UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

TESIS DOCTORAL

Advances in the study of biomarkers in canine hypothyroidism

Avances en el estudio de biomarcadores en hipotiroidismo canino

D. Luis G. González Arostegui

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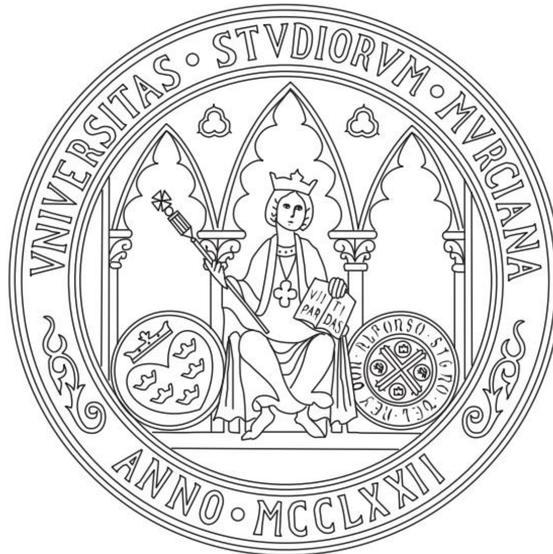

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Advances in the study of biomarkers in canine hypothyroidism

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Autor: D. Luis Guillermo González Arostegui

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“Queda prohibido no sonreír a los problemas, no luchar por lo que quieres, abandonarlo todo por miedo, no convertir tus sueños en realidad”

Pablo Neruda

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DOCTORAL THESIS AS COMPENDIUM OF ARTICLES

This Ph.D. Thesis, after the authorization of the directors of the Ph.D. Thesis and the Academic Commission responsible for the Veterinary Sciences Ph.D. Program, is presented as a compendium of six studies previously published. Therefore, the Ph.D. Thesis is composed of the following article references:

1. González-Arostegui LG, Rubio CP, Cerón JJ, et al (2021) Proteomics in dogs; a systematic review. Res Vet Sci 143:107–114. https://doi.org/10.1016/j.rvsc.2021.12.026
2. González-Arostegui LG, Muñoz-Prieto A, Rubio CP, et al (2023) Changes of the Salivary and Serum Proteome in Canine Hypothyroidism. Domest Anim Endocrinol 106825. https://doi.org/10.1016/j.domaniend.2023.106825
3. Muñoz-Prieto A, González-Arostegui LG, Rubić I, et al (2021) Untargeted metabolomic profiling of serum in dogs with hypothyroidism. Res Vet Sci 136:6–10. https://doi.org/10.1016/j.rvsc.2021.01.023
4. González-Arostegui LG, Rubio CP, Rubić I, et al (2022b) Changes in the salivary metabolome in canine hypothyroidism: A pilot study. Res Vet Sci 108947. https://doi.org/10.1016/j.rvsc.2022.08.012
5. González-Arostegui LG, Muñoz-Prieto A, Tvarijonaviciute A, et al (2022a) Measurement of Redox Biomarkers in the Whole Blood and Red Blood Cell Lysates of Dogs. Antioxidants 11:. https://doi.org/10.3390/antiox11020424
6. Arostegui LGG, Prieto AM, Marín LP, et al (2023) Changes in biomarkers of redox status in serum and saliva of dogs with hypothyroidism. BMC Vet Res 19:33. https://doi.org/10.1186/s12917-023-03586-4

Additionally, in the Annex of this document, the results related to a study carried out during the grant period is included. These data will be submitted for their possible publication in peer reviewed Journals.

ABBREVIATIONS

ABBREVIATIONS

AOPP: advanced oxidation protein products

AUC: area under the curve

BCA: bicinchoninic acid assay

CUPRAC: cupric reducing antioxidant capacity

CV: coefficient of variation

d-ROMs: reactive oxygen-derived compounds

FDR: false discovery rate

FIA: flow injection analysis

FRAP: ferric reducing ability of plasma

FRAS: ferric reducing ability of saliva

GPx: glutathione peroxidase

GO: gene ontology

ITIH4: inter-alpha-trypsin inhibitor heavy chain H4

KRT4: keratin type II cytoskeletal 4

LDH: lactate dehydrogenase

LC-MS: liquid chromatography mass spectrometry

LC-MS/MS: liquid chromatography tandem-mass spectrometry

MS: mass spectrometry

MS/MS: tandem mass spectrometry

NTH: non-thyroidal disease

PCA: partial component analysis

PLSDA: partial least square-discriminant analysis

PON-1: paraoxonase type-1

POX-Act: peroxide activity

QCs: quality controls

QEA: quantitative enrichment analysis

RBCs: red blood cells

ROS: reactive oxygen species

SHBG: sex hormone-binding globulin

SOD: superoxide dismutase

TBARS: thiobarbituric acid reactive substances

TEAC: Trolox equivalent antioxidant capacity

TMT: tandem mass tag

TOS: total oxidant status

T3: triiodothyronine

T4: thyroxine

TSH: thyroid stimulating hormone

UHPLC: ultra-high-performance liquid chromatography

WB: whole blood

INTRODUCTION

INTRODUCTION

- **Overview on canine hypothyroidism.**

Canine hypothyroidism is the most common endocrine disorder in dogs, with a prevalence ranging from 0.8 to 2.7% of all cases presented in small animal practice (Dixon and Reid, 1999; Ziener et al., 2015). This disease is characterized by an impaired production of thyroid hormones in the thyroid gland, mainly triiodothyronine (T3) and thyroxine (T4), causing weight gain, lameness and diminishing the dog's life quality (Jaiswal et al., 2018). The diagnosis of this disease is mostly limited to the determination of T4 and thyroid stimulating hormone (TSH) in serum, however, results of their determination are often confusing as these hormones are fluctuant, complicating the diagnosis of the disease (Daminet and Ferguson, 2003; E. C. Feldman et al., 2014; Jaiswal et al., 2018).

- **Importance of biomarkers in veterinary medicine**

One of the most important aspects in the diagnosis and treatment monitorization of diseases is the use of biomarkers, as they serve as key components on the evaluation of any pathology. Omics techniques, such as proteomic analyses and targeted and untargeted metabolomic analyses, are very useful for the discovery of biomarkers.

The proteome is the complete set of proteins present in a sample at a given time, constantly changing to external or internal stimuli (Aslam et al., 2017). Proteomic analyses have been performed in dogs using different types of samples, such as serum (Yang et al., 2015), saliva (Lucena et al., 2019), urine (Hormaeche et al., 2014), among others. Through the use of these analyses, several biomarkers have been identified in different diseases, for instance, apolipoproteins as a biomarker of obesity (Tvarijonaviciute et al., 2012),leishmaniasis and babesiosis (Kuleš et al., 2014), antithrombin-III as a biomarker of chronic valve disease (Kuleš et al., 2020a), retinol-binding protein as a biomarker of glomerular disease (Nabity et al., 2011), as well as many others.

While the basis of proteomic analyses is the identification of proteins, metabolomic analyses aim to identify the metabolic fingerprint that is in a sample at a given time. This fingerprint is prone to constant change, similar to the proteome (Emwas, 2015). Metabolomic analyses can be targeted or untargeted. The targeted technique is used to evaluate the presence of a defined set of metabolites, meanwhile, the untargeted technique is used to study all the metabolites present in a sample (Roberts et al., 2012; Souza and Patti, 2021). The detection of this metabolic fingerprint helps identify biomarkers while also giving a wider understanding of the pathogenesis of a disease and discovery of new therapeutic targets (Clish, 2015). The metabolic fingerprint in dogs has not been studied as widely as the proteome. Nonetheless, two studies have been performed, leading to the identification of different biomarkers of disease. One study identified phenylalanine and lactate in serum as possible biomarkers of chronic enteropathy (Walker et al., 2022), while another study identified tyrosine and branch-chained amino acids in serum as possible biomarkers of congenital portosystemic shunt (Imbery et al., 2022).

In the last years the biomarkers of redox state are gaining importance in veterinary medicine. The redox state is defined as the balance between the production of reactive oxygen species (ROS) and the antioxidant defense system (Halliwell, 2007; Liu et al., 2023). Increased production of ROS leads to cellular hypoxia, DNA damage, and, as a consequence, cell death, contributing to the development of pathologies (Çay, 2017; Poljšak and Milisav, 2012). The evaluation of the redox state helps to the understanding of the physiopathologic mechanisms of diseases while detecting specific biomarkers that can be useful to either diagnose a disease or aid in treatment monitorization (Forman and Zhang, 2021; Sies, 1991). To evaluate the redox state, antioxidants and oxidant compounds can be measured in different samples like serum, saliva, whole blood (WB) and red blood cell (RBCs) lysates, which offers a wide field of study. In dogs, biomarkers of the redox state have been evaluated in different diseases, for instance, superoxide dismutase (SOD) was shown to be a useful biomarker of in visceral leishmaniasis (Britti et al., 2008) and sarcoptic mange (Singh et al., 2011), paraoxonase type-1 (PON-1) of parvoviral enteritis (Kocaturk et al., 2015), glutathione peroxidase (GPx) was shown to be altered in dogs infected with *Dirofilaria immitis* (Dimri et al., 2012).

- **Biomarkers in Canine Hypothyroidism.**

The use of biomarkers is of high importance for an accurate diagnosis and treatment monitorization, as well as a deeper understanding of the mechanisms that lead to the onset of a disease. This Thesis was focused on providing advances in the discovery and evaluation of biomarkers of canine hypothyroidism through various methods:

  - The use of proteomic analysis in different sample types.
  - The use of targeted and untargeted metabolomic analysis in different sample types.
  - The evaluation of the redox state in different sample types.

Overall, this PhD thesis provides advances in the knowledge of canine hypothyroidism through the discovery of new possible biomarkers by proteomic and metabolomic techniques, as well the application of various biomarkers to evaluate redox status. These biomarkers were measured in serum and saliva, WB and RBCs lysates.

OBJECTIVES

OBJECTIVES

The objectives of this Ph.D. Thesis were focused on providing advances in the study of biomarkers of canine hypothyroidism through the use of proteomics, metabolomics and the evaluation of the redox status. For this purpose, the specific aims were:

- **Objective 1.** To perform a systematic review of the existing literature available on biomarkers identified through proteomic analyses in dogs (Article 1).
- **Objective 2.** To perform a proteomic analysis using serum and saliva from dogs with hypothyroidism and healthy controls in order to identify possible biomarkers of canine hypothyroidism (Article 2).
- **Objective 3.** To perform metabolomic analyses using serum and saliva from dogs with hypothyroidism and healthy controls in order to identify possible biomarkers of canine hypothyroidism (Article 3 and 4).
- **Objective 4.** To validate the determination of antioxidants and oxidants in two novel sample types, namely, WB and RBCs lysates of dogs (Article 5).
- **Objective 5.** To evaluate the redox status in serum, saliva, WB, and RBCs lysates of dogs with hypothyroidism, comparing it with the redox state of dogs with non-thyroid disease (NTH) dogs and healthy control (Article 6 and Annex 1).

EXTENDED SUMMARY

EXTENDED SUMMARY

1. Canine Hypothyroidism

Canine hypothyroidism is the most common endocrine disorder in dogs, with a prevalence of up to 2.7% (Dixon and Reid, 1999; Panciera, 1994). Hypothyroidism is mostly associated with a destruction of the thyroid gland (primary hypothyroidism), due to lymphocytic thyroiditis or an idiopathic atrophy of the gland resulting in the disruption of thyroid hormone synthesis (Graham et al., 2001; Mooney and Shiel, 2012). Lymphocytic thyroiditis is an immune-mediated inflammation characterized by diffuse infiltration of lymphocytes, macrophages and plasma cells that progressively destroy the thyroid follicles and lead to a secondary fibrosis (Mooney, 2011). While, the idiopathic atrophy is produced by an adipose tissue replacement of the lost thyroid parenchyma (Mooney, 2011).

Dogs of any age, breed or size can manifest this disease, however, most of the cases are observed in geriatric dogs, and in medium to larger breeds (Dobberman, Golden Retriever, Beagle, Dachshund, among others) are most affected (Bianchi et al., 2020, 2015). Symptoms of the disease are rather unspecific and can vary with disease progression, nonetheless, the most common signs are associated with decreased metabolic rate (lethargy, weight gain, exercise and cold intolerance) and dermatological disorders (poor hair quality, alopecia, pyoderma and seborrhea), with other signs being associated with the nervous, reproductive, ocular and hemostatic systems (Dixon and Reid, 1999).

The diagnosis of canine hypothyroidism is led by a detailed anamnesis and clinical history and the use of laboratory and diagnostic tests. The diagnostic test of choice is the measurement of T4 and TSH in serum (Boretti and Reusch, 2004; Feldman and Nelson, 2015). However, their measurement does not always confirm a diagnosis, as thyroid hormones fluctuate, and false negatives are frequent (Santhosh et al., 2020). In these cases, a thyroid recombined hormone stimulation test is recommended, but this hormone is not easily available. Also this test requires the extraction of three repeated blood samples, which produces high levels of stress in the dog (Boretti et al., 2006; Daminet et al., 2007). Some authors also suggest the measurement of free T4, but its measurement is more expensive, and its clinical value depends on the type of assay used, additionally, free T4 can rapidly degrade with sample transportation and give a false negative diagnosis. On the other hand, the use of T3 for the diagnosis of hypothyroidism offers a less sensible test than

T4 and TSH in both dogs and humans (Panciera, 1999; Rasmussen et al., 2014). In addition, thyroglobulin autoantibodies have been used for the diagnosis of hypothyroidism, although, the diagnostic efficiency of this method is reduced to subclinical cases, as its sensitivity decreases significantly with the progression of the disease (Graham et al., 2007).

2. General Methods

2.1 Ethics considerations

All the experiments performed throughout this PhD Thesis were approved by Bioethical Committee (Comité Ético de Experimentación Animal, CEEA), under the protocol number 288/2017 (canine species) and by the University of Murcia's ethic committees and the Ministry of Agriculture, Livestock, Fishing, and Aquaculture of the Region of Murcia, under the protocol number A13170503. Experiments performed were in accordance with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines. In addition, owner consent was obtained for the participation of all animals.

2.2 Sampling Procedure

All samples used in this thesis were collected after at least 12 hours of fasting. Saliva samples were taken before blood extraction and were obtained as previously reported (Parra et al., 2005). In brief, a piece of sponge is introduced into the dog's mouth until it is moist. Sponges were placed into collection tubes (Salivette, Sarstedt, Aktiengesellshacft & Co, Nümbrecht, Germany), and stored with ice. Once in the laboratory, tubes were then centrifuged at 3000 g for 20 minutes at 4ºC and samples were transferred to 1.5 mL Eppendorf® tubes (Daslab, Barcelona, Spain) and stored at -80ºC until analysis.

WB and serum samplings were performed in all dogs obtaining the blood from the jugular vein and placing into EDTA tubes and tubes containing coagulation activator gel (TapVal, Aquisel, Selecta, Barcelona, Spain) for the WB and serum samples, respectively. Each EDTA tubed sample was divided into two different aliquots for posterior WB and RBCs preparation. To obtain the WB, one aliquot was kept at -80ºC for at least two hours before analysis. To obtain the RBCs, the other aliquot was centrifuged at 3000 rpm for 10 minutes at 4ºC in order to remove plasma and buffy coat. The RBC pellet was then washed with isotonic saline (NaCl 0.9%) and centrifuged, as previously mentioned. Then, the supernatant was removed, and the process was repeated for a total of four washes. Finally,

the RBCs was reconstituted using ultrapure water in a 1:4 dilution and then stored at -80ºC until analysis. Serum tubes were left to clot at room temperature (22-24ºC) for 10 to 20 minutes and were centrifuged at 3500 g for 5 minutes at room temperature. The obtained serum was transferred to 1.5 mL Eppendorf® tubes and stored at -80ºC until analysis.

2.3 Liquid Proteomics

From each sample, 35 µg of acetone-precipitated proteins were subjected to reduction, alkylation, and digestion and were labeled using 10-plex tandem mass tag (TMT) reagents (Thermo Scientific, Rockford, IL, USA) according to manufacturer instructions (Thermo Scientific, Waltham, MA, USA). The total protein concentration of salivary samples was determined using BCA assay (Thermo Scientific, Rockford, USA). A pooled sample, generated by mixing equal protein amounts of all 20 samples, was used as an internal standard in all TMT 10-plex experiments. In short, 35 µg of the samples and internal standards were reduced with 200 mM DTT (Sigma-Aldrich, St. Louis, MO, USA), alkylated with 375 mM iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA), and precipitated with ice-cold acetone (VWR, Radnor, PA, USA) overnight. The samples were then centrifuged, and acetone was decanted. The pellets were resuspended with 50 µL of 100 mM triethylammonium bicarbonate (TEAB, Thermo Scientific, Rockford, USA) and digested with trypsin (Promega) overnight at 37ºC (enzyme-to-protein ratio 1:35, v/v). TMT 10-plex reagents were equilibrated at room temperature, resuspended in anhydrous acetonitrile (LC-MS grade, Thermo Scientific, USA), and added to each sample. The labeling reaction was incubated for one hour at room temperature and then quenched by adding 5 % hydroxylamine (Sigma- Aldrich, St. Louis, MO, USA) for 15 min. The samples were then combined in equal amounts, and 5 µg of each mixed sample set was stored at −80ºC before further liquid chromatography tandem-mass spectrometry (LC-MS/MS) analysis. The LC-MS/MS analysis was performed by using an Ultimate 3000 RSLCnano flow system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) as described previously (Muñoz-Prieto et al., 2022). Peptides were dissolved in loading solvent (1 % ACN, 0.1 % formic acid) and loaded onto the trap column (C18 PepMap100, 5 µm, 100A, 300 µm × 5 mm), desalted for 12 min at the flow rate of 15 µL/min and separated on the analytical column (PepMap™ RSLC C18, 50 cm × 75 µm) using a linear gradient of 5–45 % mobile phase B (0.1 % formic acid in 80 % ACN) over 120 min, 45 % to 90 % for 2 min, held at

80 % for 2 min and re-equilibrated at 5 % B for 20 min at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1 % formic acid in water. Ionization was achieved using nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) containing a 10 μm-inner diameter SilicaTip emitter (New Objective, Woburn, MA, USA). The MS operated in positive ion mode using DDA Top8 method. Full scan MS spectra were acquired in the range from m/z 350.0 to m/z 1800.0 with a resolution of 70,000, 110 ms injection time, AGC target 1E6, a ± 2.0 Da isolation window and the dynamic exclusion 30 s. HCD fragmentation was performed at step collision energy (29 % and 35 % % NCE) with a resolution of 17,500 and AGC target of 2E5.

Proteins were identified using Proteome Discoverer (version 2.3., Thermo Fisher Scientific), searching against a canine protein database (downloaded from Uniprot database on April 4th, 2019, 172083 sequences). The following parameters were applied: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), and TMT 6-plex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow according to the search results against a decoy database and was set at 1 % FDR. For reporting confidently identified proteins, at least two unique peptides and 5 % FDR were required. Protein quantification was achieved by correlating the relative intensities of reporter ions extracted from the tandem mass spectrum to that of the peptides selected for tandem mass spectrometry (MS/MS) fragmentation. The internal standard was used to compare relative quantification results between the experiments.

2.4 Metabolomics

- *Untargeted Metabolomics*

A total of 25 μL of each serum sample was mixed with 1000 μL of ice-cold extraction solvent (CHCl3:CH3OH:H2O = 1:3:1) to precipitate the proteins. Ten μL of each original serum sample (control and diseased) were pooled, and 25 μL from pooled sample was mixed with 1000 μL of ice-cold extraction solvent. All samples (serum samples, pooled samples, matrix blank) were quickly vortexed and were centrifuged at 13000g for 5 min at 4ºC. The supernatant (200 μL) was transferred to a screw-top vial and stored at −80ºC until analyzed using liquid chromatography mass spectrometry (LC-MS).

