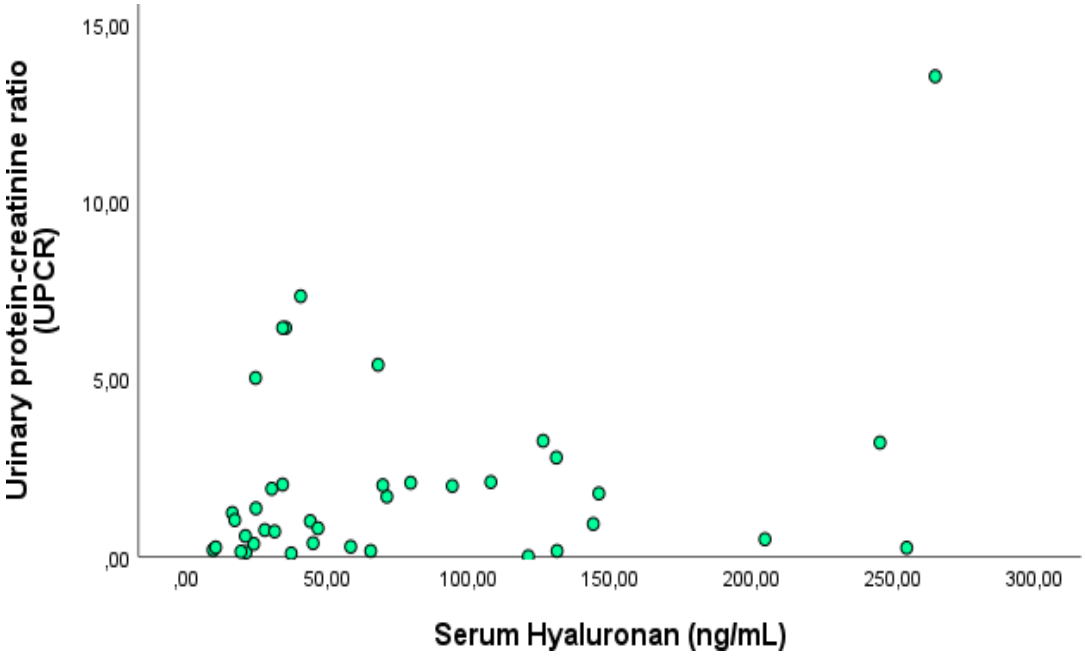
Graph 2: Scatter plot illustrating serum hyaluronan concentrations (ng/mL) between healthy dogs (Control Group) (n = 18) compared to dogs with various naturally occurring diseases. (Hypercortisolaemia n = 40; myxomatous mitral valve disease n = 27; and hypercoagulable, n = 21). Bars show minimum, mean, maximum values and interquartile ranges (Lawrence-Mills et al. 2022).



3.4.5 : Serum Hyaluronan concentrations and urine creatinine-protein ratio.

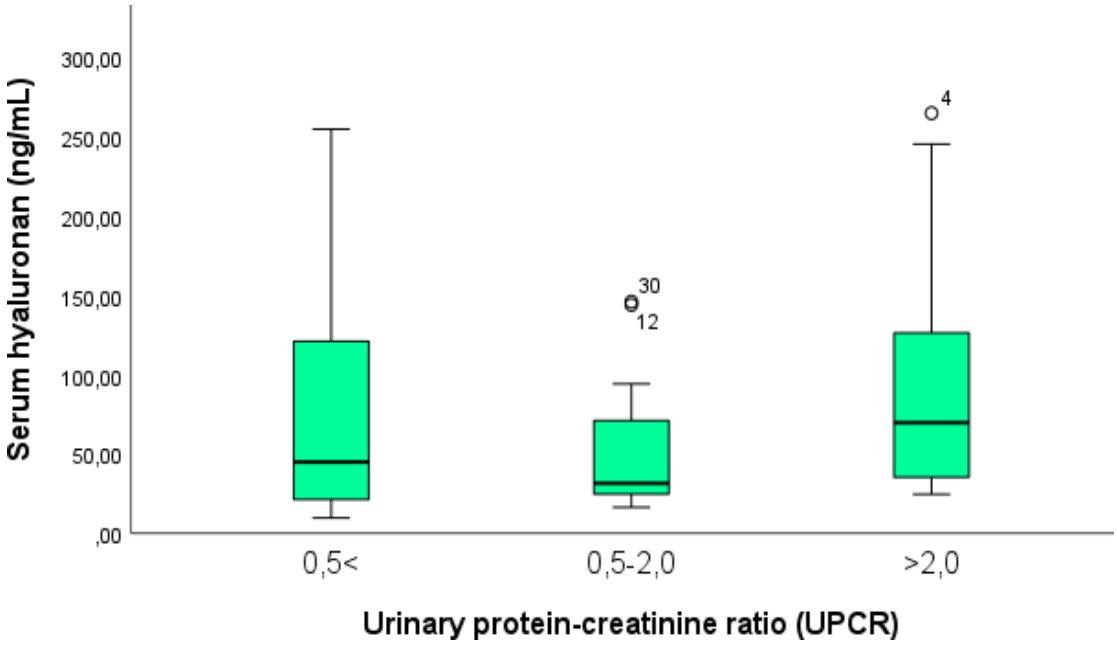
A Spearman rank correlation test with a p = 0,119 and a spread-out distribution of values in the Scattered chart (Graph 3), supports the conclusion that there is no meaningful correlation between serum hyaluronan concentration and UPC.

