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Changes of the salivary and serum proteome in canine hypothyroidism

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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 proteome 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.

1. Introduction

Hypothyroidism is the most represented endocrinopathy in dogs with up to 2.7 % of prevalence. It can affect dogs of any breed or age, although it is more often presented in geriatric and medium to large breeds [1–4]. In hypothyroidism, there is a deficiency of triiodothyronine (T3) and thyroxine (T4), leading to the development of metabolic changes that affect amino acid uptake and fatty acid metabolism, among others [5,6], causing a plethora of symptoms that are often unspecific, such as lethargy, skin disorders, and weight gain, etc. [1,7]. Diagnosis of hypothyroidism is made by the determination of T4 and thyroid-stimulating hormone (TSH), although, measurement of thyroid hormones does not always lead to a clear diagnosis, since, in some cases, hormone concentrations can be found within reference range [2,8,9].

The proteome is defined as the total set of proteins expressed at a given time, changing constantly in response to external or internal stimuli [10,11]. The use of proteomic analyses helps to better understand the mechanisms and identify possible biomarkers of the disease [12]. Proteomic analyses can be made on any sample type, including serum and saliva, as these samples have been used in dogs to identify biomarkers of several diseases like mammary tumors [13], leishmaniasis [14–16] and obesity [17,18].

In humans, the serum proteome in hypothyroidism after thyroid hormone replacement has been studied. Proteins like complement C4-A, haptoglobin, and apolipoprotein A-1 were found to be upregulated after treatment [19], meanwhile, in urine, proteins like keratin type II cytoskeletal 5 and serotransferrin were found to be upregulated after thyroid hormone replacement [20]. However, to our knowledge, there are no proteomic studies performed on canine hypothyroidism.

In this report, we hypothesize that proteins could change in the saliva and serum of dogs with hypothyroidism. Therefore, our objective was to evaluate the salivary and serum proteome changes in dogs with hypothyroidism by gel-free proteomics using TMT labeling. This study will provide knowledge about the pathophysiological changes in this

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disease, as well as help to identify possible new biomarkers of canine hypothyroidism in serum and saliva.

2. Materials and methods

This study consisted of two different experiments. (1) A liquid chromatography-mass spectrometry (LC-MS) proteomic analysis was performed in the serum and saliva of dogs with hypothyroidism ($n = 10$) and healthy controls ($n == 10$). Data referring to these populations is described in a previously published article [21]. (2) One of the proteins that changed in saliva proteome, lactate dehydrogenase (LDH) was used for the validation of the proteomic results. For this purpose, a spectrophotometric assay for the measurement of the activity of this enzyme was analytically validated and then measured in saliva samples of three groups of dogs: dogs with hypothyroidism ($n == 42$), dogs with non-thyroid disease ($n == 42$) and healthy controls ($n == 46$). This project has been approved by the Murcia University Ethics Committee with the number CEFA 288/2017

2.1. Study design and case selection.

The samples used in the proteomic study were aliquoted samples from a previous study focusing on metabolomics in hypothyroidism that were stored at -80°C [21]. Briefly, the inclusion criteria for the hypothyroid group was 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. 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.

Animals for the second part of the study were also client-owned dogs attending several Veterinary Clinics in Murcia Region (Spain). Saliva samples were obtained from three groups of dogs: (1) dogs with hypothyroidism; (2) dogs with non-hypothyroid diseases ; and (3) healthy controls. The inclusion criteria for both the hypothyroid group and the healthy controls were the same as in the previous paragraph. Meanwhile, the non-hypothyroid diseased group met the inclusion criteria described in a previous report [22]; (1) being adult dogs (>1 y); (2) having hypothyroidism as a differential diagnosis that led the practitioners to send serum samples to our laboratory for T4 and TSH measurement; (3) not having or receiving any treatment prior to 6 months of the study; (4) having T4 and TSH results indicating the absence of hypothyroidism [23]; and (5) absence of gingivitis or any disease of the oral cavity. A total of 42 hypothyroid, 42 non-hypothyroid diseased dogs and 46 healthy controls were included. Owners' consent was obtained in all cases.

2.2. Saliva sampling procedures

Saliva samples were collected from all dogs after a minimum 12 h fast as previously described [24]. In brief, sponges were introduced into the dog's mouth for one to two minutes until wet and then placed into Salivette tubes (Salivette, Sarstedt, Aktiengesellschaft & CO., Nümbrecht, Germany). All tubes were stored with ice until taken to the laboratory. Once in the laboratory, the Salivette tubes were centrifuged at 3000 × *g* for 20 min at 4°C. Saliva was collected and transferred to 1.5ml Eppendorf tubes and stored ad −80°C until analysis. None of the dogs used in this study showed any clinical signs of periodontitis.