Metabolites were analyzed using a Thermo Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an UltiMate 3000 RSLC system (Dionex, Germering, Germany). Chromatographic separation was performed using a ZIC-pHILIC column (150 × 4.6 mm, 5 µm column, Merck Sequant, Germany). The column was maintained at 25ºC and samples were eluted with a linear gradient (A = 20 mM ammonium carbonate in water, B = acetonitrile). During the 27 min, the first 15 min gradient changed of 80% A and 20% B to 95% A and 5% B. In the 17th min, the gradient started to return to initial conditions. Thermo Q-Exactive Orbitrap was operated in positive and negative modes at a mass resolution of 70.000 with an m/z range of 70–1050. The MS analysis for positive electrospray ionization was accomplished using a source voltage of +3.8 kV, sheath gas of 40 (arbitrary units), auxiliary gas of 5 (arbitrary units), and capillary temperature of 320ºC. The MS settings for negative electrospray ionization used a source voltage of 3.8 kV. Quality control samples were a mixture of metabolites provided by Glasgow Polyomics, UK, and used to check the quality of chromatographic separation. For metabolite detection, a standard mix (provided by Glasgow Polyomics, UK), which contains about 200 metabolites, was used. Pooled samples were used to check signal reproducibility.

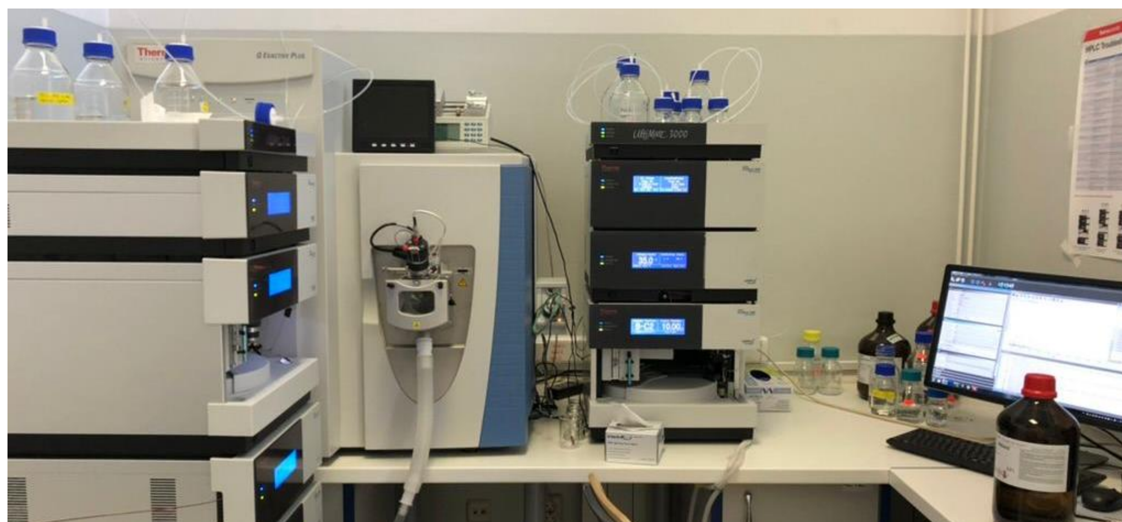

Figure 1. Thermo Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) and UltiMate 3000 RSLC system (Dionex, Germering, Germany)

- *Targeted Metabolomics*

Samples were prepared using the AbsoluteIDQ p400 HR kit (Bio-crates Life Sciences AG, Innsbruck, Austria) following the guidelines from the company (Hengstl et al. 2016; Biocrates Life Sciences 2020). This kit has been previously applied in the saliva of dogs where metabolites were accurately quantified (Muñoz-Prieto et al., 2021). A volume of 10 µL of each saliva sample, quality control samples, blank, and PBS were added to the plate containing the internal standard mix. The plate was then dried for 30 min with a vacuum manifold. Samples were then derivatized using 50 µL of 5% derivatization solution (phenylisothiocyanate (PITC) in water: ethanol: pyridine (1:1:1, v/v/v)) for 20 min and subsequently dried for 60 min using a vacuum manifold. Metabolites were then extracted with 300 µL of 5 mM ammonium acetate solution in methanol by shaking for 30 min at 450 rpm, then eluted using a vacuum manifold. A total of 150 µL of extracts was transferred from the capture plate to another empty 96-deep well plate and diluted with water (50:50, v/v) for the LC-MS analysis. The original plate was diluted with 250 µL of the flow injection analysis (FIA) mobile phase (10 mL ampule Biocrates FIA mobile phase in 290 mL of methanol) for FIA and MS. Both the LC and FIA plates were securely covered with silicon mats and shaken for 5 min at 500 rpm before the analysis.

Metabolites were measured with a targeted metabolomics approach using a Dionex Ultimate 3000 ultra-high-performance liquid chromatography (UHPLC) system coupled with a Thermo Scientific Q Exactive Orbitrap mass spectrometer with the LC-MS instrument method and parameters provided along with the AbsoluteIDQ p400 HR kit (Biocrates Life Sciences AG, Innsbruck, Austria). The assay quantifies up to 408 metabolites from eleven compound classes that allowed the measurement of 21 amino acids, 21 biogenic amines, one monosaccharide (including glucose), 172 phosphatidylcholines, 24 lysophosphatidylcholines, 31 sphingomyelins, 9 ceramides, 55 acylcarnitine, 14 cholesteryl esters, 18 diglycerides, and 42 triglycerides. For quantitation, calibration standards in seven concentration levels and stable isotope-labeled internal standards (ISTD) were used by confirming the correct peak retention times and peak area integration. On the other hand, the FIA and MS analysis used a single-point calibrator with representative internal standards. The internal standards were partially integrated into the filters of the Kit plate. A total of 3 replicates of blank PBS samples were used to calculate the lower limits of detection (LOD). The analytical performance is validated using the

quality controls (QCs) provided by the manufacturer at three concentration levels. Metabolites were identified and quantified using a standard workflow, using the MetIDQ™ software provided by Biocrates Life Sciences AG, Innsbruck, Austria.

2.5 Redox Biomarkers

A wide panel of redox state biomarkers were measured in all samples obtained, these were analyzed using the Olympus AU400 Automatic Chemistry Analyzer (Olympus Europe GmbH), unless stated otherwise.

Figure 2. Olympus AU400 Automatic Chemistry Analyzer (Olympus Europe GmbH).

- *Antioxidant biomarkers*

The cupric reducing ion antioxidant capacity (CUPRAC) assay was based on the reduction of Cu^{2+} into Cu^{1+} by the nonenzymatic antioxidants in the sample (Campos et al., 2009). Evaluation of CUPRAC was made following a previously validated protocol for its use in serum of dogs (Rubio et al., 2016c). Results are expressed in millimoles per liter (mmol/L).

The determination of the ferric reducing ability of plasma (FRAP) and saliva (FRAS) assay was based on the reduction of ferric-tripyridyltriazine (Fe^{3+}-TPTZ) to the ferrous (Fe^{2+}) form (Benzie and Strain, 1996). Its determination was made following a

previously validated method for the serum of dogs (Almela et al., 2018; Rubio et al., 2018). Results are expressed in mmol/L.

Measurement of the Trolox equivalent antioxidant capacity (TEAC) assay was based on the enzymatic generation of 2,2'-azino-bis(3-ethylbenz-thiazoline-5-sulfonic acid) (ABTS) radical and its reduction by non-enzymatic antioxidants present in the sample (Arnao et al., 1996). This assay was performed following a previously validated protocol for its use in the serum of dogs (Rubio et al., 2016a). Results are expressed in mmol/L.

The determination of total thiol is based on the reaction of thiols within the sample with 5,5'0dithiobis-(2-nitrobenzoic acid) (DTNB) (Jocelyn, 1987). The total thiol determination was performed following previously described methods performed in canine serum samples (Almela et al., 2018). Results are expressed in micromoles per liter (µmol/L).

Measurement of PON-1 was based on the hydrolysis of phenyl acetate into phenol and it was determined following a previously validated protocol for it use in canine serum (Asta Tvarijonaviciute et al., 2012). Results are expressed in units per milliliter (IU/mL).

Measurement of GPx was based on the use of commercially available assays following manufacturer's instructions (RANDOX, Crumlin, UK), as used in previous studies (Kapun et al., 2012; Verk et al., 2017). Results are expressed in IU/mL.

- *Oxidant biomarkers*

Determination of the total oxidant status (TOS) assay was based on the ability of oxidants in the sample to oxidize Fe^{2+}-*o*-dianisidine complex to Fe^{3+} (Erel, 2005). This assay was performed following the previously described method for serum performed in dogs (Rubio et al., 2016b). Results are expressed in µmol/L.

Evaluation of the peroxide activity (POX-Act) assay was based on the determination of total peroxides through a peroxide-peroxidase reaction using tetramethylbenzidine as the chromogenic substrate (Tatzber et al., 2003). Determination of POX-Act was measured following a validated method for human sera (Tatzber et al., 2003). Results are expressed in µmol/L.

Measurement of the reactive oxygen-derived compounds (d-ROMs) assay was based on the reaction of the sample in an acidic medium in the presence of f *N,N,-diethyl-para*-phenylenediamine (DEPPD), and it was made following a previously described method (Alberti et al., 2000). Results are expressed in Carratelli Units (U.CARR).

Determination of advanced oxidation protein products (AOPP) was based on the oxidized albumin and di-tyrosine containing cross-linked proteins and previously described (Witko-Sarsat et al., 1996), and measured in canine serum (Rubio et al., 2018). Results are expressed in µmol/L.

Determination of the thiobarbituric acid reactive substances (TBARS) assay was based on the reaction of the sample to a Trichloroacetic acid, thiobarbituric acid and *N* hydrochloric acid in heated conditions. TBARS was measured following a previously described method (Rubio et al., 2017) using a microplate reader (Powerwave XS, Biotek Instruments). Results are expressed in µmol/L.

2.6 Analytical Validation of Assays

The analytical validation of the assays performed during this PhD thesis were assessed by calculating precision, accuracy and sensitivity, following previously reported protocol (Tiwari and Tiwari, 2010) that has been applied in other analytical validations performed in dogs (Rubio et al., 2016c, 2016a; Asta Tvarijonaviciute et al., 2012)

- *Precision*

Precision was evaluated through the assessment of the intra and inter-assay variation, by using three different samples with different concentrations of the analyte (high, medium, low concentrations). These samples were measured in five replicates times during the same day for the intra-assay precision, while the inter-assay precision was obtained through the repeated measurement of the three samples in five different days. In order to avoid the effects of freezing and thawing the samples continually, five aliquots for each sample were prepared and stored at -80ºC until analysis. The intra and inter-assay variation was expressed as the coefficient of variation (CV).

- *Accuracy*

Accuracy was evaluated by assessing: (1) linearity under dilution and (2) spiking recovery. Linearity under dilution was evaluated by performing serial dilution of two samples with different concentrations of the analyte. All samples were diluted using ultrapure water with the exemption of RBCs lysates that were diluted using a Phosphate buffer pH 7.5. The spiking recovery was evaluated using two samples (one with high concentrations and one with low concentrations of the analyte) that were mixed at different percentages (87.5 %, 75 %, 50 %, 25 % and 12.5 % of the sample with high concentration with 12.5 %, 25 %, 50 %, 75 % and 87.5 % of the sample with low concentration of each method, respectively), the ratios of the measured values to the expected values of each method were then calculated.

- *Sensitivity*

Sensitivity was evaluated by assessing (1) LOD and (2) lower limit of quantification (LLOQ).

LOD for each method was calculated with the data from 20 replicate determinations of ultrapure water and/or Phosphate buffer pH 7.5. LLOQ was defined as the lowest concentration of each method quantified with a CV of less than 20%.

3. Experimental Design, Results and Discussion

The experimental design, results, and discussion will be presented according to the different articles published during this Thesis.

3.1 Objective 1

In order to fulfill the Objective 1, a systematic review was performed to gather the existing knowledge on proteomic analyses performed in dogs to the date. In addition, the realization of this review facilitated the planification of the proteomic analysis performed during this PhD Thesis.

<u>3.1.1 Systematic Review.</u> *Proteomics in dogs; a systematic review.*

- **Aims and experimental design**

The investigation in new biomarkers has proven to be a field of high interest, as it helps clinicians and other scientist in the diagnosis of a disease, while also providing new knowledge on the physiological and pathological aspects associated with the development of a disease (Adaszek et al., 2014).

The use of proteomic analysis as a novel method for the search of new biomarkers of different diseases is currently on the high as it has been increasingly used in humans, as well as, in veterinary medicine (Miller et al., 2020). For instance, biomarkers have been discovered in several diseases like cancer (Hayward et al., 2016; Kirby et al., 2011; Pawlak et al., 2013; Ploypetch et al., 2020), cardiovascular diseases (Kuleš et al., 2020a; Locatelli et al., 2016; Powers and Recchia, 2018), infectious diseases (Escribano et al., 2017; Franco-Martínez et al., 2020c; Hormaeche et al., 2014; Martinez-Subiela et al., 2017), as well as, metabolic (Lucena et al., 2019) and reproductive disorders (Franco-Martínez et al., 2020b; Kuleš et al., 2020b).

The proteome is defined as the total set of proteins that are expressed within a sample at a given time, this set being sensible to external and internal stimuli, which means that if a dog is undergoing a specific disease, a specific set of proteins will be expressed differently than in a healthy dog (Krause et al., 2009).

The purpose of this review was to evaluate the advances made through the use of proteomics and to provide a perspicuous updated view of the available proteomic studies in dogs and the biomarkers identified in different samples, such as plasma, serum, saliva, and urine.

  - *Search Methodology*

This systematic review was performed by undergoing a search of the veterinary literature published in the last 10 years (2010 – 2020). This search was performed three separate times in order to ensure all information gathered was correct, following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses Protocol (PRISMA) 2015 guidelines (Shamseer et al., 2015). This review was made specifically for research

studies made in dogs, where the proteome was evaluated using samples such as serum, plasma, saliva and urine, all other samples, as well as, findings in animals different than dogs were excluded from this review. A total of three electronic databases were chosen for the literature research: PubMed, Web of Science, and Wiley Online Library. The complete search strategy performed for this review is shown in Table 1.

Table 1. Keywords used for each search engine.

Database	Search strategy
PUBMED	((Canine[Abstract] OR Dogs[Abstract]) AND ("proteome"[MeSH Terms] OR "proteome"[All Fields])) AND ("proteomics"[MeSH Terms] OR "proteomics"[All Fields]) AND ("2010/01/01"[PubDate]: "2020/12/31"[PubDate])
Web of Science	(((ALL = (canine OR dog)) AND ALL = (proteome OR proteomics)) AND ALL = (protein OR peptide)) AND PY = (2010–2020)
Wiley Online Library	[[Abstract: dogs] OR [Abstract: canine]] AND [[Abstract: proteome] OR [Abstract: proteomics]] AND [Earliest: (01/01/2010 TO 12/31/2020)]

- *Inclusion criteria*

The inclusion criteria for this review were based on the PICO questions: Population (P), only studies made on dogs were included; Intervention (I), studies involving the development of proteomic analyses were included; Comparison (C), studies where a diseased population was compared with a healthy were also included; Outcomes (O), studies where the similarities and differences in proteomic profiles found in different canine diseases and the number of proteins identified are explained were included. All textbook chapters, letters, reviews, non-canine studies and those not pertaining proteomic analysis and the report of proteins were excluded. Only English language studied were included.

- *Review Methodology*

Articles were first screened in accordance with their title and abstract, then the selected articles were extracted from peer-review articles and entered in an Excel sheet. Excel sheets included all data referring to the public information of the article and authors, population characteristics, method characteristics, and the reporting of the results obtained. All of the papers were also assessed for bias using the Cochrane Collaboration tool (Boutron et al., 2019).

- **Results and discussion**

From the literature search, a total of 704 articles were found throughout all three electronic databases. After duplicates were removed, 557 articles were screened for relevance, then after title screening, a total of 492 articles were excluded, and finally, after evaluating the abstract of each paper, a total of 52 articles were evaluated in full, from which eight articles did not meet the inclusion criteria and were excluded, resulting in 44 publications included in this review. A flow diagram of the search strategy undertaken in this review, as well as the screening procedure performed in this review, is explained in Figure 3.

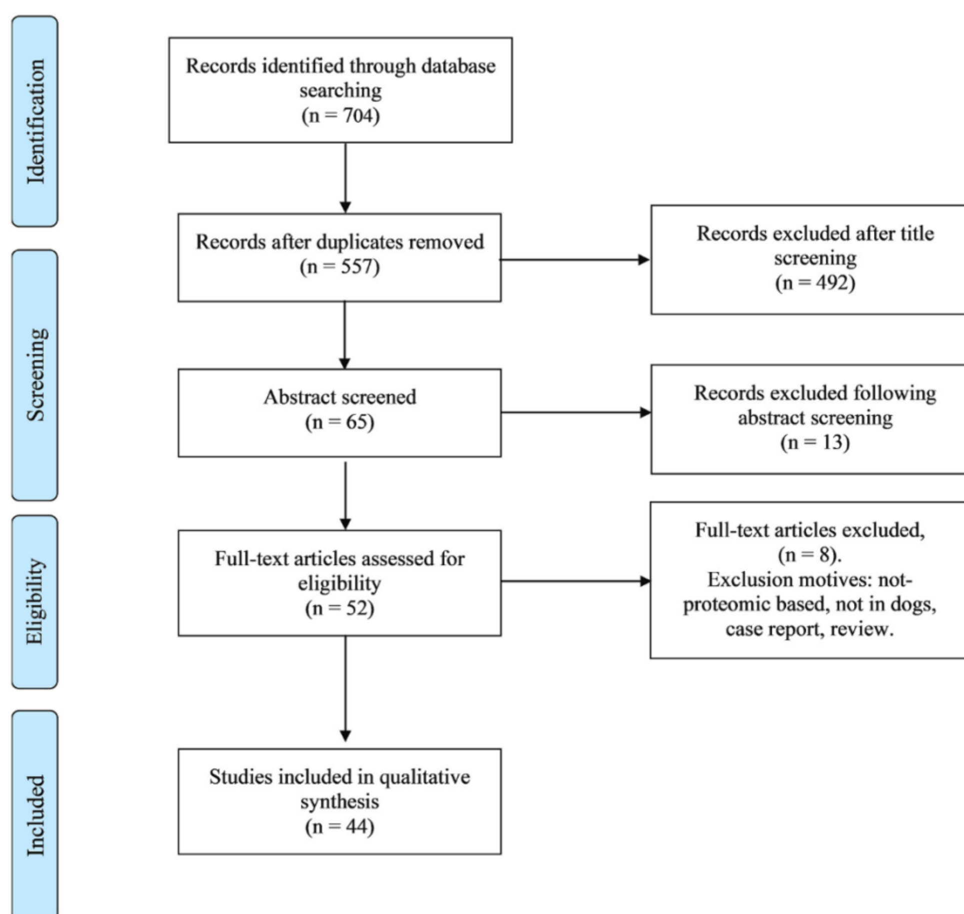

Figure 3. PRISMA (2009) flow diagram of the search strategy undertaken in a systemic review to identify and screen publications with information on proteomic studies made on dogs in serum, saliva, urine, and plasma, resulting in the identification of 44 included publications.

All the studies included were separated into two groups: (1) studies made on healthy dogs under physiological conditions, and (2) studies made on dogs with a specific pathology. The sample type found to be most used was serum, followed by saliva, urine and plasma, with one article using both serum and saliva (Franco-Martínez et al., 2020a). The most commonly used techniques were two-dimensional gel electrophoresis (2-DE) and LC-MS/MS.

Through the use of proteomic analyses, a variety of biomarkers have been identified in different diseases. For instance, Apolipoprotein A-1 as a possible biomarker of leishmaniasis, as it has been shown to be downregulated in diseased dogs (Franco-Martínez et al., 2020c), however, more importantly, after treatment, the concentrations of this protein were shown to increase in diseased dogs (Escribano et al., 2016). Another important finding, is the identification of haptoglobin and transferrin, two acute phase proteins in all of the publications that studied infectious diseases (Escribano et al., 2017; Franco-Martínez et al., 2018; Kuleš et al., 2014; Winiarczyk et al., 2019). In urine and serum, the evaluation of the proteome has led to the discovery of a new potential biomarker for the diagnosis of kidney disease, in this case, retinol-binding protein, which was shown to be present in dogs with early stage kidney disease before the development of azotemia (Chacar et al., 2017; Ferlizza et al., 2020; Nabity et al., 2011).

3.2 Objective 2

In order to fulfill the Objective 2, two experiments corresponding to article nº2 were carried out. First, a proteomic analysis using saliva and serum of hypothyroid and healthy dogs was performed using gel-free proteomics, in order to identify new possible biomarkers. After developing the proteomic analysis, a potential salivary biomarker was selected, and an analytical and clinical validation of this biomarkers was performed.