Graphs 3: Scatter plot of UPCR values by serum hyaluronan concentration



To investigate the differences between the severity of proteinuria and serum hyaluronan concentration, three groups of UPC, categorized by severity (<0.5 – non-proteinuric; 0.5-2.0 – proteinuric; >2.0 – overly proteinuric), were compared based on each animal's serum hyaluronan concentration (Graph 4). With a p = 0.062, no significant associations between the two variables were found. Although median hyaluronan concentration was slightly increased in the overtly proteinuric group, this was not statistically significant.

Graphs 4: Box plot representing Serum Hyaluronan Concentration in each UPCR category. Bars show minimum, mean, maximum values and interquartile ranges



3.4.6 : Serum Hyaluronan Concentration: Correlation with Blood Pressure and Cholesterol in Dogs with Cushing´s syndrome.

Regarding the possible correlation between serum hyaluronan concentrations and the Systolic blood pressure, cholesterol and triglycerides, all of the following p values were above 0,05 demonstrating no statical relevancy between the variables in question (Figure 2).

No significant difference was found between the median values for hypertensive versus normotensive dogs (p = 0.114) or hypercholesterolaemic versus normocholestorolaemic (p = 0.655). The result (p = 0.266) indicates that there is insufficient evidence to establish a statistically significant relationship between hypertension and a high cholesterol status in this study population.