3.2.1 Experiment 1. *Changes of the salivary and serum proteome in canine hypothyroidism.*

- **Aims and experimental design**

The aim of this study was to evaluate the salivary and serum proteome of dogs with hypothyroidism and compare it with the salivary and serum proteome of healthy dogs in order to identify new possible biomarkers of canine hypothyroidism.

- *Animals*

Saliva and serum samples were obtained as described in the General Methods section. A total of 10 dogs with hypothyroidism and 10 healthy controls were used for the proteomic analysis. The inclusion criteria for the hypothyroid group were as follows: (1) adult dogs (>1 y), (2) absence of any other disease, (3) no history of treatment six month previous to the diagnosis, (4) having clinical signs of the specific disease (lethargy, tiredness, weight gain), (5) having serum T4 and TSH concentrations indicative of the disease, with values of T4 lower than 10.0 ng/mL and values of TSH higher than 0.040 ng/mL and (6) absence of gingivitis or any disease of the oral cavity. Meanwhile, the healthy control group included in this study was formed of adult dogs (>1 y) with no previous history of treatment or disease six months prior to the study and T4 and TSH concentrations within reference values.

Saliva and serum samples were obtained as described in the General Methods section.

Serum thyroid hormones were measured using a competitive chemiluminescent enzyme immunoassay using the Immulite analyzer (Immulite/Immulite 1000 Canine Total T4, Immulite/Immulite 1000 Canine TSH, Siemens Medical Solutions Diagnostics, Deerfield, IL, USA.) Owners' consent was obtained in all cases.

- **Results and discussion**
  - *Changes in the salivary proteome*

In the salivary proteome a total of seven proteins showed differences in their abundances in dogs with hypothyroidism compared to controls. From these, six proteins were downregulated and one upregulated in hypothyroid dogs. The most downregulated proteins were moesin (MSN), heat shock cognate 71 kDa protein (HSPA8), and L-lactate dehydrogenase A chain (LDH). On the other hand, the only upregulated protein was keratin type II cytoskeletal 4 (KRT4). No gender differences were observed in these proteins. Results regarding the salivary proteome are shown in Table 4.

Table 4. Statistically significant expression changes of protein in the saliva of dogs with hypothyroidism (n = 10) in relation to healthy dogs (n = 10).

UniProt AC	Protein Name	Gene	P-Value	Log_2FC	Regulation in Hypo
A0A8C0N5H8	Hyaluronidase-1	HYAL1	0.016	-0.394	Down
A0A8C0PRK9	Coronin-1A	CORO1A	0.016	-0.379	Down
A0A8C0LZQ6	Heat shock cognate 71 kDa protein	HSPA8	0.018	-0.446	Down
A0A8C0Z495	Glucose-6-phosphate 1-dehydrogenase	G6PD	0.036	-0.427	Down
A0A8C0TCI6	Moesin	MSN	0.036	-0.471	Down
A0A8C0PCN9	L-lactate dehydrogenase A chain	LDHA	0.036	-0.444	Down
A0A8C0RP12	Keratin, type II cytoskeletal 4	KRT4	0.046	1.883	Up

Gene ontology (GO) annotations revealed that the differently abundant proteins found in saliva are associated with diverse cellular processes, like the regulation of cell shape, its biogenesis, and structural activity, among others. The GO annotations for the proteins identified in saliva are observed in Figure 4.

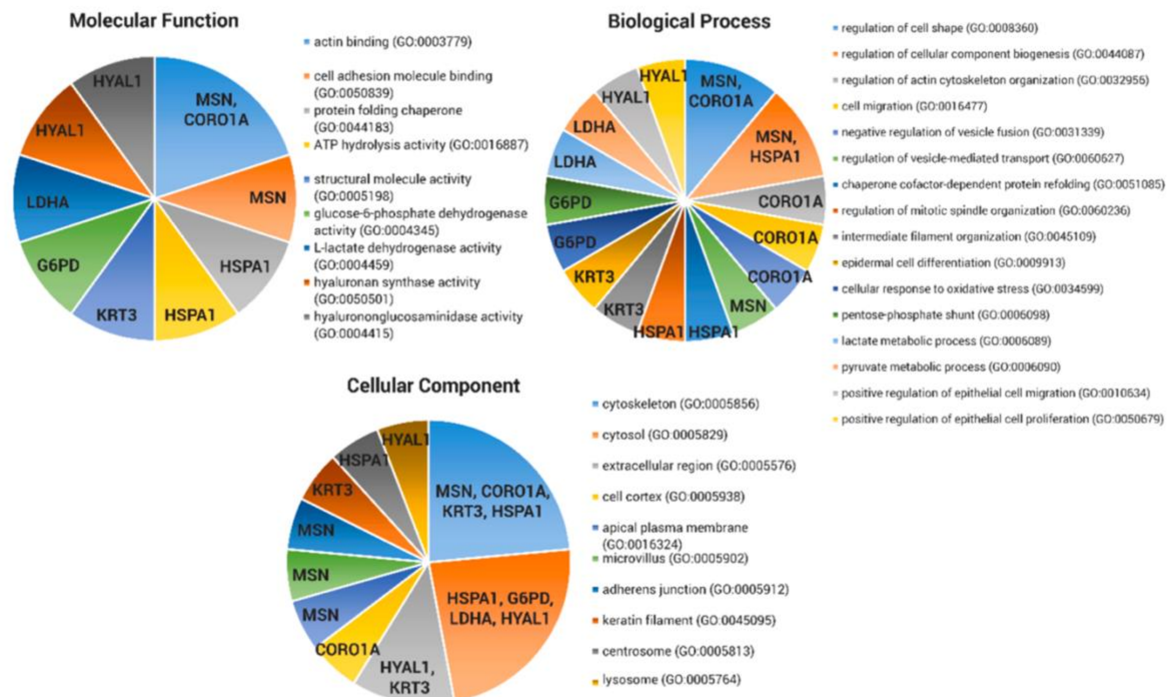

Figure 4. Pie charts showing GO terms from the three ontologies (molecular function, biological process and cellular component) associated to differentially expressed proteins in the saliva of dogs with hypothyroidism. GO terms in the legend follow a clockwise order. GO annotation for each gene was obtained with PANTHER. For dog proteins lacking annotations, we used those of their human orthologs. For clarity, the most relevant GO terms for each protein were manually selected.

In saliva, concentrations of hyaluronidase-1 and KRT4, two proteins related to the skin function were found to be downregulated in hypothyroid dogs. Thyroid hormones stimulate the synthesis of hyaluronidase-1, therefore the lack of hyaluronidase in hypothyroidism leads to one of the common signs seen in this disease, the myxedema, which develops as a consequence of the accumulation of hyaluronic acid in the skin (DeMartino and Goldberg, 1981; Pouyani et al., 2013). On the other hand, thyroid hormones influence the function of keratins in the epithelial tissue, these proteins are important for the epidermal regeneration, for this reason, an alteration in the thyroid physiology leads to an incorrect balance between skin proliferation and differentiation, contributing to the development of alopecia and other skin disorders (Mancino et al., 2021; Safer, 2011).

Additionally, in saliva, two proteins; coronin-1A and moesin were found to be downregulated in hypothyroid dogs. These two proteins contribute with the immune response, as coronin-1A is associated with T lymphocyte activation, regulation and survival (Föger et al., 2006; Kaminski et al., 2011; Mueller et al., 2008), while moesin regulated the immune response (Pore and Gupta, 2015). A decreased thyroid hormone synthesis intervenes directly in the regulation of the immune system, which could be the reason for the downregulation of these two proteins (Graham et al., 2007). Another downregulated protein was LDH, which could be associated with the changes in the redox status of hypothyroid dogs, as LDH has been shown to decrease due to the effects of oxidative stress (Avezov et al., 2014).

- *Changes in the serum proteome*

In the serum proteome profile, a total of six proteins differ in their abundance in dogs with hypothyroidism in comparison with controls. From these, five proteins were downregulated and one upregulated in the serum of hypothyroid dogs. The most downregulated proteins were sex hormone-binding globulin, vitamin D-binding protein, apolipoprotein M, complement component C7, and antithrombin-III. On the other hand, the upregulated protein was inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4). No gender differences were observed in these proteins. Results regarding the serum proteome are shown in Table 5.

Table 5. Statistically significant expression changes of protein in dogs' serum with hypothyroidism (n = 10) in relation to healthy dogs (n = 10).

UniProt AC	Protein Name	Gene	P-Value	Log_2FC	Regulation in Hypo
A0A8I3PLX4	Sex hormone-binding globulin	SHBG	0.0129	-0.486	Down
A0A8C0M760	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4	0.019	0.518	Up
A0A8I3NHZ1	Antithrombin-III	SERPINC1	0.027	-0.207	Down
A0A8I3MQ72	Complement component C7	C7	0.029	-0.230	Down
A0A8I3NFP8	GC vitamin D-binding protein	GC	0.032	-0.200	Down
A0A8C0MAQ8	Apolipoprotein M	APOM	0.035	-0.134	Down

GO annotations in serum revealed that the proteins identified were extracellular/secreted proteins, some of these are serin protease inhibitors while the others are involved in the transport of vitamin D, androgens and lipids. The GO annotations for the proteins identified in serum are observed in the Figure 5.

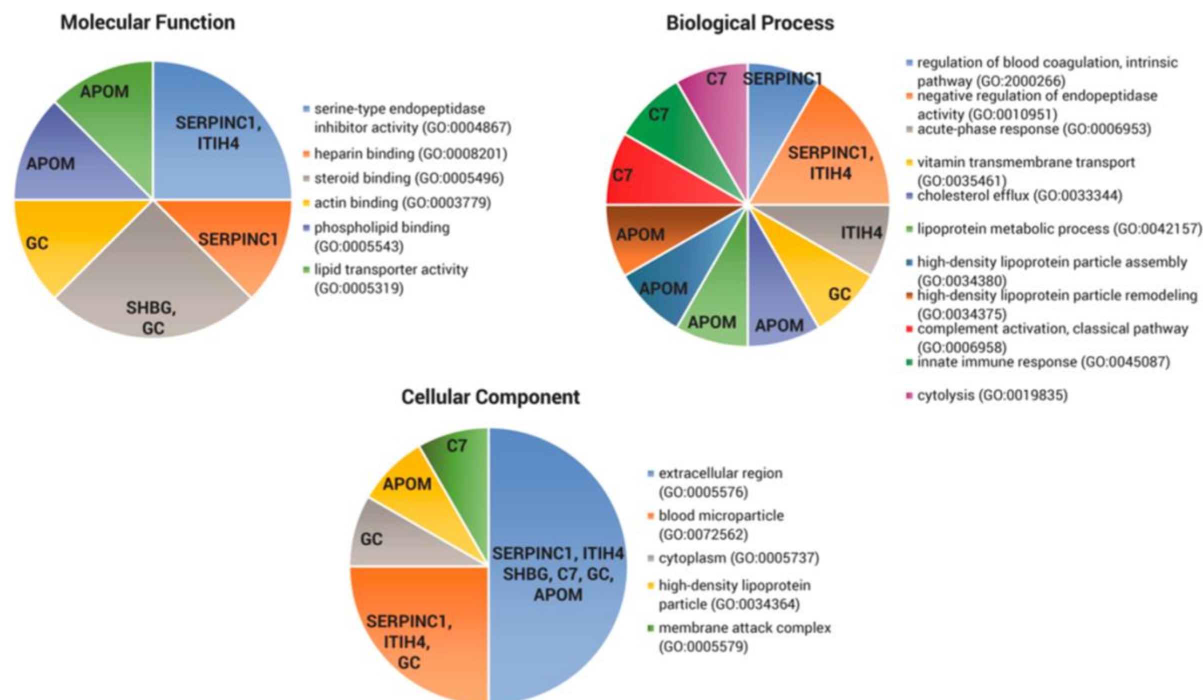

Figure 5. Pie charts showing GO terms from the three ontologies (molecular function, biological process and cellular component) associated to differentially expressed proteins in serum. GO terms in the legend follow a counterclockwise order. GO annotation for each gene was obtained with PANTHER. For dog proteins lacking annotations, we used those of their human orthologs. For clarity, the most relevant GO terms for each protein were manually selected.

In serum, sex hormone binding globulin and vitamin D-binding protein were found to be downregulated. The downregulation of these two proteins could be associated with a lower thyroid function as these proteins are synthesized by the liver and are influenced by thyroid hormones (Anderson, 1974; Selby, 1990). Additionally, vitamin D-binding protein has been reported to be decreased in Hashimoto's thyroiditis (Christoffersen et al., 2012). Antithrombin III was also found to be downregulated in serum. The decrease in this protein could be associated with the hypercoagulable state found in the disease, as this protein is key to the regulation of the coagulation process (Hsu and Moosavi, 2022; Rennie et al., 1978).

ITIH4 was found to be upregulated in hypothyroid dogs, meanwhile apolipoprotein M was found to be downregulated. These two proteins are associated with lipid metabolism, and their change could be associated with the dyslipidemia caused by a decreased availability of thyroid hormones (Christoffersen et al., 2012; Fujita et al., 2004; Huang et al., 2015; Yao et al., 2021).

3.2.2 Experiment 2. *Clinical and analytical validation of salivary lactate dehydrogenase activity measurement*

- **Aims and experimental design**

The aim of this study was to perform the analytical and clinical validation of the measurement of LDH in saliva. For this purpose, the precision, accuracy and LOQ were evaluated and the LDH determination in dogs with hypothyroidism, NTH dogs and healthy controls was performed.

- *Animals*

In this case-control study a total of 42 hypothyroid, 42 NTH dogs and 46 healthy controls were included. The inclusion criteria for the hypothyroid group and control group are described in Objective 2. The NTH group had the same inclusion criteria, except for having negative results for specific thyroid diagnostic tests. Owners' consent was obtained in all cases.

- *Lactate dehydrogenase activity analysis*

LDH activity in saliva was measured using a commercially available kit for the measurement of human LDH (Lactate Dehydrogenase spectrophotometry kit, REF 11581, Byosystems, Barcelona, Spain) using an Olympus A400 automated analyzer (Olympus Diagnostica GmbH, Ennis, Ireland). Concentrations are expressed in International Units per liter (IU/L).

- **Results and discussion**
  - *Analytical validation of the LDH assay*

The determination of the salivary LDH proved to be precise and sensible, with an intra-assay and inter-assay precision of 2.34 % and 3.81 % respectively, a limit of detection of 3.75 IU/L and a high linearity (R = 1.0) after serial dilutions.

  - *LDH changes in hypothyroid dogs.*

Salivary concentrations of LDH are shown in Figure 6. Concentrations were significantly lower in dogs with hypothyroidism (median; min – max; 55.35 IU/L; 4.70–115.60) compared to both controls (204.80 IU/L; 17.2–1506 *and* $P = 0.0001$) and NTH dogs (314.50 IU/L; 4.30–2374 *and* $P < 0.0001$). The receiving operating characteristic (ROC) curve analysis showed an AUC of 0.88 (CI: 0.79-0.96 *%;* $P < 001$) establishing a cut-off value of 74.25 U/L (sensitivity: 87.50 %; specificity: 70.59 %) to discriminate dogs with hypothyroidism from dogs with NTH and healthy dogs.

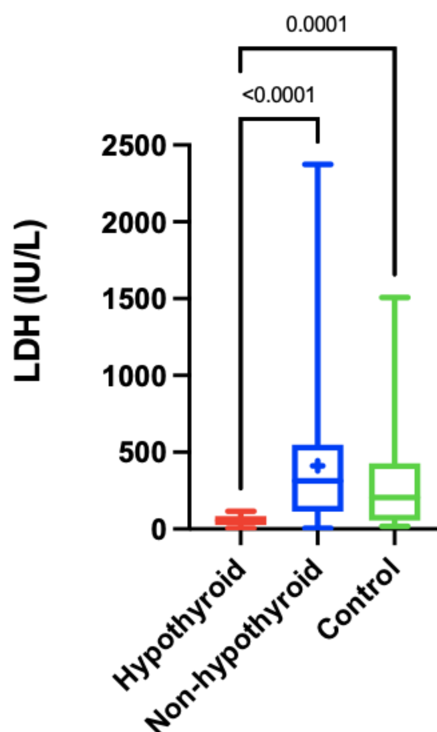

Figure 6. Lactate dehydrogenase in the saliva of dogs with hypothyroidism (n = 42) compared with dogs with non-hypothyroid disease (n = 42) and healthy control dogs (n = 46). The plot shows the median (line within the box), the interquartile range (10 and 90), and the range (whiskers).

The decrease shown in LDH in hypothyroid dogs presented values of area under the curve (AUC) higher than 0.8. Therefore, the measurement of LDH has a good ability to distinguish between dogs with hypothyroidism and dogs with other diseases and clinical signs compatible with hypothyroidism or healthy dogs. LDH could be a possible biomarker for the diagnosis of canine hypothyroidism, although this protein is highly sensible to gingivitis, a main confounding factor for salivary biomarker interpretation. Nevertheless, in this study, only dogs with healthy gums were included.

3.3 Objective 3

In order to fulfill Objective 3, two experiments corresponding to article nº3 and article nº4 were carried out. First, an untargeted metabolomic analysis using the serum of hypothyroid and healthy dogs and secondly, a targeted metabolomic analysis using the saliva of hypothyroid and healthy dogs was performed in order to identify new possible biomarkers.

3.3.1 Experiment 1. *Untargeted metabolomic profiling of serum in dogs with hypothyroidism.*

- **Aims and experimental design**

The aim of this study was to evaluate the metabolomic profile of dogs with hypothyroidism and to compare it with the metabolomic profile of healthy controls in order to identify possible biomarkers of canine hypothyroidism through the use of untargeted metabolomic analysis.

- *Animals*

In this case-control study a total of 20 hypothyroid dogs and 20 healthy controls were included following the inclusion criteria stated in Objectives 2 and 3. Owners' consent was obtained in all cases.

- **Results and discussion**

Through an untargeted metabolomic approach, a total of 2160 metabolites were revealed, among these, 15 metabolites showed statistically significant *($P < 0.05$)* changes between dogs with hypothyroidism and healthy controls. The principal component analysis (PCA)and the partial-least-squared discriminant analysis (PLSDA) indicated a clear separation between the hypothyroid and control groups. The PCA and PLSDA analysis are shown in Figures 7 and 8.

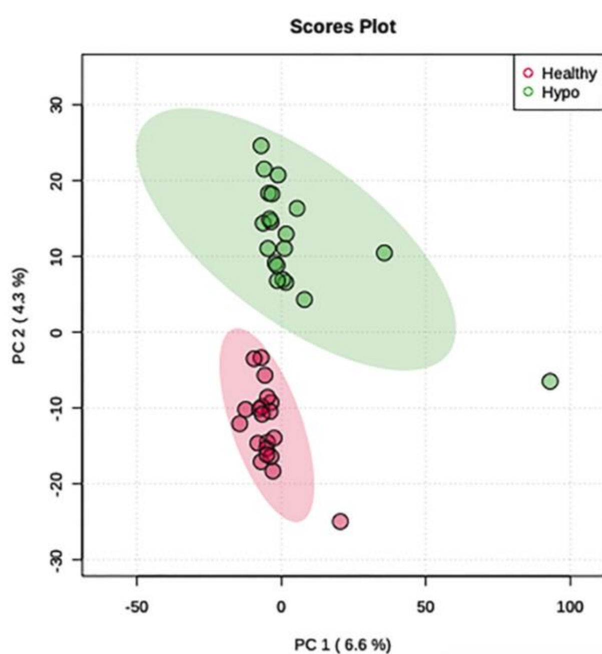

Figure 7. PCA score plots for the data from hypothyroidism (green) and healthy (red) dogs.

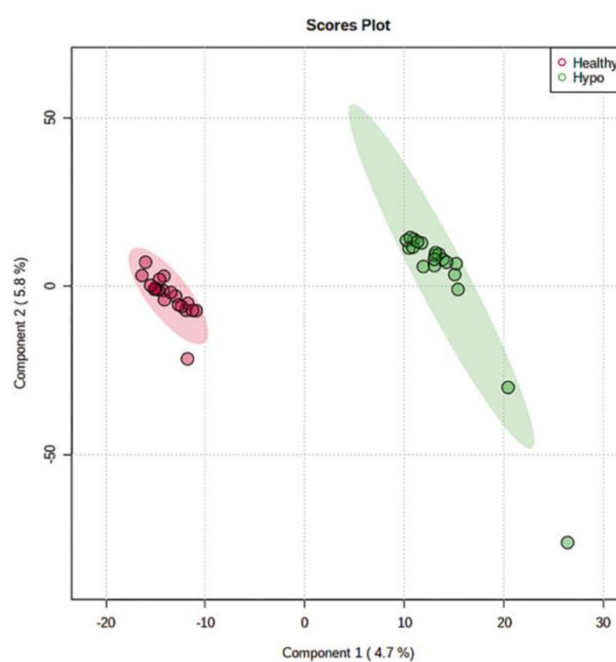

Figure 8. PLSDA score plot from the hypothyroidism (green) and healthy (red) dogs.