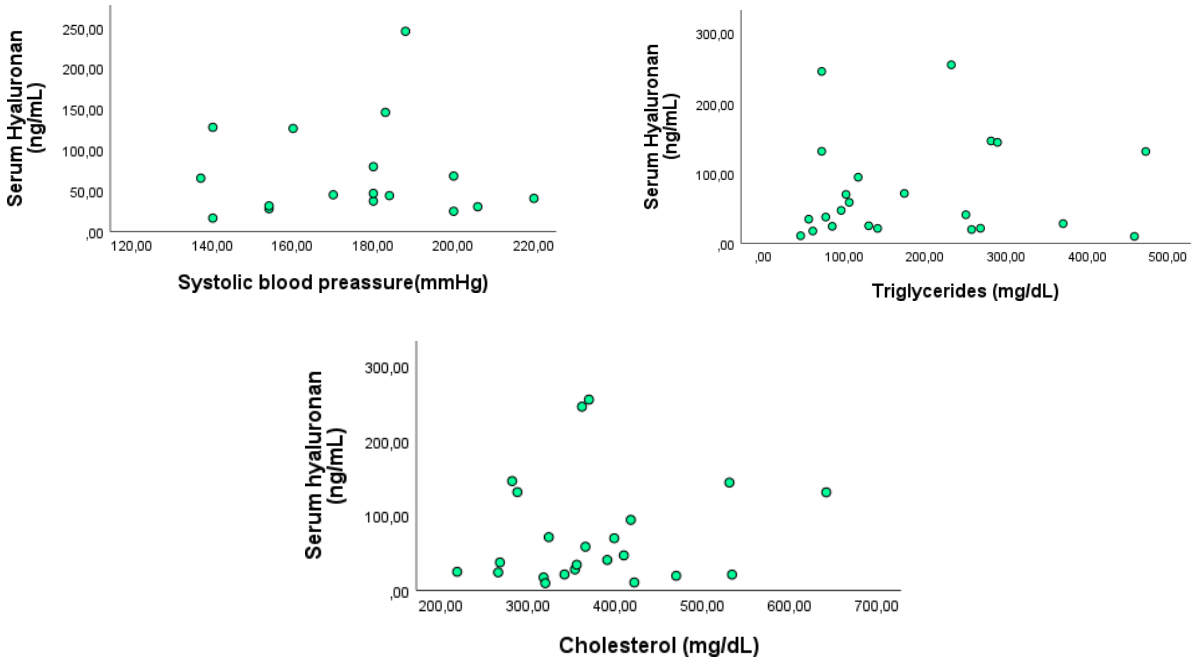


Figure 2: Scatter Plot representation of the correlation between Serum Hyaluronan concentrations and Systolic blood pressure, Triglycerides and Cholesterol. All the graphs show no correlation between the variables.

3.5 : Discussion

This study opens new insights on the potential role of the endothelial glycocalyx breakdown in the pathophysiology of proteinuria in dogs with naturally occurring hypercortisolism.

In this study, the hypercortisolaemic group exhibited elevated serum hyaluronan levels, indicating a possible increased eGlx breakdown in patients with hypercortisolism. Delving deeper in the subject, a statistical analysis of cholesterol levels in hypercortisolaemic dogs was conducted, given its known association in human patients (Meuwese et al. 2009). In this study, no significant correlation was found, suggesting that in dogs, eGlx shedding in hypercortisolism is not explained by hypercholesterolemia. Additional assessments of systolic blood pressure and triglycerides were performed, yet no statistically relevant associations were identified, leaving the mechanisms behind eGlx breakdown in dogs with hypercortisolism still unclear.

Taking into account the historical controls in healthy dogs, serum hyaluronan concentrations differed between the control group and hypercortisolaemic patients, with the latter showing an overall higher distribution of hyaluronan levels. In humans, eGlx breakdown has been predominantly associated with ischemic and inflammatory conditions, but it is also linked to hypercholesterolemia (Meuwese et al. 2009), chronic kidney and cardiac disease (Padberg et al. 2014; Liew et al. 2021). In Veterinary medicine, all the scarce data regarding the eGLx breakdown have shown its shedding in diseases such as septic peritonitis (Shaw et al. 2021), septic shock models (YINI et al. 2015) and hypercoagulable states (Lawrence-Mills et al. 2022), linking the inflammatory and ischemic states as a possible cause for the eGlx disruption (YINI et al. 2015; Sieve et al. 2018; Shaw et al. 2021). Dogs with MMVD are known to have endothelial disfunction (Jones et al. 2012) most likely leading to eGlx breakdown (Lawrence-Mills et al. 2022). When comparing data from this study with historic data in dogs with hypercoagulable state it was shown that serum hyaluronan concentrations ranges were very similar across both groups. This suggests a similar degree of eGlx breakdown and shedding between the two groups. As previously mentioned, over 80% of dogs suffering from hypercortisolism display at least one altered parameter from the proposed hypercoagulability panel (Pace et al. 2013). Antithrombin (previously known as Antithrombin III) is an hepatic synthesized protein that functions as broad-spectrum plasma serine protease inhibitor mediating the intrinsic, extrinsic, and common coagulation pathways. In addition to its function as a specific thrombin inhibitor, antithrombin has the capacity to inhibit the contact between factors XIIa and XIa. It also inhibits the activity of factor IXa of the intrinsic clotting system, preventing the activation of the factor X (Vervloet* et al. 1998). Antithrombin can circulate in

the bloodstream as a monomer, but it´s also found in platelets and in particular, linked to heparan sulfate, (Opal et al. 2002) a very densely located protein in the extracellular matrix of the endothelial cells (eGlx) (Pries et al. 2000). The binding of heparan sulfate to antithrombin triggers endothelial cells to produce prostacyclin, a powerful inhibitor of platelet aggregation that also acts as an anti-inflammatory agent by suppressing pro-inflammatory cytokines through a cyclic adenosine monophosphate mediated pathway (Opal et al. 2002). Regarding this information, the present study findings suggest a potential link between the hypercoagulable state observed in hypercortisolaemic patients and the breakdown of the eGlx. This breakdown could diminish the availability of antithrombin and prostacyclin therefore contributing to the development of an hypercoagulable state in these patients.

This data shows no statistical significance when comparing serum hyaluronan concentrations with the degree of proteinuria, assessed via UPC ratio, in hypercortisolaemic patients. This suggests no clear link between the proteinuric state of these patients and the breakdown of eGlx. However, it is important to note that most studies investigating the relationship between eGlx degradation and potential protein loss in urine have relied solely on albumin as the protein marker (Hayashi et al. 1990; Jacob et al. 2007; Salmon et al. 2012; Alphonsus and Rodseth 2014; Rabelink and Zeeuw 2015). Considering that the UPC ratio assesses the total protein content in urine (Rossi et al. 2016), a potential correlation between eGlx shedding in hypercortisolaemic patients and albuminuria is yet to be explored.

As previously shown in a study, a 42-day treatment with exogenous glucocorticoids in dogs was enough to induce microscopic alterations in the kidneys, particularly within the glomeruli, leading to structural modifications across all components and layers of the glomerulus (Waters et al. 1997). These changes could be either reversible or irreversible, depending on the severity and duration of hypercortisolemia (Schellenberg et al. 2008). Consequently, the filtration barrier, composed of the glomerular endothelial cells, basement membrane, and podocytes (Toblli et al. 2012) may become disrupted. Conditions that impair the function of these glomerular structures can lead proteinuria (Boute et al. 2000; Miner 2011) as well as a disruption of glomerular eGlx integrity (Satchell and Tooke 2008; Vera et al. 2008).

3.5.1 : Limitations

Regarding the limitations of this study, since the majority of the data connecting eGlx breakdown and proteinuria only analysed urine albumin content, a potential statistical correlation might have been achieved if the background noise caused by other proteins had been excluded. Therefore, a urine protein content analysis by electrophoresis would have been ideal. A more robust statistical analysis could have been generated if the data on the serum

hyaluronan concentrations from an historical control group, the hypercortisolaemic group, and the MMVD group had been available. Additionally, a parallel evaluation with others eGlx breakdown biomarkers such as heparan sulfate or the direct evaluation could give a better inside on the Glycocalyx structure. Unknown comorbidities or conditions like age, breeds, undiagnosed diseases, or even other non-endothelial hyaluronan sources could potentially alter the serum acid hyaluronic concentrations.

3.5.2 : Future prospects

In this study, serum hyaluronan concentrations were evaluated in forty dogs. A comprehensive dataset of values was generated for future search in this area. Potential future research opportunities could include, analysing and comparing hyaluronan levels between dogs with PDH and ADHC, other glycocalyx degradation biomarkers, such as heparan sulfate, or directly visualizing the glycocalyx itself. Moreover, comparing hyaluronan distribution patterns with specific thromboelastographic charts could offer better insides on the hypercoagulation state in hypercortisolaemic patients, and the role of the eGlx breakdown. Further investigation into the specific glomerular changes induced by glucocorticoids, and the potential treatment role of glycocalyx-replenishing drugs like Sulodexide would be of great interest (Broekhuizen et al. 2010). Curiously, some studies demonstrated that the Renin-Angiotensin-Aldosterone system is critically involved in the upregulation of glomerular heparanase activity (Hoven et al. 2009). The increased activity of heparanase observed in human diabetic patients may contribute to the degradation of the glycocalyx in this pathological context, thereby promoting proteinuria in diabetic human individuals (Mahtal et al. 2021). Based on this information, controlling proteinuria in dogs with hypercortisolism by inhibiting the Renin-Angiotensin-Aldosterone System (Fowler et al. 2021), may also be associated with the restoration of normal eGlx within the kidney glomeruli.

3.5.3 : Conclusion

This study identified elevated serum hyaluronan concentrations in dogs with hypercortisolism, suggesting eGlx damage in these disease states. It also raises the potential link between the hypercoagulable state seen in hypercortisolism and eGlx breakdown. No apparent correlation was found between eGlx breakdown and UPC values, despite both being elevated in hypercortisolaemic patients.