Two metabolites that matched with a known standard were identified; D-gluconic acid and L-isoleucine, both metabolites were found to be upregulated as shown in Figure 9. The increase in D-gluconic acid could be associated with dyslipidemia and other complications associated with hypothyroidism, as this metabolite is involved in the pentose phosphate pathway that plays a role in lipid metabolism (Baquer et al., 1976; Spégel et al., 2013). The evaluation of this metabolite as a biomarker could be considered useful in the early detection of dyslipidemia and associated complications. On the other hand, the increase in L-isoleucine, could be related to the effect of thyroid hormones in the inhibition

of cellular amino acid uptake (Morrison et al., 1988), as well as producing an insulin resistance, as this metabolite is a branched-chain amino acid and has been associated with the development of diabetes mellitus type 2 in people (Floegel et al., 2013).

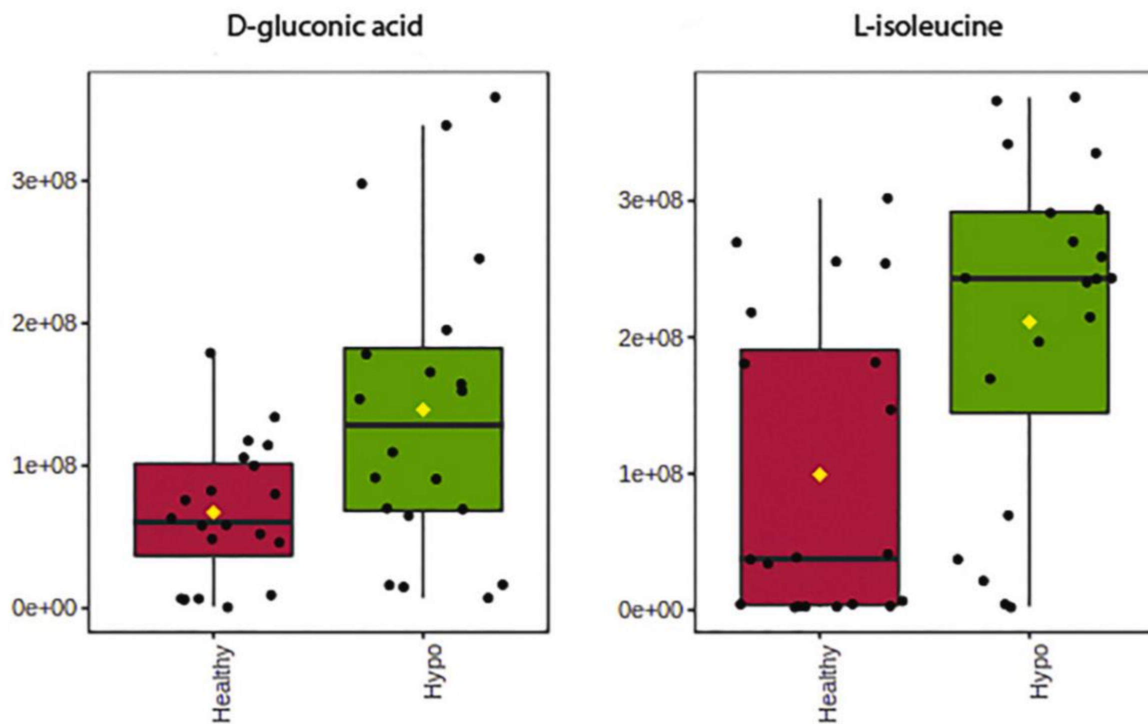

Figure 9. Concentrations of identified metabolites in hypothyroidism (green) and control groups (red). The plot shows the median (line within the box), the interquartile range (25 and 75), and the range (yellow point).

Octanoic acid is associated with fatty acids pathways and was found to be reduced in hypothyroid dogs. It can be hypothesized that the decrease of this metabolite could be due to the reduced lipolysis that is given as a consequence of an impaired thyroid function (Sinha et al., 2018). Meanwhile, the downregulation found in calcitriol, an active form of vitamin C, could be associated with the development of hypothyroidism as it has been described to be decreased in people with hypothyroidism (Tokić et al., 2017).

3.3.2 Experiment 2. *Changes in the salivary metabolome in canine hypothyroidism: A pilot study*

- **Aims and experimental design**

The aim of this study was to evaluate the metabolomic profile of dogs with hypothyroidism and to compare it with the metabolomic profile of healthy controls in order to identify possible biomarkers of canine hypothyroidism, through the use of untargeted metabolomic analysis.

- *Animals*

In this case-control study, a total of 10 hypothyroid dogs and 10 healthy controls were included following the inclusion criteria stated in Objectives 2 and 3. Owners' consent was obtained in all cases.

- **Results and discussion**

The targeted metabolomic approach led to the identification of 88 different salivary metabolites present in both hypothyroid and healthy dogs. To identify which metabolites were differentiators between the two groups, a PLSDA was depicted, as shown in Figure 10, which shows a clear and strong separation among the two groups.

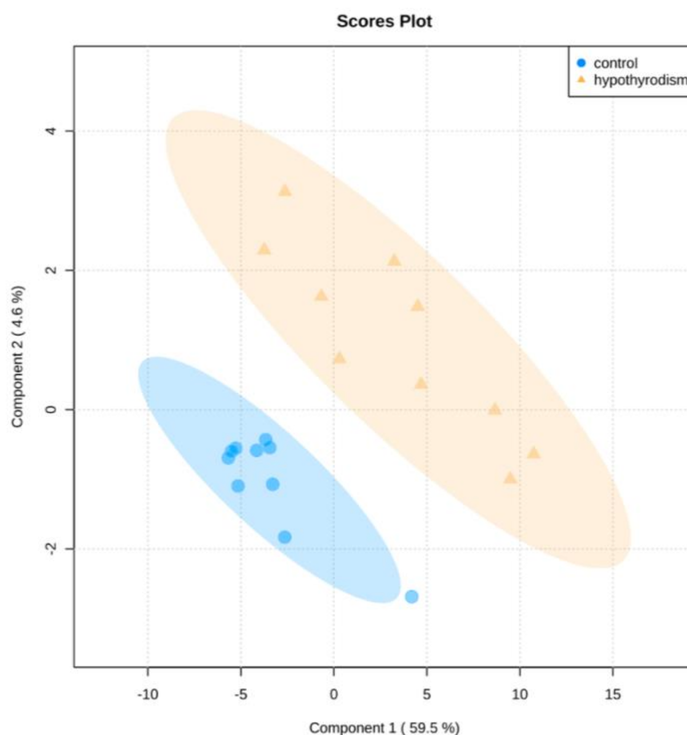

Figure 10. Partial least square-discriminant analysis (PLSDA), showing a strong separation between the groups (hypothyroidism vs control). Each circle point in the plot corresponds to a control saliva sample and each triangle point to a hypothyroid saliva sample. The shaded areas indicate the 95% confidence regions.

Among the 88 identified metabolites, 23 were found to be downregulated between hypothyroid and healthy dogs, these metabolites are listed in Table 6. These include seven amino acids, one triglyceride, two cholesterol esters and 12 phosphatidylcholins.

Metabolite class	Metabolite	FC	log2(FC)	P value (adjusted)	-Log10(p)	VIP values	Regulation in hypothyroidism
Amino acid	Histamine	4.22	2.07	0.02	1.56	1.65	Down
	Tyrosine	3.77	1.91	0.02	1.56	1.65	Down
	Lysine	2.94	1.55	0.03	1.50	1.55	Down
	Ornithine	3.34	1.74	0.02	1.69	1.53	Down
	Citrulline	3.45	1.79	0.03	1.50	1.51	Down
	Phenylalanine	3.96	1.98	0.03	1.50	1.5	Down
	Glutamine	2.28	1.19	0.02	1.56	1.29	Down
Triglycerides	TG(56:7)	2.63	1.39	0.02	1.56	1.2	Down
Cholesterol Ester	CE(20:4)	5.23	2.38	0.02	1.69	1.73	Down
	CE(18:2)	4.47	2.16	0.02	1.69	1.69	Down
Phostatidylcholines	PC(40:6)	3.54	1.82	0.02	1.56	1.63	Down
	PC(36:4)	3.52	1.81	0.03	1.50	1.63	Down
	PC(38:6)	3.42	1.77	0.03	1.50	1.63	Down
	PC(34:1)	3.07	1.61	0.04	1.39	1.50	Down
	PC(38:5)	3.20	1.67	0.03	1.50	1.48	Down
	PC(36:1)	3.49	1.80	0.02	1.69	1.46	Down
	PC(38:4)	2.63	1.39	0.02	1.56	1.44	Down
	PC(34:2)	3.24	1.69	0.02	1.56	1.41	Down
	PC(36:5)	3.84	1.94	0.04	1.39	1.33	Down
	PC(40:5)	2.60	1.47	0.04	1.33	1.28	Down
	PC(36:2)	3.49	1.80	0.04	1.39	1.23	Down
	PC(32:1)	2.11	1.08	0.03	1.50	1.17	Down
	PC(32:4)	2.75	1.46	0.04	1.39	1.08	Down

Table 6. Volcano plot data comparing hypothyroid versus control dogs. VIP values were obtained from multivariate analysis of the partial least square-discriminant analysis (PLSDA) model. The targeted metabolomic analysis revealed a decrease in seven amino acids, one triglyceride (TG), two cholesterol esters (CE), and 13 phosphatidylcholines (PC) in dogs with hypothyroidism compared with healthy dogs.

The metabolome of dogs with hypothyroidism showcased an alteration of several metabolic pathways, as shown in Figure 11. For instance, the metabolic pathway involving the metabolism of histidine was shown to be altered, this pathway being represented by the downregulation of histamine, a proinflammatory mediator that requires the metabolism of T3 in order to be released by the mast cells (Csaba and Pállinger, 2009; Landucci et al., 2019; Thangam et al., 2018). The metabolic pathway for the synthesis of thyroid hormones, as well as, tyrosine metabolism were impaired, as a possible consequence of the reduction of tyrosine, as this metabolite serves as a substrate for thyroid hormone synthesis and its reduction is often associated with a diminished functionality of the thyroid gland (Laidlaw and Kopple, 1987; Young et al., 1978).

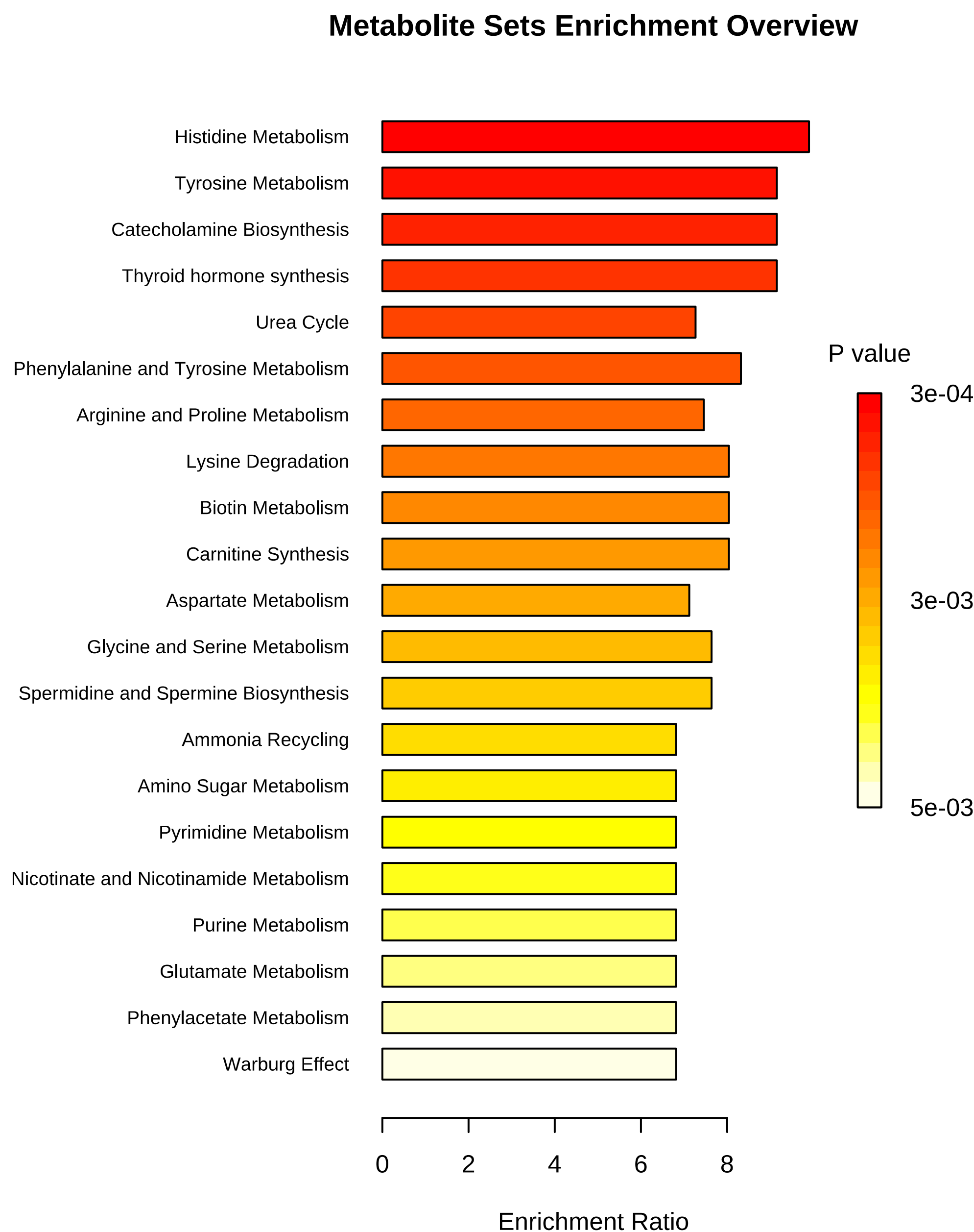

Figure 11. Quantitative enrichment analysis (QEA) of hypothyroidism. The horizontal bar graph summarizes 21 metabolic pathways using metabolites that were significantly altered in dogs with hypothyroidism.

Canine hypothyroidism has a strong impact in lipid metabolism, as this disease is known for causing an exacerbated lipogenesis with an impaired catabolism of these lipids, leading to clinical dyslipidemia (Pucci et al., 2000; Rizos et al., 2011). In this study, the reduction of three metabolites associated with lipoproteins, one triacylglycerol (TG) and two cholesterol esters (CE) was evident, raising the hypothesis that a decreased lipid

metabolic rate could be associated with the reduction of this metabolites, while providing importance on the effects that a lack of thyroid hormones have on the lipid profile.

3.4 Objective 4

In order to fulfill Objective 4, an experiment corresponding to article nº4 was carried out. In this experiment, a wide panel of redox biomarkers was validated for the first time in both WB and RBCs lysates of dogs.

3.4.1 Experiment 1. *Analytical validation of redox biomarkers in whole blood and red blood cells lysates*

- **Aims and experimental design**

The redox status is of high importance to life, as it is involved in important biological process (Sies et al., 2017). This status is mostly evaluated in serum, nonetheless the use of other samples opens a new field of research, for this reason an analytical validation of a wide panel of redox biomarkers was made for the first time in WB and RBCs lysates of dogs. This panel of redox biomarkers included five antioxidants (CUPRAC, FRAP, TEAC, thiol and PON-1) and five oxidants (TOS, POX-Act, d-ROMs, AOPP and TBARS).

After the analytical validation, these biomarkers were subjected to an "in vitro" test, in which the changes of the redox status in two samples was evaluated when exposed to different concentrations of ascorbic acid, as it is a chain-breaking antioxidant that can also act as a pro-oxidant depending on the dosage used (Chakraborthy et al., 2014).

- *In vitro test*

For this test, blood from seven male adult Beagle dogs was collected. Each sample was divided into three groups: (1) control; (2) ascorbic acid 10 mM (VC10); and (3) ascorbic acid 60 mM (VC60). Vials were mixed with NaCL 0.9% (control) and ascorbic acid at the specified concentration and incubated for 2 hours, before analysis.

- **Results and discussion**
    - *Analytical validation*
    - Whole blood

For WB samples, all assays showed and intra-assay CV between 0.01% and 15.89% and an inter-assay CV between 0.01% and 13.62%. Serial dilution of WB resulted in linear regression higher than 0.96. Recovery in all cases was between 92.77% and 122.49%.

- Red blood cells lysates

For RBCs lysates, all assays showed an intra-assay CV between 1.02% and 15.98% and an inter-assay CV between 0.58% and 12.6%. Serial dilution of RBCs lysates resulted in linear regression higher than 0.96 for all assays studied. Recovery in all cases was between 87.5% and 113.9%.

- *In vitro test*
- Antioxidant status

Results for the antioxidant status in the "in vitro" test are shown in Figure 12. After the addition of ascorbic acid at 10 mM and 60 mM, CUPRAC, FRAP and TEAC, showed significantly higher levels than the control group when measured in WB (except for CUPRAC in WB VC10). On the contrary, these changes were not evident in RBCs lysates; moreover, a decrease in these three antioxidants biomarkers was evident when using the higher dosage of ascorbic acid. These results reflect the potential of WB and RBCs lysates as new samples for the evaluation of antioxidant status.

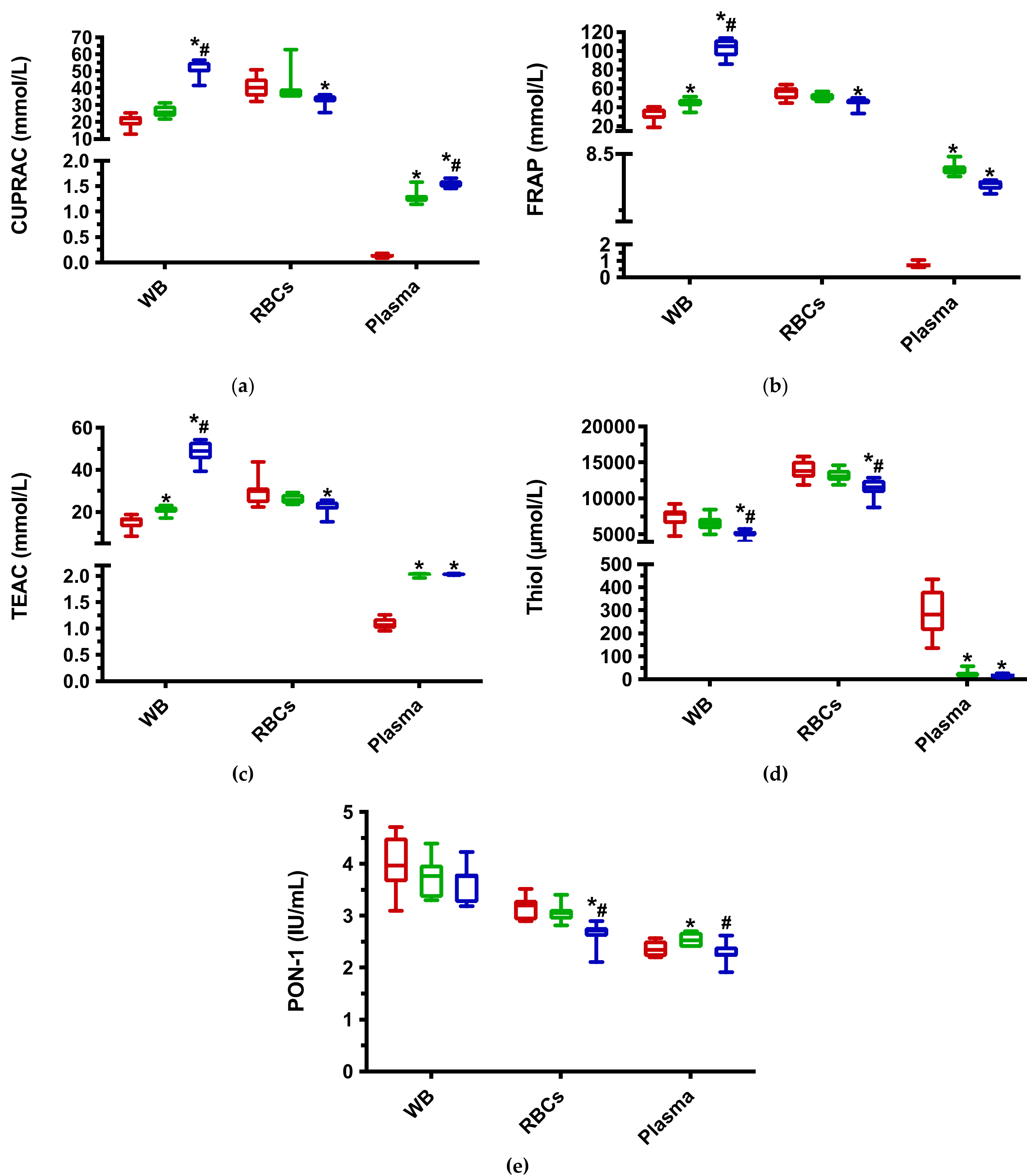

Figure 12. Results of the "in vitro" test on antioxidant biomarkers. (a) Cupric reducing antioxidant capacity (CUPRAC); (b) ferric reducing ability of plasma (FRAP); (c)Trolox equivalent antioxidant capacity (TEAC); (d) thiol, and (e) paraoxonase type 1 (PON-1) results obtained during the "in vitro" study for whole blood (WB), red blood cells (RBCs) lysates and plasma samples. Probability levels of $p < 0.05$ were regarded as significant and

marked with an asterisk (*: vs. control) and a hashtag (#: VC10 vs. VC60). Red box: control group; green box: VC10 group; and blue box: VC60 group**.**

- Oxidant status

Results for the oxidant status in the "in vitro" test are shown in Figure 13. In WB, a decrease in POX-Act and d-ROMs occurred when lower dose of ascorbic acid was used. Meanwhile, when adding a higher dose of ascorbic acid, a decrease of TOS, POX-Act and d-ROMs was shown in WB. In RBCs lysates a similar decrease was found in POX-Act and AOPP. These results could indicate the inhibition of peroxide formation by the added antioxidant and the capacity that these two samples have to detect the changes produced by the addition of ascorbic acid.

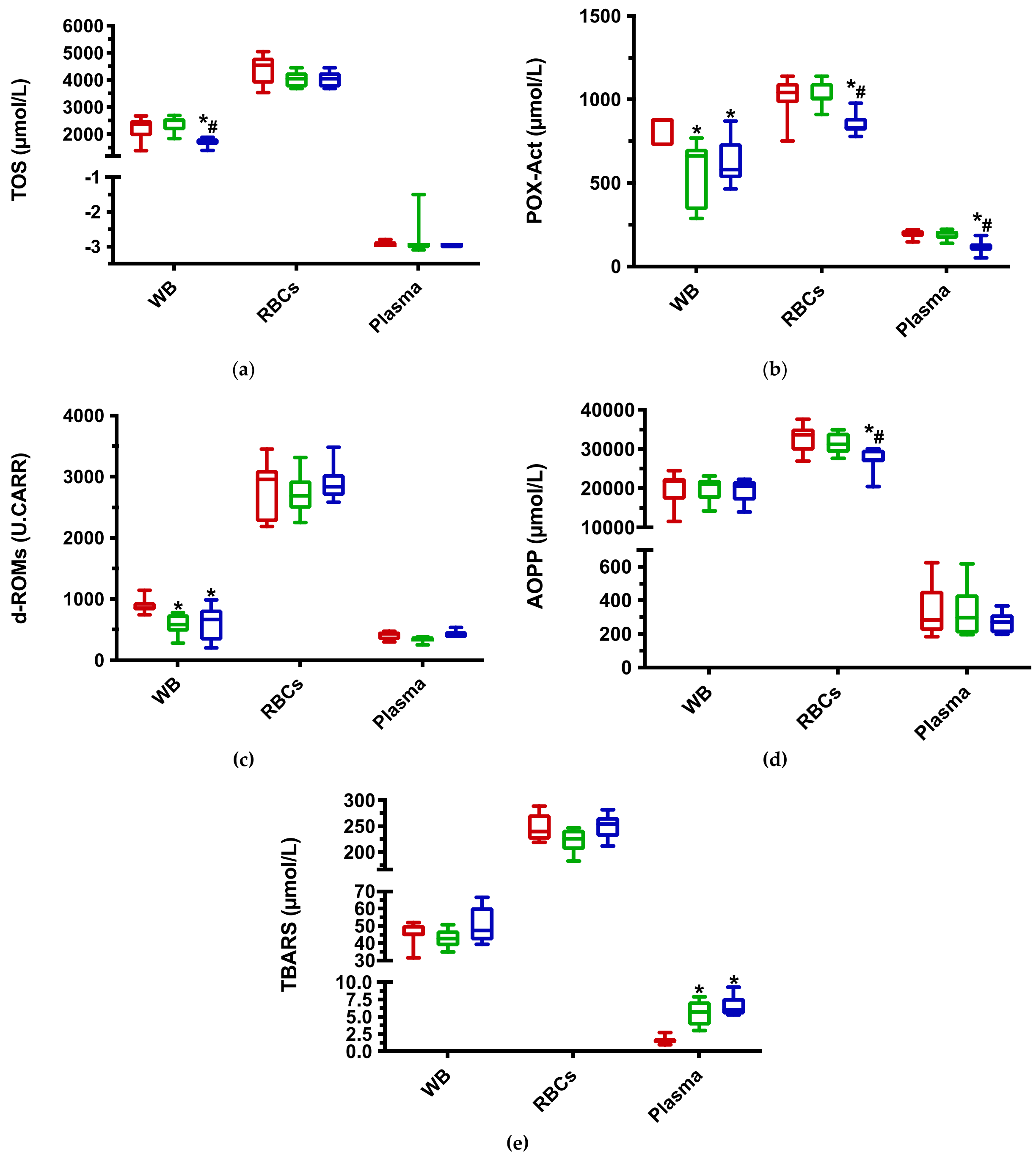

Figure 13. Results for the "in vitro" test on oxidant biomarkers. (a) Total oxidant status (TOS); (b) peroxide-activity (POX-Act); (c) reactive oxygen-derived compounds (d-ROMs); (d) advanced oxidation protein products (AOPP), and (e) thiobarbituric acid reactive substances (TBARS) results obtained during the "in vitro" study for whole blood (WB), red blood cells (RBCs) lysates and plasma samples. Probability levels of $p < 0.05$ were regarded as significant and marked with an asterisk (*: vs. control) and a hashtag (#: VC10 vs. VC60). Red box: control group; green box: VC10 group; and blue box: VC60 group.

Processing of WB is easier than RBCs lysates, plasma, serum and saliva, as for the obtention of WB no centrifugation or aspiration is needed, while the preparation of RBCs lysate takes around one or two hours. WB could be considered a promising sample for the evaluation of the redox status in dogs due to its high sensitivity to detect changes and its easy sample preparation.

3.5 Objective 5

In order to fulfill Objective 5, two experiments corresponding to article nº6 and Annex 1 were carried out. In the first experiment, a wide panel of redox biomarkers was evaluated for the first time in the serum and saliva of dogs with hypothyroidism. In the experiment performed in Annex 1, redox biomarkers were evaluated for the first time in WB and RBCs lysates of dogs with hypothyroidism. Both experiments were made to compare the redox state of hypothyroid dogs with the redox state of dogs with NHT and healthy dogs, in order to identify new possible biomarkers.

3.5.1 Experiment 1. *Changes of biomarkers of redox status in serum and saliva of dogs with hypothyroidism*

- **Aims and experimental design**

Thyroid hormones have an important effect on the redox status, as these hormones are enzymatic and non-enzymatic free radical scavengers and also promote catabolic and anabolic reactions that can lead to the consumption of oxygen and regulate the development of ROS (Chainy and Sahoo, 2020; Venditti et al., 2003; Villanueva et al., 2013). As a consequence of a lower thyroid hormone synthesis, a decreased metabolic rate is expected in canine hypothyroidism, which could heavily influence the redox state.

For this reason, in order to evaluate the redox status of hypothyroid dogs, a wide panel of six antioxidants (CUPRAC, FRAP, TEAC, thiol, PON-1, GPx) and five oxidants (TOS, POX-Act, d-ROMs, AOPP and TBARS) were measured in serum and three antioxidants (CUPRAC, FRAS and TEAC) and two oxidants (AOPP and TBARS) were measured in saliva, and compared with the serum and salivary redox state of dogs with NTH and healthy controls.

- *Animals*

In this case-control study, client-owned dogs attending different Veterinary Clinics of Murcia Region (Spain) between March 2021 and May 2022. These dogs were divided into three groups; (1) hypothyroid group (n = 23), (2) NTH group (n = 21) and (3) Control group (n = 16), following the inclusion criteria stated in Objectives 2 and 3. Owner's consent was obtained in all cases.

- **Results and discussion**
  - *Changes of the redox state in serum*

In serum, dogs with hypothyroidism showed an increase in TEAC and PON-1 when compared with NTH dogs (P = 0.049 and P = 0.007, respectively) and healthy controls (P = 0.042 and P = 0.007, respectively) (Figure 14).

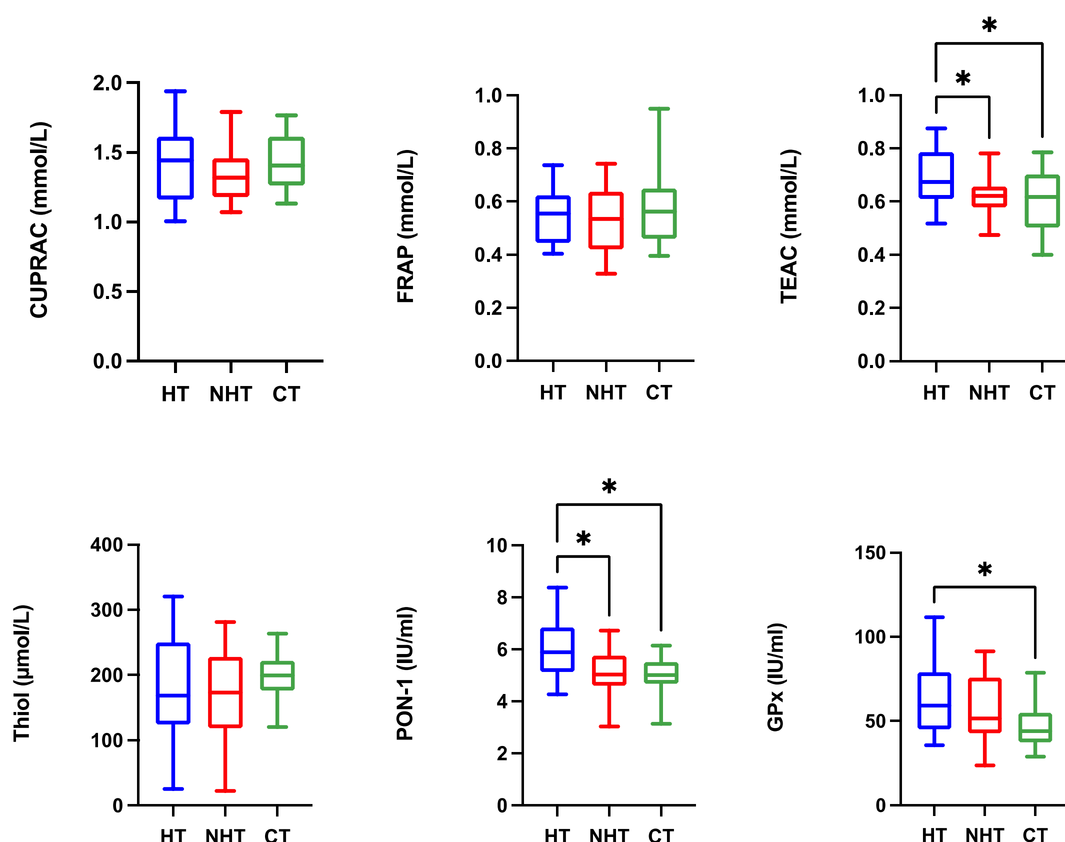

Figure 14. Results of antioxidant biomarkers in serum. Cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), thiol, paraoxonase type-1 (PON-1), and glutathione peroxidase (GPx) in dogs with hypothyroidism (HT), non-thyroid disease (NHT) dogs and controls (CT). Asterisks indicate significant differences between groups. *P ≤ 0.05.

Results for the oxidant biomarkers for serum are shown in Figure 15. Hypothyroidism caused an increase of TOS when compared to both NTH dogs and healthy controls (P = 0.032), meanwhile, POX-Act and d-ROMs increased in hypothyroid dogs when compared to healthy controls (P = 0.0003 and P = 0.007, respectively).

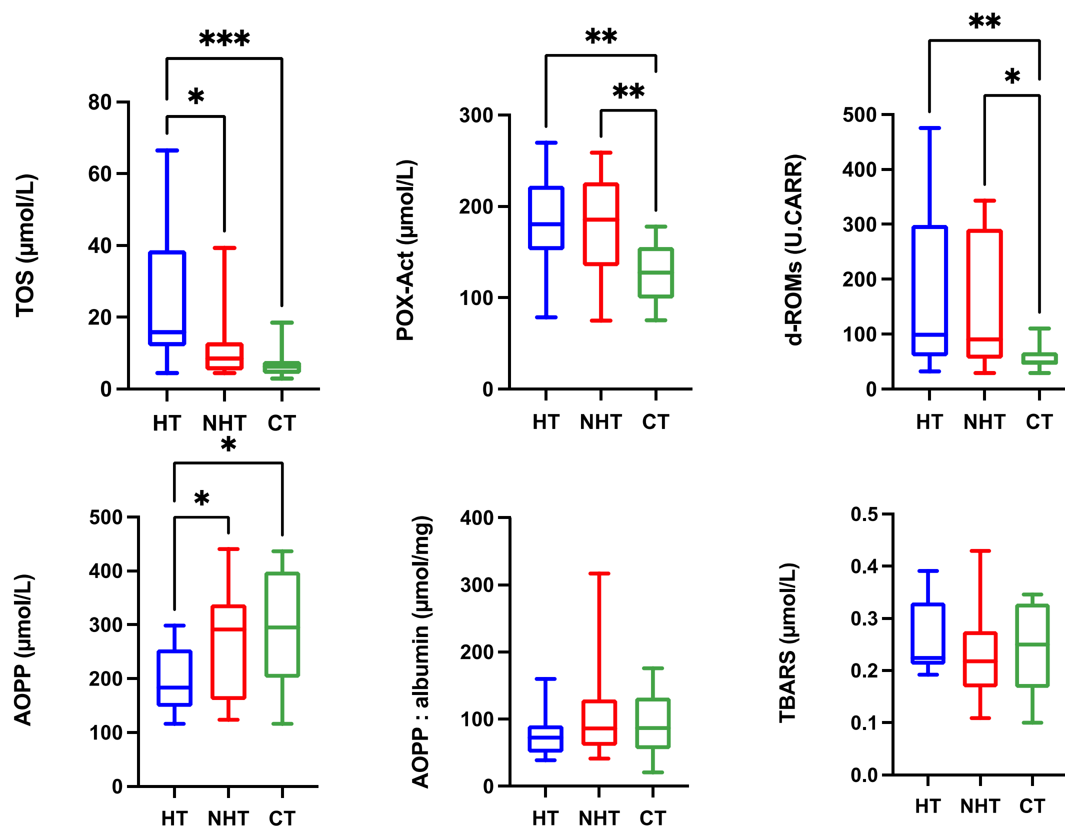

Figure 15. Results of oxidant biomarkers in serum. Total oxidant status (TOS); peroxide-activity (POX-Act), reactive oxygen-derived compounds (d-ROMs), advanced oxidation protein products (AOPP), thiobarbituric acid reactive substances (TBARS) and AOPP: albumin ratio in dogs with hypothyroidism (HT), non-thyroid disease (NHT) dogs and controls (CT). Asterisks indicate significant differences between groups. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

- *Changes of the redox state in saliva*

In saliva, dogs with hypothyroidism had lower concentrations of FRAS than NTH dogs (P = 0.003) and healthy controls (P = 0.016) (Figure 16). However, once data were corrected by total protein, no statistically significant differences were observed.

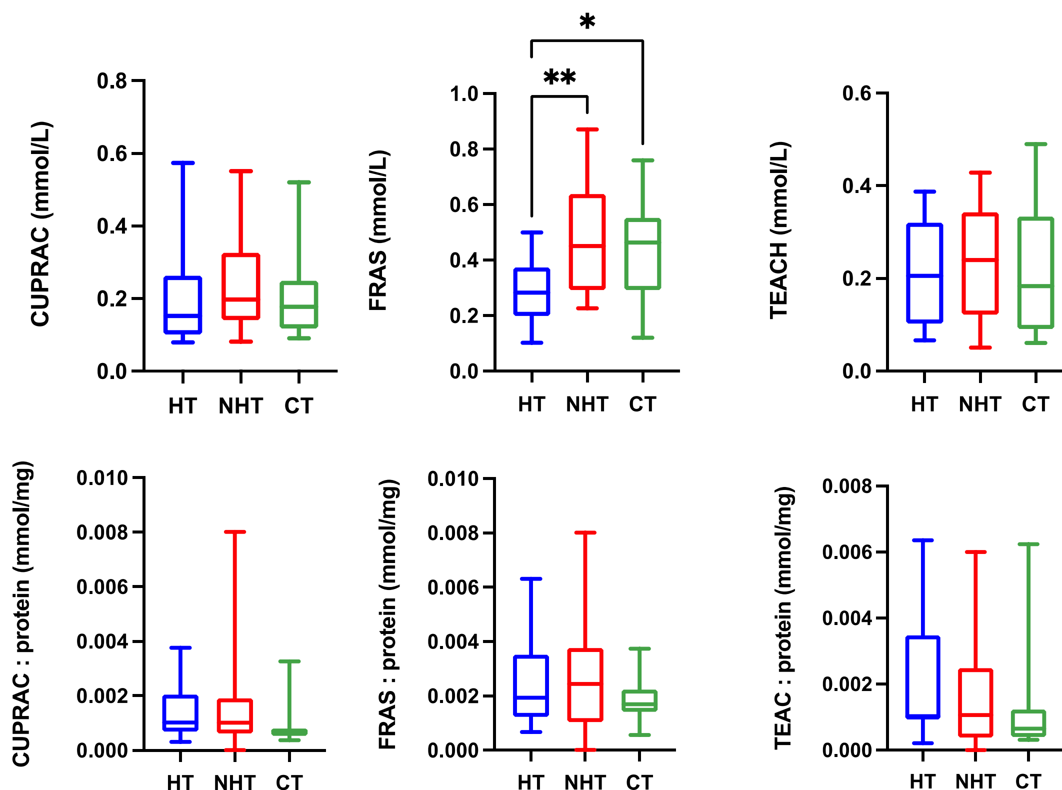

Figure 16. Results of the measurement of antioxidant biomarkers in saliva (above), and its ratioed value with salivary protein concentration (below). Cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of saliva (FRAS), and Trolox equivalent antioxidant capacity (TEAC) in dogs with hypothyroidism (HT), non-thyroid disease (NHT) dogs and controls (CT). Asterisks indicate significant differences between groups. $*P \leq 0.05$, $**P \leq 0.01$.

Similarly, when evaluating the oxidative biomarkers (Figure 17), AOPP was shown to be significantly lower in dogs with hypothyroidism in comparison to NHT dogs (P = 0.011) and healthy controls (P = 0.011). After the results have been corrected by microalbumin, no significant differences were shown.