Section 4: Annexes

Annex i: Poster presented at the Congress of the European College of Veterinary Internal Medicine (ECVIM) – Companion Animals (5-7 September 2024)

Characterization of hyaluronic acid concentrations in dogs with naturally-occuring hypercortisolism and possible implications

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BACKGROUND

- Proteinuria is common in dogs with naturally-occurring hypercortisolism - Incidence can be as high as 75%.
- Hypertension and changes on glomerular filtration rate may contribute to proteinuria but its etiology is still poorly understood.
- Emerging studies have revealed a correlation between endothelial glycocalyx (EG) breakdown and proteinuria in humans.
- Serum circulating hyaluronan has been considered a reliable biomarker of EG shedding.

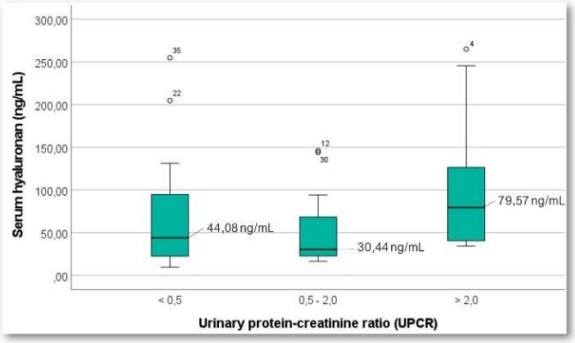
AIM

To evaluate serum hyaluronan concentration (SHC) in dogs with naturally-occurring hypercortisolism and its possible relationship with proteinuria.

MATERIALS AND METHODS

- A multicentric cross-sectional study was performed using leftover serum samples (stored and frozen -20 or -80°C) from 40 dogs with naturally-occurring hypercortisolism (diagnosed from 2022 to 2024) before starting trilostane treatment.
- Serum hyaluronan was measured using a kit previously validated in dogs (Hyaluronan Quantikine ELISA, R&D Systems)[1].
- Paired urinary protein-creatinine ratio (UPCR) and urine specific gravity (USG) values for each animal were obtained from medical records.
- Values were compared with historic controls from previous studies[1], including healthy dogs and dogs in hypercoagulable states.
- Dogs were grouped regarding their degree of proteinuria: proteinuric (0.5 < UPCR < 2), non-proteinuric (UPCR < 0.5) and overtly proteinuric (UPCR > 2).
- Non-parametric tests were used for statistical analysis.

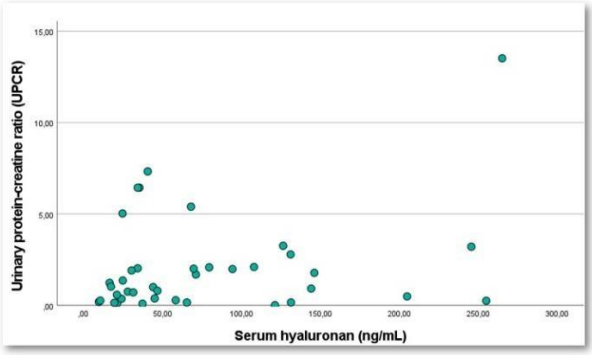
RESULTS:



Graph 1 - Serum hyaluronan concentrations in each category of UPCR. No significant differences were found among the 3 groups (p=0.062; Kruskal Wallis test).

- Median SHC was higher in the overtly-proteinuric group than in the non-proteinuric and the proteinuric group.
- No significant difference in SHC was found among the 3 groups.
- No significant correlation was observed between UPCR and SHC.

- The SHC (median [range]) in dogs with hypercortisolism was 45.94 [9.70-265.07] ng/mL. The median value was similar to those previously obtained healthy dogs (45.7 [8.7-80.2] ng/mL).
- The SHC range was similar to those previously obtained in dogs in hypercoagulable states (92.40 [16.9-247.6] ng/mL).



Graph 2 – Scatter plot of UPCR values by serum hyaluronan. No correlation was found between UPCR and SHC (p = 0,119; Spearman rank correlation).

DISCUSSION:

- This study shows endothelial dysfunction, as measured through SHC, does not appear to be driving proteinuria in dogs with Cushing's syndrome.
- Given the high variability of the values, SHC might have a more relevant role in other facets of Cushing's syndrome, such as helping to identify hypercoagulable states individually, or in proteinuria with other etiologies.

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