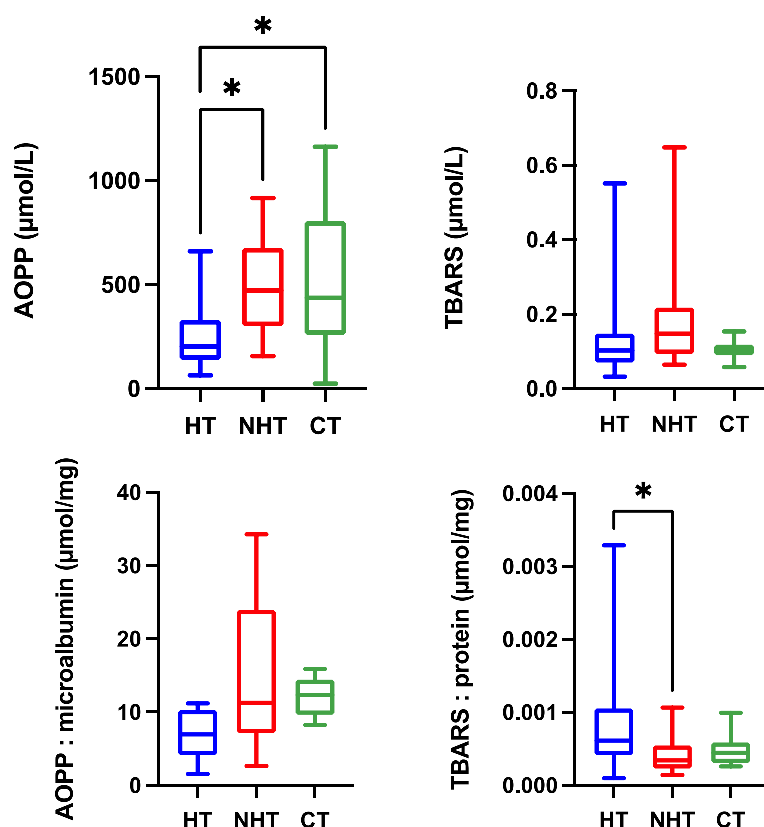

Figure 17. Results of the measurement of oxidant biomarkers in saliva (above), and its ratioed value with salivary microalbumin and protein concentration for AOPP and TBARS, respectively (below). Advanced oxidation protein products (AOPP) and thiobarbituric acid reactive substances (TBARS) in dogs with hypothyroidism (HT), dogs with non-thyroid disease (NHT) dogs and controls (CT). Asterisks indicate significant differences between groups. $*P \leq 0.05$.

Additionally, a correlation study was performed to evaluate the possible correlation between salivary and serum biomarkers. The results showed a positive correlation (r = 0.418, $P \leq 0.0002$) for AOPP between serum and saliva corrected by albumin.

These results showcase that in canine hypothyroidism the redox state is altered with an increase in serum antioxidants like TEAC, PON-1 and GPx being presumably associated with the influence of an increased TSH stimulation as well as an increased lipid mobilization as a consequence of the disease. On the other hand, an increase in serum oxidants like TOS, POX-Act and d-ROMs indicate an overproduction of oxidants contributing to the onset of oxidative stress. Meanwhile, in saliva, a decrease in FRAS and AOPP was observed in dogs with hypothyroidism, however, these values were normalized once values were corrected by salivary protein concentration. Nonetheless, after correction, salivary TBARS was shown to be increased indicating of the occurrence of lipid peroxidation.

3.5.2 Experiment 2. *Changes in biomarkers of the redox status in whole blood and red blood cell lysates in canine hypothyroidism (ANNEX 1)*

- **Aims and experimental design**

The objective of this study was to investigate if the biomarkers of redox status are changed in WB and RBCs of dogs with hypothyroidism. For that a wide panel of antioxidant and oxidant biomarkers was measured in WB and RBCs lysates of dogs with hypothyroidism and results were compared with the redox status of dogs with NTHs and healthy controls.

  - *Animals*

In this case-control study, client-owned dogs attending different Veterinary Clinics of Murcia Region (Spain) between March and October 2022. These dogs were divided into three groups; (1) hypothyroid group (n = 30), (2) NTH group (n = 26) and (3) Control group (n = 15), following the inclusion criteria stated in Objectives 2 and 3. Owner's consent was obtained in all cases.

- **Results and discussion**
  - *Changes in the antioxidant status*

Results for the biomarkers of the total antioxidant capacity (CUPRAC, FRAP and TEAC) are shown in Figure 18. Dogs with hypothyroidism showed significantly lower levels of CUPRAC (P = 0.003) and TEAC (P = 0.005) compared to healthy controls in WB. Meanwhile, in RBCs lysates, significantly higher concentrations of FRAP ($P = 0.002$) and lower TEAC ($P = 0.011$) were found. These results are indicative of a decreased WB antioxidant capacity in dogs with hypothyroidism, being associated with an impaired thyroid function, as thyroid hormones are part of the antioxidant defense system (Chainy and Sahoo, 2020; Venditti et al., 2003; Villanueva et al., 2013).

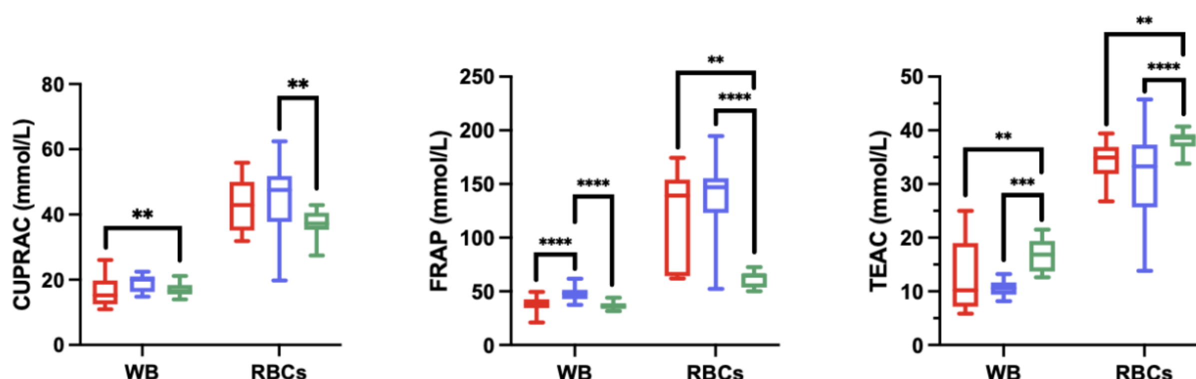

Figure 18. Results for the antioxidant biomarkers for whole blood (WB) and red blood cells (RBCs) lysates. Cupric reducing antioxidant capacity (CUPRAC); ferric reducing ability of plasma (FRAP) and Trolox equivalent antioxidant capacity (TEAC) in dogs with hypothyroidism (red box), non-thyroid disease (NTH) (blue box) and controls (green box). Asterisks indicate significant differences between groups. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$, $^{****}P \leq 0.0001$.

Results for thiol and PON-1 concentrations are shown in Figure 19. Thiol concentrations in WB were significantly lower in dogs with hypothyroidism compared to healthy dogs ($P \leq 0.001$). PON-1 values in RBC lysates were statistically significantly higher in dogs with hypothyroidism compared to the Control group ($P = 0.049$). Dogs with hypothyroidism showed statistically significantly higher PON-1 concentrations compared to dogs with non-thyroidal disease in both WB and RBC lysates ($P = 0.011$ and $P \leq 0.001$, respectively). These results could be associated with an increased lipid mobilization, as PON-1 is transported by low density lipoproteins (Navab et al., 1997)(Ozler et al., 2016) and thiol has been associated with lipid accumulation

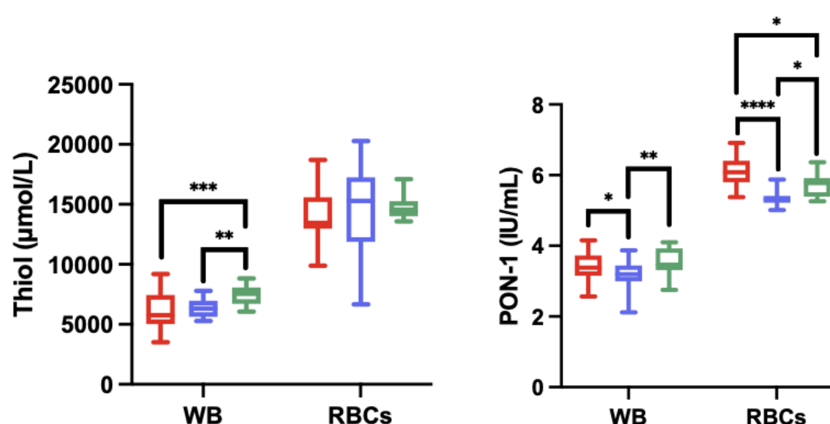

Figure 19. Results for antioxidant biomarkers for whole blood (WB) and red blood cells (RBCs) lysates. Thiol and paraoxonase type-1 (PON-1) in dogs with hypothyroidism (red box), non-thyroid disease (blue box) and controls (green box). Asterisks indicate significant differences between groups. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

- *Changes in the oxidant status*

Results for the biomarkers of the total oxidant status (TOS, POX-Act, d-ROMs) are shown in Figure 20. Dogs with hypothyroidism were found to have significantly lower WB concentrations of TOS ($P \leq 0.0001$ and $P = 0.009$, respectively) and POX-Act ($P \leq 0.0001$ and $P = 0.0005$, respectively) when compared to NTH dogs, while concentrations of d-ROMs were found to be significantly lower in RBCs lysates.

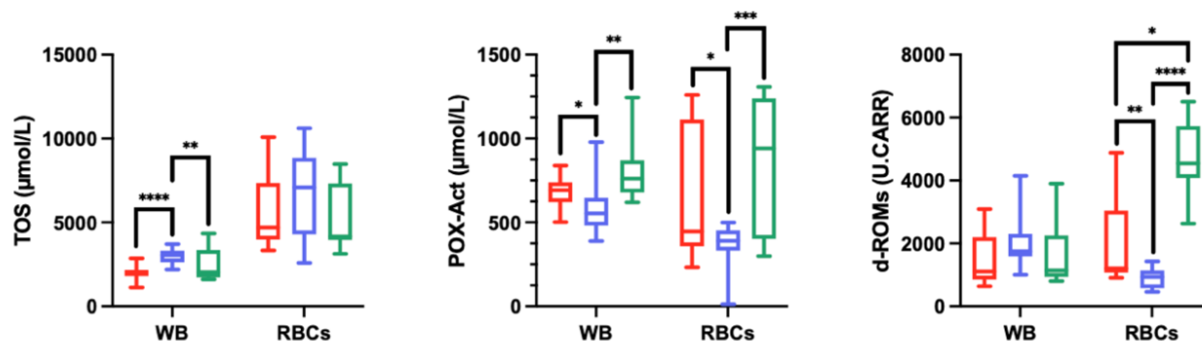

Figure 20. Results for oxidant biomarkers for whole blood (WB) and red blood cells (RBCs) lysates. Total oxidant status (TOS); peroxide-activity (POX-Act) and reactive oxygen-derived compounds (d-ROMs) in dogs with hypothyroidism (red box), non-thyroid disease (blue box) and controls (green box). Asterisks indicate significant differences between groups. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$

Results for the measurement of AOPP and TBARS are shown in Figure 21. Dogs with hypothyroidism showed a significantly higher AOPP and TBARS in WB ($P = 0.002$ and $P = 0.034$, respectively) when compared to healthy dogs. The increase in these two biomarkers are representative of the presence of both protein and lipid peroxidation, and could be a consequence of a lowered thyroid hormone action in the metabolic rate (Nanda et al., 2008; Torun et al., 2009).

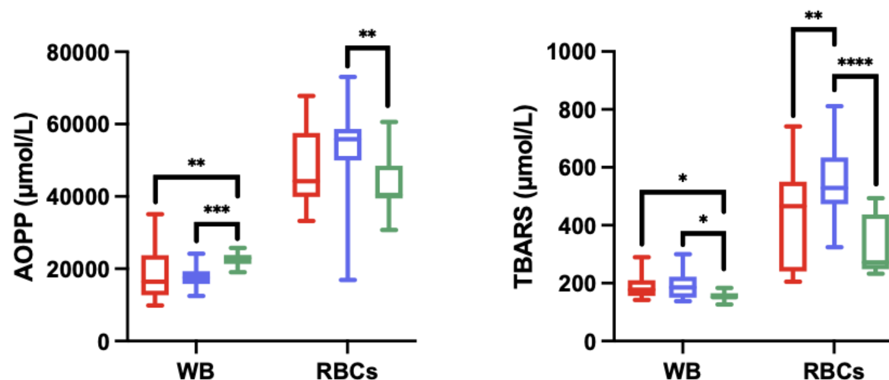

Figure 21. Results for oxidant biomarkers for whole blood (WB) and red blood cells (RBCs) lysates. Advanced oxidation protein products (AOPP) and thiobarbituric acid reactive substances (TBARS) in dogs with hypothyroidism (red box), non-thyroid disease (blue box) and controls (green box). Asterisks indicate significant differences between groups. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$.

Additionally, a correlation study was carried out between serum, WB, and RBC lysates. A negative correlation between serum and WB for TEAC and AOPP was found. Between serum and RBCs lysates a positive correlation for CUPRAC and PON-1 and a negative correlation for FRAP was found. On the other hand, a strong positive correlation for FRAP, PON-1, and TOS and a strong negative correlation for d-ROMs and AOPP were found between WB and RBC lysates.

Canine hypothyroidism produces changes in the redox status in these two novel sample types, and the changes are mainly associated with the effects of a lowered metabolic rate due to impaired thyroid hormone synthesis. Results were consistent in the two types of samples. However, most of the changes were observed in WB when compared with RBC lysates and serum, making this sample useful for future applications.

ARTICLES COMPENDIUM

ARTICLE 1

Proteomics in dogs; a systematic review

(Published)

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Journal: Research in Veterinary Sciences

Abstract: Proteomic analysis is having a rapid development as a method for the detection of biomarkers of diseases in dogs. Dogs in addition to their importance as companion animals, serve as important animal models for research. This study aims to systematically review evidence regarding the studies performed in proteomics in dogs, and spe- cifically those made in serum, saliva, urine and/or plasma. Information searched in October 2020, January 2021 and August 2021, for English language publications of the last decade (2010−2020) were obtained from elec- tronic databases. Screening, data extraction and risk of bias assessment were undertaken by two investigators. The risk of bias was evaluated using the Review Manager (RevMan 5) tool. Meta-analysis and case report studies were not included in this review.

Through the screening process a total of 557 publications were identified after the removal of duplicates. Out of these, 65 were fully evaluated and 44 of these were included in the review. Most studies evaluated the pro- teome of disease and compared it with a healthy population, and most of the articles included were made on serum, followed by saliva. The overall risk of bias for all studies was high, due to an absence in the generation of random sequence. Overall proteomic analysis has allowed the discovery of new physiopathological pathways of diseases and potential biomarkers in the dog, which are addressed in this review.

URL: https://doi.org/10.1016/j.rvsc.2021.12.026

ARTICLE 2

Changes of the salivary and serum proteome in canine hypothyroidism

(Published)

Luis Guillermo González-Arostegui[a], Alberto Muñoz-Prieto[a], Camila Peres Rubio[a], José Joaquín Cerón[a], Luis Bernal[a], Ivana Rubić[b], Vladimir Mrljak[b], Juan Carlos González-Sánchez[c], Asta Tvarijonaviciute[a]

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Journal: Domestic Animal Endocrinology

Abstract: In this study, changes in salivary and serum proteome of dogs with hypothyroidism were studied using tandem mass tags (TMT) labelling and liquid chromatography-mass spectrometry (LC-MS/MS). Saliva and serum pro- teome from 10 dogs with hypothyroidism were compared with 10 healthy dogs. In saliva, a total of seven proteins showed significant changes between the two groups, being six downregulated and one upregulated, meanwhile, in serum, a total of six proteins showed significant changes, being five downregulated and one upregulated. The altered proteins reflected metabolic and immunologic changes, as well as, skin and coagulation alterations, and these proteins were not affected by gender. One of the proteins that were downregulated in saliva, lactate dehydrognease (LDH), was measured by a spectrophotometric assay in saliva samples from 42 dogs with hypothyroidism, 42 dogs with non-thyroid diseases and 46 healthy dogs. The activity of LDH was lower in the saliva of hypothyroid dogs when compared to non-thyroid diseased dogs and healthy controls.

This study indicates that canine hypothyroidism can produce changes in the proteome of saliva and serum. These two sample types showed different variations in their proteins reflecting physiopathological changes that occur in this disease, mainly related to the immune system, metabolism, skin and coagulation. In addition, some of the proteins identified in this study, specially LDH in saliva, should be further explored as potential biomarkers of canine hypothyroidism.

URL: https://doi.org/10.1016/j.domaniend.2023.106825

ARTICLE 3

Untargeted metabolomic profiling of serum in dogs with hypothyroidism

(Published)

Alberto Muñoz-Prieto[a], Luis Guillermo González-Arostegui[b], Ivana Rubić[a], José Joaquín Cerón[b], Asta Tvarijonaviciute[b], Anita Horvatić[c], Vladimir Mrljak[a]

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Journal: Research in Veterinary Science

Abstract: Hypothyroidism is one of the most commonly diagnosed endocrine disease in dogs. The clinical signs are caused by a deficiency of the active thyroid hormones triiodothyronine (T3) and thyroxine (T4) and have a negative impact on dog's quality of life. We hypothesized that serum metabolic profile varies between healthy dogs and dogs with hypothyroidism. Twenty serum samples from dogs with hypothyroidism and 20 from healthy dogs were used for untargeted metabolomics analysis performed by LC/MS analysis. Fifteen metabolites showed significant changes between hypothyroid and healthy dogs, being the pentose phosphate pathway (PPP), aminoacyl-tRNA biosynthesis and pyrimidine metabolism the principal pathways altered in hypothyroidism. Specifically, metabolites such as D-gluconic acid and L-Isoleucine may potentially act as biomarkers of disease.

URL: https://doi.org/10.1016/j.rvsc.2021.01.023

ARTICLE 4

Changes in the salivary metabolome in canine hypothyroidism: A pilot study

(Published)

Luis Guillermo González-Arostegui[a], Camila Peres Rubio[a,b], Ivana Rubić[c], Renata Barić Rafaj[d], Jelena Gotić[e], José Joaquín Cerón[b], Asta Tvarijonaviciute[a], Vladimir Mrljak[c], Alberto Muñoz-Prieto[a,c]

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Journal: Research in Veterinary Science

Abstract: Hypothyroidism is the most commonly diagnosed endocrine disorder in dogs. It produces a deficiency of thyroid hormones which impacts negatively the dog's quality of life. The objective of this study is to evaluate the possible changes in the salivary metabolic profile in dogs with hypothyroidism. For this purpose, targeted metabolomics analysis performed by LC/MS analysis was made in saliva samples from a group of dogs with hypothyroidism and a group of healthy dogs. Twenty-three metabolites showed a significant decrease between hypothyroid and healthy dogs, most of these associated with thyroid hormone synthesis, catecholamine synthesis, and tyrosine and phenylalanine metabolism. Based on the results, it can be stated that hypothyroidism produces changes in the metabolome of saliva and some of them can reflect the metabolic changes presented in the disease and could serve as a potential biomarker of this condition.

URL: https://doi.org/10.1016/j.rvsc.2022.08.012

ARTICLE 5

Measurement of redox biomarkers in the whole blood and red blood cell lysates of dogs

(Published)

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Journal: Antioxidants

Abstract: The evaluation of the biomarkers of oxidative status is usually performed in serum, however, other samples, such as red blood cells (RBCs) lysates or whole blood (WB), can be used. The objective of this study was to evaluate if a comprehensive panel of redox biomarkers can be measured in the WB and RBCs of dogs, and their possible changes "in vitro" after the addition of different concentrations of ascorbic acid. The panel was integrated by biomarkers of the antioxidant status, such as cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), thiol and paraoxonase type 1 (PON-1), and of the oxidant status, such as total oxidant status (TOS), peroxide-activity (POX-Act), reactive oxygen- derived compounds (d-ROMs), advanced oxidation protein products (AOPP) and thiobarbituric acid reactive substances (TBARS). All the assays were precise and accurate in WB and RBCs lysates. In addition, they showed changes after ascorbic acid addition that are in line with previously published results, being WB more sensitive to detect these changes in our experimental conditions. In conclusion, the panel of assays used in this study can be measured in the WB and RBCs of the dog. In particular, the higher sensitivity to detect changes in our experimental conditions and its easier sample preparation makes WB a promising sample for the evaluation of redox status in dogs, with also potential applications to other animal species and humans.

URL: https://doi.org/10.3390/antiox11020424

ARTICLE 6

Changes in biomarkers of redox status in serum and saliva of dogs with hypothyroidism

(Published)

Luis Guillermo González-Arostegui[a], Alberto Muñoz-Prieto[b], Luis Pardo Marín[a], Gregorio García López[a], Asta Tvarijonaviciute[a], José Joaquín Cerón[a], Camila Peres Rubio[c]

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Journal: BMC Veterinary Research

Abstract: Hypothyroidism is the most common endocrine disorder diagnosed in dogs, leading to deleterious effects on a dog's life quality. This study aims to evaluate changes in the redox status in canine hypothyroidism. For this purpose, a comprehensive panel of antioxidants and oxidants biomarkers were measured in serum and saliva of 23 dogs with hypothyroidism, 21 dogs with non-thyroidal illness, and 16 healthy dogs. Among the antioxidants, cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), thiol, paraoxonase type 1 (PON-1) and glutathione peroxidase (GPx) were determined in serum and CUPRAC, ferric reducing ability of saliva (FRAS) and TEAC in saliva. The oxidant biomarkers included were total oxidant status (TOS), peroxide-activity (POX-Act), reactive oxygen-derived compounds (d-ROMs), advanced oxidation protein products (AOPP), and thiobarbituric acid reactive substances (TBARS) in serum and AOPP and TBARS in saliva. Results showed a significantly higher TEAC, PON-1, GPx, TOS, POX-Act, and d-ROMs, and a significantly lower AOPP in serum of dogs with hypothyroidism. Meanwhile, significantly lower FRAS and AOPP were observed in saliva of dogs with hypothyroidism. Once salivary concentrations were corrected based on their total protein con- centrations, the only analyte showing significant changes was TBARS which was significantly higher in dogs with hypothyroidism. Our results show that dogs with hypothyroidism present alterations in the redox status in both serum and saliva. This study should be considered a preliminary study and further research addressing these changes should be made using larger populations.

URL: https://doi.org/10.1186/s12917-023-03586-4

CONCLUSIONS

The conclusions of this study were:

1. In the review of proteomic studies published in dogs, it was found that several sample types, especially serum and saliva, have been used to evaluate the proteome in this species. The use of proteomic analyses to evaluate diseases can lead to the detection of new biomarkers and to clarify or discover new physiopathological pathways, especially in the field of endocrine disorders.
2. Hypothyroidism induced changes in the serum and salivary proteome of dogs. These changes reflected a lower metabolic rate, a situation of oxidative stress, and immunologic and dermatologic alterations in saliva; meanwhile, changes in serum could reflect reduced protein synthesis, coagulation, and hypercholesterolemia. In particular, LDH activity, which can be easily measured by spectrophotometric assays, was reduced in the saliva of dogs with hypothyroidism when compared with dogs with other different diseases and healthy dogs and serves as a new potential biomarker of canine hypothyroidism.
3. Hypothyroidism induces changes in the serum and salivary metabolome of dogs. Salivary changes are directly associated with thyroid hormone synthesis and impaired lipid metabolism. Some of the identified metabolites, like tyrosine and phenylalanine, could serve as potential biomarkers of canine hypothyroidism. Serum changes are associated with the pentose phosphate pathway, aminoacyl-tRNA biosynthesis acid, and pyrimidine metabolism. Some of the identified metabolites, like D-gluconic acid and L-isoleucine, could be potential biomarkers of canine hypothyroidism.
4. Antioxidants and oxidants can be measured in WB and RBC lysates in dogs. Dogs with hypothyroidism showed a decrease in CUPRAC, TEAC, and thiol, and an increase in AOPP and TBARS in WB. They also have a decrease in TEAC and d-ROMs and an increase in PON-1 in RBC lysates. In addition, hypothyroidism induces changes in the redox state of saliva, with decreased FRAS and GPX. These data indicate that hypothyroidism can produce changes in oxidative status biomarkers, which can be assessed using different samples.

RESUMEN

1. Introducción y Objetivos

El hipotiroidismo canino es una de las enfermedades endocrinas más comunes en el perro, con una prevalencia de 0,8 a 2,7% de los casos presentados en clínicas de pequeños animales (Dixon and Reid, 1999; Ziener et al., 2015). Esta enfermedad se caracteriza por una alteración en la producción de hormonas tiroideas, principalmente triyodotironina (T3) y tiroxina (T4), provocando aumento de peso, letargia y afectando negativamente a la calidad de vida de los perros (Jaiswal et al., 2018). El diagnóstico del hipotiroidismo se limita mayormente a la determinación de T4 y de la hormona estimulante de tiroides (TSH) en suero, no obstante, los resultados de sus determinaciones pueden ser confusas, ya que estas hormonas pueden fluctuar, complicando el diagnóstico de la enfermedad (Daminet and Ferguson, 2003; E. Feldman et al., 2014; Jaiswal et al., 2018).

Uno de los aspectos mas importantes en el diagnóstico y monitorización de los tratamientos de las enfermedades es el uso de biomarcadores, los cuales sirven como compuestos claves para la evaluación de cualquier enfermedad. La identificación de estos biomarcadores se ve potenciado suele llevarse a cabo por medio del uso de técnicas ómicas, como son los análisis proteómicos y los análisis metabolómicos dirigidos y no dirigidos.

El proteoma se define como el conjunto de proteínas que se encuentran en una muestra en un tiempo concreto, este siendo sujeto a constante cambio debido a estímulos externos o internos (Aslam et al., 2017). Los análisis proteómicos han sido aplicados en perros usando diferentes tipos de muestras, como son el suero (Yang et al., 2015), la saliva (Lucena et al., 2019) y la orina (Hormaeche et al., 2014), entre otros. A través de estos análisis, una gran variedad de biomarcadores ha sido identificado en varias enfermedades, como por ejemplo, las apolipoproteínas como biomarcadores de obesidad (Escribano et al., 2016), leishmaniosis (Escribano et al., 2016) y babesiosis (Kuleš et al., 2014), la antitrombina-III como biomarcador de la enfermedad valvular crónica (Kuleš et al., 2020a), y la proteína unida a retinol (Nabity et al., 2011), así como muchos otros.

Mientras que la base de los análisis proteómicos es la identificación de proteínas, los análisis metabolómicos permiten identificar la huella metabólica que se encuentra en una muestra en un tiempo concreto, esta huella es sensible a los cambios fisiopatológicos, del mismo modo que el proteoma (Emwas, 2015). Los análisis metabolómicos pueden ser dirigidos o no dirigidos, la técnica dirigida es usada para evaluar la presencia de un conjunto

de metabolitos definidos, mientras que, la técnica no dirigida es usada para estudiar todos los metabolitos presentes en una muestra (Roberts et al., 2012; Souza and Patti, 2021). La detección de esta huella metabólica ayuda a la identificación de biomarcadores, además de también ofrecer un mayor entendimiento de la patogénesis de una enfermedad y el descubrimiento de nuevas rutas de tratamiento (Clish, 2015). La huella metabólica en los perros no ha sido estudiada en tanta profundidad como el proteoma, algunos estudios han sido producidos conduciendo a la identificación de distintos biomarcadores de enfermedad. Un estudio identificó la fenilalanina y el lactato en suero como posible biomarcadores de la enteropatía crónica (Walker et al., 2022), mientras que otro estudio identificó la tirosina y los aminoácidos de cadena ramificada en suero como posibles biomarcadores de shunt portosistémico congénito (Imbery et al., 2022), demostrando el potencial de esta técnica en esta especie.

En los últimos años los biomarcadores del estado redox han ganado importancia. El estado redox se define como el balance entre la producción de especies reactivas de oxígeno (ROS) que actúan como oxidantes y el sistema de defensa antioxidante (Halliwell, 2007; Liu et al., 2023). Un aumento en la producción de ROS conduce a la hipoxia celular, al daño en el ADN y como consecuencia, la muerte celular, contribuyendo al desarrollo de patologías (Çay, 2017; Poljšak and Milisav, 2012). La evaluación del estado redox ayuda al entendimiento de los mecanismos fisiopatológicos de las enfermedades, detectando biomarcadores específico que pueden ser útil para el diagnóstico de una enfermedad o para ayudar a la monitorización de un tratamiento (Forman and Zhang, 2021; Sies, 1991). Para evaluar el estado redox, los compuestos antioxidantes y oxidantes pueden ser evaluados en distintas muestras, como suero, saliva, sangre entera (WB) y lisados eritrocitarios (RBCs), lo que ofrece un amplio campo de estudio. En perros, los biomarcadores del estado redox han sido evaluados en distintas enfermedades, como el superóxido dismutasa (SOD) en leishmaniosis visceral (Britti et al., 2008) y sarna sacróptica (Singh et al., 2011), la paraoxonase tipo-1 (PON-1) en entertitis parvoviral (Kocaturk et al., 2015), el glutatión peroxidasa (GPx) en perros infectados con *Dirofilaria immitis* (Dimri et al., 2012), entre otros.

Esta tesis se enfocará en proveer avances en el descubrimiento y la evaluación de biomarcadores de hipotiroidismo canino por medio de varios métodos:

- El uso de análisis proteómicos en diferentes tipos de muestras.
- El uso de análisis metabolómicos en diferentes tipos de muestras.
- La evaluación del estado redox en diferentes tipos de muestras.

En base a los datos indicados en la introducción, los **objetivos** de esta tesis doctoral fueron los siguientes:

1. Realizar una revisión sistemática sobre la literatura disponible existente sobre los biomarcadores identificados en perros por medio de análisis proteómicos(**Artículo 1**).

2. Realizar un análisis proteómico usando suero y saliva de perros hipotiroideos y sanos, para identificar posibles biomarcadores de hipotiroidismo canino (**Artículo 2**).

3. Realizar análisis metabolómicos usando suero y saliva de perros hipotiroideos y sanos para identificar posibles biomarcadores de hipotiroidismo canino (**Artículo 3 y 4**).

4. Validar la determinación de antioxidantes y oxidantes en dos tipos de muestra novedosos, como son; la sangre entera (WB) y lisados eritrocitarios (RBCs) (**Artículo 5**).

5. Evaluar el estado redox en suero, saliva, WB y lisados RBCs de perros con hipotiroidismo, comparándolo con el estado redox de perros con enfermedades no tiroideas (NTH) y perros sanos (**Artículo 6 y Anexo 1**)

2. Materiales y Métodos

2.1 Ético biomédica.

Todos los experimentos desarrollados durante esta Tesis doctoral fueron aprobados por el Comité de Bioética (Comité Ético de Experimentación Animal, CEEA), bajo el número de protocolo 288/2017 (especies caninas) y por el comité de ética de la Universidad de Murcia y el Ministerio de Agricultura, Ganadería, Pesca y Acuicultura de la Región de Murcia, bajo el número de protocolo A13170503. Los experimentos fueron desarrollados con las guías del ARRIVE (Animal Research: Reporting of In Vivo Experiments). Además, el consentimiento de los propietarios fue obtenido para la participación de todos los animales.

2.2 Obtención de muestras.

La obtención de saliva se realizó introduciendo una esponja de tamaño pequeño en la boca de cada perro, hasta que la esponja estuviera suficientemente húmeda (Parra et al., 2005). Posteriormente, se colocaron en tubos de recolección (Salivette, Sarstedt, Aktiengesellshacft & Co, Nümbrecht, Alemania) y se almacenaron en hielo hasta la llegada al laboratorio, donde fueron centrifugadas a 3000 g durante 20 minutos a 4ºC. Las muestras de saliva obtenidas fueron transferidas a un tubo Eppendorf® (Daslab, Barcelona, España) de 1.5 mL y se almacenaron a -80ºC hasta el análisis.

Las muestras de suero y WB se obtuvieron mediante venopunción yugular. La sangre fue colocada en tubos con EDTA y en tubos con gel activador de la coagulación (TapVal, Aquisel, Selecta, Barcelona, España) para las muestras de WB y suero. Los tubos de EDTA se dividieron en dos alícuotas distintas para la preparación de WB y lisados RBCs. Para obtener la WB, una alícuota fue almacenada a -80ºC dos horas antes del análisis. Para obtener los lisados RBCs, la alícuota restante fue centrifugada a 3000 rpm durante 10 minutos a 4ºC para poder descartar el plasma y la capa blanca. Posteriormente, se procedió al lavado de los glóbulos rojos con solución salina isotónica (NaCl 0.9%) y al centrifugado con las especificaciones mencionadas anteriormente. Luego, se descartó el sobrenadante y se repitió este proceso para un total de cuatro lavados, finalmente los glóbulos rojos fueron reconstituidos usando agua ultrapura a una dilución de 1:4 y los lisados se almacenaron a -80ºC hasta el análisis. Los tubos de suero se dejaron a temperatura ambiente (22-24ºC) por unos 10 a 20 minutos para que coagulara, luego los tubos se centrifugaron a 3500 g durante 5 minutos a temperatura ambiente, el suero obtenido fue transferido a tubos Eppendorf® de 1,5 mL y almacenados a -80ºC hasta el análisis.

2.3 Proteómica Líquida.

Para cada muestra, 35 µg de proteínas precipitadas en acetona fueron sujetas a reducción, alquilación y digestión y fueron etiquetadas usando reactivos etiquetado de masa tándem (TMT) 10-plex (Thermo Scientific, Rockford, IL, USA). La concentración total de proteínas en saliva fue determinada por medio el ensayo BCA (Thermo Scientific, Rockford, USA). Se generó una muestra compuesta de la mezcla de concentraciones iguales de proteínas de las 20 muestras como estándar interno en los experimentos. De forma resumida, 35 µg de las muestras y el estándar interno fueron reducidas con 200 mM

de DTT (Sigma-Aldrich, St. Louis, MO, USA), alquiladas con 375 mM de iodoacetamida (Sigma-Aldrich, St. Louis, Mo, USA) y precipitadas con acetona fría (VWR, Radnor, PA, USA) durante la noche. Las muestras fueron centrifugadas y la acetona fue descartada. Los pellets fueron resuspendidos en una solución de 50 µL de 100mM de bicarbonato trietilamonio (TEAB, Thermo Scientific, Rockford, USA) y digeridos con tripsina (Promega) durante la noche a 37ºC. Los reactivos TMT 10-plez fueron equilibrados a temperatura ambiente, y resuspendidos en acetonitrilo anhidro (LC-MS grade, Thermo Scientific, USA) y agregados a cada muestra. La reacción de etiquetado fue incubada durante una hora a temperatura ambiente y detenida agregando hidroxilamina al 5% (Sigma-Aldrich, St. Louis, MO, USA) durante 15 minutos. Las muestras fueron mezcladas en cantidades iguales y 5 µg de cada conjunto de muestras fue almacenada a -80ºC hasta el análisis. El análisis de cromatografía líquida y espectrometría de masas tándem (LC-MS/MS) fue realizado usando el dispositivo Ultimate 3000.

El sistema de flujo RSLCnano (Dionez, Germering, Alemania) fue acoplado a espectrofotómetro de masas Q Exactive Pluss (Thermo Fisher Scientific, Bremen, Alemania). Los péptidos de disolvieron en una solución de carga, y colocados en una columna trampa, desalinizados durante 12 minutos a una tasa flujo de 15 µL/min y separadas en una columna analítica (PepMap™ RSLC C18, 50 cm x 75 µm) usando un gradiente linear en fase móvil B de 5-45% (ácido fórmico 0,1% en ACN 80%) durante 120 minutos, a un 45 a 90% durante dos minutos, sostenido a 80% durante dos minutos y reequilibrado a 5% B durante 20 minutos a una tasa de flujo de 300 nL/min. La fase móvil A consistió en ácido fórmico 0,1% en agua. La ionización fue producida usando una fuente de ion nanospray Flex (Thermo Fisher Scientific, Bremen, Alemania). La MS fue operada en modo de ion positivo usando el método DDA Top8.

Las proteínas se identificaron usando Proteome Discoverer (versión 2.3., Thermo Fisher Scientific), buscando en la base de datos de proteínas caninas (descargado desde la base de datos Uniprot el 4 de Abril de 2019, 172083 secuencias). Los siguientes parámetros fueron aplicados: 2 sitios de anclajes faltantes de tripsina, tolerancias de fragmentos y precursores de 100 ppm y 0.02 Da, respectivamente, modificación fija del péptido carbamidometil (C), oxidación (M), y modificaciones dinámicas de TMT 6-plex (K, péptido N-terminus). La tasa de descubrimiento falso (FDR) para la identificación de proteínas fue calculada usando el algoritmo Percolator en el programa Proteome Discoverer

según los resultados de la búsqueda contra la base de datos, y fue ajustado a un FDR de un 1%. Para el reporte adecuado de las proteínas identificadas, al menos dos péptidos únicos y un FDR de 5% es requerido. La cuantificación de proteínas fue obtenida por medio de la correlación de las intensidades relativas de iones extraídos del espectro de masa tándem a la de los péptidos elegidos para la fragmentación espectrometría de masas tándem (MS/MS). Para la comparación de la cuantificación relativa de los resultados entre los experimentos se utilizo el estándar interno.

2.4 Metabolómica.

- *Metabolómica no dirigida*

Un volumen de 25 µL de cada muestra de suero fue mezclada con 1000 µL de solvente de extracción frío (CHCl3:CH3OH:H2) = 1:3:1) para precipitar las proteínas. 10 µL de cada muestra de suero fueron agrupadas y 25 µL de las muestras agrupadas fueron mezcladas con 1000 µL de solvente de extracción frío. Todas las muestras fueron pasadas por el vortex y centrifugadas a 13000 g durante 5 min a 4ºC. El sobrenadante (200 µL) fue transferido a un vial con rosca y almacenado a -80ºC hasta el análisis.

Los metabolitos fueron analizados usando un espectrofotómetro de masas Thermo Q-Exactive Orbitrap acoplado a un sistema UltiMate 3000 RSLC (Dionex, Germering, Alemania). La separación cromatográfica fue realizada usando una columna ZICpHILIC (150 x 4.6 mm, 5 µm, Merck Sequant, Alemania). La columna fue mantenida a 25ºC y las muestras fueron eluídas usando un gradiente linear (A = 20 mM de carbonato de amonio en agua, B = acetonitrilo). Durante 27 min, los primeros 15 minutos el gradiente cambió de 80% de A y 20% de B, a 95% de A y 5% de B. En el minuto 17, el gradiente regresó a las condiciones iniciales. El Thermo Q-Exactive Orbitrap fue operado en modos positivo y negativo a una resolución de masas de 70.000 con rango m/z de 70-1050. El análisis MS para la ionización positiva en electrospray fue realizada usando una fuente de voltaje de +3.8 kV, gas envolvente 40, gas auxiliar de 5 y temperatura capilar de 320 ^{O}C. Los ajustes del MS para ionización negativa en electrospray fueron los mismos, con una fuente de voltaje de 3.8 kV. Las muestras de control de calidad fueron una mezcla de los metabolitos provistos por Glasgow Polyomics, UK y usados para evaluar la calidad de la separación cromatográfica. Para la detección de metabolitos, un mix estándar (Glasgow Polyomics, UK), el cual contiene cerca de 200 metabolitos, fue usados. Las muestras agrupadas fueron usadas para evaluar la reproducibilidad de la señal.

- *Metabolómica dirigida*

Las muestras fueron preparadas usando el kit AbsoluteIDO p400 HR (Bio-crates Life Sciences AG, Innsbruck, Austria). Un volumen de 10 µL de cada muestra de saliva, controles y PBS fueron agregados a la placa con la mezcla de estándar interno. La placa fue secada durante 30 minutos en un colector de vacío. Las muestras fueron derivatizadas usando 50 µL de 5% de solución de derivatización en agua durante 20 minutos y luego secadas durante 20 minutos usando un colector de vacío. Los metabolitos fueron extraídos usando 300 µL de solución de 5 mM de acetato de amonio en metanol por medio de agitación durante 30 min a 450 rpm, y luego eluídos usando un colector de vacío. Un total de 150 µL de los extractos fueron transferidos de la placa de captura a una placa vacía y diluidos con agua (50:50, v/v) para el análisis LC-MS. La plata original fue diluida con 250 µL de la fase móvil FIA para el análisis de espectrofotometría de masas con inyección en flujo (FIA-MS). Ambas placas fueron protegidas con cobertores de silicona y agitados durante 5 min a 500 rpm antes del análisis.

Los metabolitos fueron medidos usando una cromatografía líquida de alto rendimiento en el Dionex Ultimate 3000 acoplado a un espectrofotómetro de masas Thermo Scientific Q Exactive Orbitrap con el instrumento de LC-MS y los parámetros provistos con el kit AbsoluteIDO p400 HR. El ensayo cuantifica un total de 408 metabolitos de 11 clases de compuestos, lo que permitió la medición de 21 aminoacidos, 21 aminas biogénicas, 1 monosacárido (incluida la glucosa), 172 fosfatidilcolinas, 24 lisofosfatidilcolinas, 31 esfinomielinas, 9 ceramidas, 55 acilcarnitinas, 41 esteres de colesterol, 18 diglicéridos y 42 triglicéridos. Para la cuantificación, los estándares de calibración en 7 niveles de concentración y los estándares internos fueron usados confirmando el pico correcto de retención de tiempo y el área del pico de integración. Por otro lado, el análisis FIA-MS usó un calibrador de punto único con los estándares internos. Los estándares internos fueron integrados parcialmente a los filtros de la placa del Kit. Un total de 3 muestras replicadas de PBS como blanco fueron usadas para calcular el límite mas bajo de detección (LOD). El rendimiento analítico fue validado usando los controles de calidad provistos por el fabricante. Los metabolitos fueron identificados y cuantificados usando un flujo de trabajo estándar con el software MetIDO™ (Biocrates Life Sciences AG, Innsbruck, Austria).

2.5 Biomarcadores Redox

Un panel de biomarcadores del estado redox fue evaluado en todas las muestras obtenidas, estos fueron analizados usando el analizador Olumpus AU400 Automatic Chemistry Analizer (Olumpus Europe GmbH), a menos que se diga lo contrario.

Los biomarcadores antioxidantes y oxidantes fueron medidos siguiendo protocolos previamente descritos para su medición en suero. Las mediciones de TBARS se realizaron usando un lector de placas (Powerwave XS, Biotek Instruments). Los resultados de la capacidad antioxidante reductora del ión de cobre (CUPRAC), habilidad reductora férrica del plasma y saliva (FRAP/FAS) y la capacidad antioxidante equivalente en Trolox (TEAC) fueron expresados en mmol/L, los del tiol, estado oxidante total (TOS), la actividad de peróxido (POX-Act), los productos avanzados de la peroxidación proteica (AOPP) y las sustancias reactivas de ácido tiobarbitúrico (TBARS) en µmol/L, los de PON-1 y GPx en IU/mL, y los compuestos derivados de oxígeno reactivo (d-ROMs) en U.CARR.

2.6 Validación Analítica de los Ensayos.

La validación analítica los ensayos validados en esta tesis fueron calculados por medio de la precisión, exactitud y sensibilidad, siguiendo un protocolo previamente descrito (Tiwari and Tiwari, 2010) que ha sido aplicado en otras validaciones analíticas en perros (Rubio et al., 2016a; Asta Tvarijonaviciute et al., 2012).

La precisión fue calculada a través de la evaluación de la variación intra e inter-ensayo, esta fue expresada como coeficiente de variación (CV). La exactitud fue evaluada por medio de la linealidad bajo dilución y de la recuperación de picos. La precisión se determinó por medio del LOD y límite mas bajo de cuantificación (LLOQ). El LOD para cada método se calculo tras la determinación de 20 muestras de agua ultra pura y/o buffer Fosfato pH 7.5. El LLOQ se definió como la concentración cuantificada mas baja de cada método con un CV menor del 20%.

3. Compendio de Artículos

Los objetivos planteados en esta tesis se abordaron mediante la realización de una revisión sistemática y 6 experimentos incluidos en 5 artículos publicados en revistas de prestigio internacional y 1 experimento presentado en el anexo, el cual ha sido enviado para su posible publicación. Los artículos incluidos en la presente tesis doctoral fueron los siguientes.

3.1 Artículo 1. Proteomics in dogs; a systematic review.

El uso de los análisis proteómicos ha tenido un desarrollo de gran interés como método útil para la detección de biomarcadores en enfermedades de perros, ya que estos, además de servir como animales de compañía, tienen gran utilidad como modelos animales para la investigación. El objetivo de este estudio es el de revisar sistemáticamente la evidencia científica existente sobre el uso de los análisis proteómicos en perros, especialmente aquellos desarrollados en suero, saliva, orina y/o plasma.

Para esto, se desarrolló una búsqueda bibliográfica de artículos publicados en inglés durante la última década (2010-2020), usando bases de datos electrónicas, evaluando también el riesgo de seso existente. A través de un proceso de selección, un total de 557 publicaciones fueron identificadas tras eliminar duplicados, de estos, 65 fueron evaluados completamente y 44 de estos artículos fueron incluidos en la revisión.

Dentro de los estudios incluidos, se observó que la mayoría de estos son desarrollados con la finalidad de evaluar cambios en el proteoma por enfermedades, usando suero, seguido de saliva. El grupo de enfermedades mas estudiadas por medio de estos análisis fueron las enfermedades de tipo infecciosas, permitiendo el descubrimiento de nuevos posibles biomarcadores que permitan entender nuevos mecanismos fisiopatológicos de las enfermedades y favorecer a la elaboración de nuevas técnicas de diagnóstico.

3.2 Artículo 2. Changes of the salivary and serum proteome in canine hypothyroidism.

En este estudio, los cambios en el proteoma salival y sérico de perros con hipotiroidismo fueron estudiados usando etiquetado TMT y LC-MS/MS. La saliva y el suero de 10 perros con hipotiroidismo fueron comparados con 10 perros sanos. En la saliva,

un total de 8 proteínas mostraron cambios significativos entre los grupos, 7 de estas mostrando valores disminuidos y una mostrando valores aumentados en los perros con hipotiroidismo, mientras que, en suero, un total de 7 proteínas mostraron cambios significativos, 6 de estas mostrando valores disminuidos y una mostrando valores aumentados en los perros con hipotiroidismo.

Las proteínas alteradas en los perros con hipotiroidismo reflejaron los cambios asociados al metabolismo y los cambios inmunológicos, así como las alteraciones en la piel y en el sistema de coagulación, las proteínas identificadas no se vieron afectadas por el sexo de los perros. Una de las proteínas que mostró valores disminuidos en saliva, la LDH, fue validada y medida por medio de un ensayo espectrofotométrico en muestras de saliva de 42 perros con hipotiroidismo, 42 perros con NTH y 46 perros sanos. La actividad de la LDH presento concentraciones mas bajas en la saliva de los perros hipotiroideos en comparación a los perros con NTH y perros sanos.

Por lo que se concluyó que el hipotiroidismo canino produce cambios en el proteoma de la saliva y del suero, reflejando cambios fisiopatológicos asociados al sistema inmune, al metabolismo, la piel y la coagulación. Además, el uso de la LDH en saliva podría ser considerado como un posible biomarcador de hipotiroidismo canino.

3.3 Artículo 3. Untargeted metabolomic profiling of serum in dogs with hypothyroidism.

El objetivo de este estudio fue el de evaluar el metaboloma del suero de perros con hipotiroidismo y compararlo con el de perros sanos, utilizando un análisis metabolómico no dirigido. Para esto, se tomó el suero de 20 perros con hipotiroidismo y 20 perros sanos. Los resultados mostraron cambios significativos en 15 metabolitos entre perros hipotiroideos y sanos.

Los cambios en los metabolitos identificados se ven asociados a la vía pentosa fosfato, a la biosíntesis del aminoacil-tARN y al metabolismo de la pirimidina. Además de esto, algunos metabolitos como el ácido D-glucónico y la L-isoleucina mostraron cambios significativos, resaltándose como posibles biomarcadores de la enfermedad. El ácido D-glucónico podría estar asociado con la dislipidemia que típicamente ocurre en pacientes con hipotiroidismo, mientras que la L-isoleucina podría asociarse con la falta de hormonas tiroideas y sus efectos en el consumo celular de aminoácidos.

Por lo que se concluyó que en el hipotiroidismo canino ocurren cambios metabólicos que conducen al cambio del metaboloma sérico, presentándose alteraciones que van asociadas al metabolismo y la biosíntesis de compuestos, así como a las alteraciones en el perfil lipídico en la enfermedad.

3.4 Artículo 4. Changes in the salivary metabolome in canine hypotbyroidism: A pilot study.

En este estudio se tomaron muestras de saliva de 10 perros con hipotiroidismo y 10 perros sanos, con la finalidad de evaluar los posibles cambios que puede provocar la enfermedad en el metaboloma salival. Para esto, las muestras recolectadas fueron evaluadas a través del uso de un análisis metabolómico dirigido.

Los resultados del análisis metabolómico dirigido permitieron la identificación de 88 metabolitos diferentes en ambos grupos. De estos 88, 23 fueron encontrados con valores disminuidos en los perros hipotiroideos en comparación a los perros sanos, incluyendo 7 aminoácidos, 1 triglicérido, 2 esteres de colesterol y 12 fosfatidilcolinas.

El metaboloma de los perros con hipotiroidismo demostró alteraciones en varias vías metabólicas. Entre estas, la vía del metabolismo de la histidina, la cual esta representada por la histamina, un mediador proinflamatorio que requiere del metabolismo de la hormona T3 para poder ser liberada a través de las células (Csaba and Pállinger, 2009; Landucci et al., 2019; Thangam et al., 2018). Por otro lado, la vía metabólica para la síntesis de hormonas tiroideas, así como la vía metabólica de la tirosina se vieron alteradas, estas como consecuencia a la posible reducción de la tirosina, la cual es un substrato para síntesis de hormonas tiroideas (Laidlaw and Kopple, 1987; Young et al., 1978). Además, se observó una reducción en 3 metabolitos asociados a a lipoproteínas, reflejando el impacto que tiene el hipotiroidismo en el metabolismo de lípidos.

Por lo que se concluyó que el hipotiroidismo canino produce cambios en el metaboloma salival de los perros, conduciendo a alteraciones metabólicas que caracterizan el desarrollo de la enfermedad y que pueden representar nuevas alternativas como posibles biomarcadores de enfermedad.

3.5 Artículo 5. Analytical validation of redox biomarkers in whole blood and red blood cell lysates

El estado redox es de gran importancia para la vida, ya que este se encuentra implicado en la mayoría de los procesos biológicos (Sies et al., 2017). Normalmente el estado redox es evaluado en suero, no obstante, el uso de otras muestras para su medición permite abrir nuevos campos de investigación, por esta razón el objetivo fue el de validar analíticamente la medición de un amplio panel de antioxidantes y oxidantes en WB y lisado RBCs. El panel incluyó 5 antioxidantes (CUPRAC, FRAP, TEAC, tiol y PON-1) y 5 oxidantes (TOS, POX-Act, d-ROMs, AOPP y TBARS).

La validación de los métodos en WB mostró una CV intra e inter-ensayo de 0.01% a 15.89% y 0.01% a 13.62%, respectivamente. La dilución seriada de WB resultó en una regresión lineal mayor a 0.96. La recuperación en todos los métodos se mostró entre 92.77% y 122.49%. Para los lisados RBCs, la precisión intra e inter-ensayo presentó un CV de 1.02% a 15.98% y 0.58% a 12.6%, respectivamente. La dilución seriada de lisados resultó en una regresión lineal mayor a 0.96. La recuperación en todos los métodos se mostró entre 87.5% y 113.9%.

Posterior a la validación analítica se desarrolló un estudio *in vitro* en el que las muestras de WB y lisados RBCs de 7 perros de raza Beagle se sometieron a 2 concentraciones distintas de ácido ascórbico y a 1 tratamiento control con solución salina (NaCl 0.9%). A través de este estudio se evidenció la sensibilidad que presentan ambos tipos de muestra al ser expuestos a distintas concentraciones de ácido ascórbico, y el potencial que tienen estas 2 muestras para la evaluación del estado redox y el estudio de biomarcadores de estrés oxidativo.

3.6 Artículo 6. Changes of biomarkers of redox status in serum and saliva of dogs with hypothyroidism

Las hormonas tiroideas tienen un efecto importante en el estado redox, ya que estas sirven como limpiadores enzimáticos y no enzimáticos de radicales libres, promoviendo también reacción anabólicas y catabólicas que pueden conducir al consumo de oxígeno y regular la síntesis de ROS (Chainy and Sahoo, 2020; Venditti et al., 2003; Villanueva et al., 2013). Es por esto, que el objetivo de este estudio fue el de evaluar el estado redox en perros con hipotiroidismo, usando un panel compuesto por 6 antioxidantes (CUPRAC, FRAP, TEAC, tiol, PON-1 y GPx) y por 5 oxidantes (TOS, POX-Act, d-ROMs, AOPP y TBARS), los cuales fueron medidos en suero, mientras que en saliva se midieron 3

antioxidantes (CUPRAC, FRAS y TEAC) y 2 oxidantes (AOPP y TBARS). Estos fueron comparados con el estado redox de perros con NTH y perros sanos.

Los resultados en suero presentaron un aumento significativo en las concentraciones TEAC y PON-1 en perros hipotiroideos en comparación a los otros grupos. Del mismo modo, se observó un aumento en las concentraciones de TOS en perros hipotiroideos en comparación a los otros 2 grupos, y de POX-Act y d-ROMS en perros hipotiroideos en comparación a los perros sanos.

Los resultados en saliva mostraron un descenso significativo en las concentraciones de FRAS antes de ser corregido por el valor de proteína en saliva, en perros hipotiroideos en comparación a los perros sanos, no obstante, una vez estos valores fueron corregidos, no se observaron diferencias significativas. Estos resultados también se vieron tras la medición del AOPP, los cuales mostraron un descenso significativo en perros hipotiroideos en comparación a los otros 2 grupos, pero una vez se hizo la corrección por concentración de microalbúmina, no se mostraron diferencias significativas. En cambio, las concentraciones de TBARS se vieron aumentadas en los perros hipotiroideos en comparación a los perros sanos, tras los valores ser corregidos por el valor de proteína en saliva.

Por esto se concluyó que el hipotiroidismo canino conduce a cambios en el estado redox, estos cambios siendo asociados posiblemente al incremento en la estimulación de TSH, así como el aumento en la movilización de lípidos, como consecuencia a la enfermedad.

3.6 Anexo 1 (Resultados enviados para publicar). Changes of biomarkers of redox status in serum and saliva of dogs with hypothyroidism

Tras la validación de la medición de biomarcadores del estado redox en WB y en lisados RBCs, un nuevo campo de investigación esta disponible, con esto y tras observar los cambios en el estado redox en suero y saliva, como consecuencia al hipotiroidismo canino, la investigación de los posibles cambios en estos biomarcadores resulta de interés. Por esta razón, la medición del panel validado de antioxidantes y oxidantes en WB y lisados RBCs fue evaluado en perros con hipotiroidismo y comparado con el estado redox de perros con NTH y perros sanos, con la finalidad de identificar nuevos posibles biomarcadores de enfermedad.

Los resultados en la medición de biomarcadores del estado antioxidante mostraron un descenso significativo de CUPRAC, TEAC, tiol y PON-1 en WB de perros hipotiroideos en comparación a perros sanos. Mientras que en los lisados RBCs se evidenció un aumento significativo de FRAP y PON-1, y un descenso significativo de TEAC en perros hipotiroideos en comparación a perros sanos. Estos resultados pueden ser indicativos de la perdida de la capacidad antioxidante como consecuencia a la disfunción tiroidea, ya que estas hormonas forman parte del sistema de defensa antioxidante (Chainy and Sahoo, 2020; Venditti et al., 2003; Villanueva et al., 2013).

Los resultados de la medición de biomarcadores del estado oxidante mostraron un descenso significativo de TOS y POX-Act, y un aumento significativo de AOPP y TBARS en WB de perros hipotiroideos en comparación a perros sanos. Por otro lado, en los lisados RBCs se evidenció un descenso significativo de d-ROMs. Estos resultados pueden ser indicativos de la presencia de peroxidación de proteínas y lípidos, así como el aumento en la liberación de ROS, como consecuencia a la disminución de la actividad metabólica por la pérdida de hormonas tiroideas (Nanda et al., 2008; Torun et al., 2009).

Por esto se concluyó que el hipotiroidismo canino produce cambios en el estado redox en WB y en lisados RBCs. Estos cambios podrían asociarse a los efectos de la pérdida de las hormonas tiroideas, y por tanto, una disminución de la capacidad antioxidante, así como, una actividad metabólica alterada que conduce a la liberación y síntesis de oxidantes. Los resultados entre ambas muestras fueron consistentes, no obstante, la mayoría de los cambios se observaron en WB, lo que resalta el interés en el uso de este tipo de muestra en el futuro.

3. Conclusiones

Las conclusiones de este estudio son las siguientes:

1. En la revisión de los estudios de análisis proteómicos publicados en perros, se encontró que una diversidad de muestras, especialmente el suero y la saliva, han sido utilizadas para evaluar el proteoma en esta especie. El uso de los análisis proteomicos para evaluar enfermedades puede llevar a la detección de nuevos biomarcadores y a esclarecer o descubrir nuevos mecanismos fisiopatológicos, especialmente en el campo de los desordenes endocrinos.

2. El hipotiroidismo produce cambios en el proteoma de la saliva y el suero de los perros. Estos cambios reflejan un descenso en la tasa metabólica, una situación de estrés oxidativo, y alteraciones inmunológicas y dermatológicas en saliva, mientras que, los cambios en suero podrían reflejar una reducción en la síntesis de proteínas, la coagulación y la presencia de hipercolesterolemia. En particular, la actividad de la LDH, la cual puede ser medida fácilmente por medio de ensayos espectrofotométricos, se vio reducida en la saliva de perros con hipotiroidismo al comparar con perros con otras enfermedades y perros sanos, y podría servir como un nuevo biomarcador potencial de hipotiroidismo canino.

3. El hipotiroidismo produce cambios en el metaboloma de la saliva y el suero de los perros. Los cambios en saliva están directamente asociados con la síntesis de hormonas tiroideas y la alteración en el metabolismo de lípidos. Algunos de los metabolitos identificados como la tirosina y la fenilalanina, podrían servir como biomarcadores potenciales de hipotiroidismo canino. Los cambios en suero están asociados con la vía pentosa fosfato, la biosíntesis del acido aminoacyl-tARN, y el metabolismo de pirimidina. Algunos de los metabolitos identificados, como el ácido D-glucónico y la L-isoleucina, podrían servir como biomarcadores potenciales de hipotiroidismo canino.

4. Los antioxidantes y oxidantes pueden ser medidos en WB y lisados RBCs de perros. Los perros con hipotiroidismo presentan un descenso en CUPRAC, FRAP, TEAC, tiol y TOS, y un aumento en PON-1, AOPP y TBARS en la WB. También muestran un descenso en TEAC y d-ROMs, y un aumento en FRAP y PON-1 en lisados RBCs. Además, el hipotiroidismo produce cambios en el estado redox de la saliva, con un descenso en FRAS y un aumento en TBARS. Estos datos indican que el hipotiroidismo canino puede producir cambios en el estado redox y que este puede ser determinado usando distintos tipos de muestras.

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ANNEX 1

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The following experiment was performed during this thesis and is currently submitted or planned to be submitted for possible publication.

ANNEX 1

Changes in biomarkers of the redox status in whole blood and red blood cell lysates in canine hypothyroidism

(Submitted for possible publication)

Hypothyroidism is the most commonly diagnosed endocrine disease in dogs. The objective of this study was to evaluate the changes in the redox status in canine hypothyroidism using whole blood (WB) and red blood cell lysates (RBCs). For this purpose, a panel of five antioxidants and five oxidants biomarkers was measured in WB and RBCs of 30 dogs with hypothyroidism, 26 dogs with non-thyroidal illnesses and 15 healthy dogs. The antioxidants measured were cupric reducing antioxidant capacity (CUPRAC), ferric reducing ability of plasma (FRAP), Trolox equivalent antioxidant capacity (TEAC), thiol and paraoxonase type- 1 (PON-1). Oxidants measured include the total oxidant status (TOS), peroxide-activity (POX-Act), reactive oxygen-derived metabolites (d-ROMs), advanced oxidation protein products (AOPP) and thiobarbituric acid reactive substances (TBARS). WB showed a significant decrease of the antioxidants CUPRAC, TEAC and thiol, and also an increase in TBARS and a decrease in AOPP in dogs with hypothyroidism compared to healthy dogs. Meanwhile, RBCs showed a significant decrease in TEAC and d- ROMS, and an increase in PON-1 in dogs with hypothyroidism. The changes in the redox biomarkers in this study show that WB in canine hypothyroidism had a higher number of changes in biomarkers of the redox status than RBCs lysates, making it a promising sample type for the evaluation of the redox status in this disease. In addition, WB is easier and simpler to process than RBCs and unlike serum, it does not have any hemolysis interference.