2.3. Liquid chromatography-tandem mass spectrometry (LC/MS-MS)

From each sample, 35 μg of acetone-precipitated proteins were subjected to reduction, alkylation, and digestion and were labeled using 10-plex 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 37 °C (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 −80 °C before further 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 [25]. 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 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 MS/MS fragmentation. For comparison of relative quantification results between the experiments, the internal standard was used.

2.4. Lactate dehydrogenase analysis

LDH activity measurement in saliva was made using a commercial 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). This assay showed 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) to the measurement of salivary LDH after serial dilutions of two saliva samples from dogs.

2.5. Statistical analysis

Clustering analysis of protein abundances in samples within the same group was done to identify clear outlier samples. Accordingly, saliva sample #2 from dogs with hypothyroidism and saliva samples #13 and #17 from control dogs were disregarded in subsequent analysis. Fold changes in protein abundance between disease and healthy groups were calculated as the log2(Mean(hypothyroidism group)/Mean(control group)). The Shapiro-Wilk test was first used to assess whether data were normally distributed. Under the assumption of a non-normal distribution, the statistical significance of the relative abundance differences between groups was assessed with the non-parametric Mann-Whitney U rank test. The receiver operating characteristic (ROC) curve was calculated for LDH concentrations to determine the cut-off that distinguished between groups. Then, the cut-off values were determined. To evaluate the influence of gender, the animals were divided into two groups, one of males and one of females, and the differences were studied using the non-parametric Mann-Whitney test. Statistical significance was considered when P-value < 0.05. Statistical analyses of proteomics data were implemented using Python3 and the SciPy library [26].

Validation data were analysed using GraphPad Prism software (GraphPad Software Inc., version 9.3 for MacOS) and Excel (Microsoft, 2020). Arithmetic means, medians, and intra- and inter-assay coefficients of variation (CVs) were calculated. Linearity under dilution was investigated by means of linear regression. For the evaluation of LDH concentration differences in the saliva of dogs with hypothyroidism between groups, the Kruskall-Wallis test was used, followed by Dunn's multiple comparison test as a posthoc analysis.

2.6. Bioinformatics

Proteins were mapped to UniProt entries (Bateman et al., 2021) and annotated with gene symbols, protein names, and descriptions. To functionally characterize those proteins determined to be differentially expressed, Gene Ontology (GO) terms were retrieved for each using PANTHER (version 17.0) [27].

3. Results

3.1. Dog characteristics

Details referring the dogs included in the first part of the study are described in a previously published article [21]. For the first part of the study, a population of 20 dogs was included. The hypothyroid group was integrated by four males and six females, with a mean age of 10.4 years (range 3.0–14.0 years), a mean BCS of 4/5 (range 3–5/5) and being mongrel the most representative breed. The Control group was integrated by four males and six females, with a mean age of 9.4 years (range 5.0–14.6), a mean BCS of 3/5 (range 3–4/5) and being mongrel the most representative breed. A complete individual description of the dogs included are presented in supplementary data (Supplementary Table 1). There were no statistically significant differences between groups.

A total of 130 dogs were included in the second part of the study. The hypothyroid group was integrated by 37 males and 5 females, with a mean age of 9.4 years (range 4.0–14), a mean BCS of 4.0/5 (range 3–5/5), and these belonged to 16 breeds being Beagle the most representative ($n == 12$). The non-thyroid diseased group was integrated by 29 males and 19 females, with a mean age of 8.2 years (range 2–14), a mean BCS of 3.8/5 (range 2–5/5), being mongrel ($n == 10$) the most common within 15 breeds. The Control group was integrated by 24 males and 22 females, with a mean age of 6.79 (range 2–14.6), a mean BCS of 3.4/5 (range 2–4/5), being beagle ($n == 13$) the most common within 13 breeds. A complete individual description of the dogs included are presented in supplementary data (Supplementary Table 2). The different clinical manifestations presented in the hypothyroid group and the diseases found on the non-thyroid group are shown in the Table 1 and 2, respectively. There were no statistically significant differences between groups related to age or BCS.

3.2. Salivary proteomic profile

A total of seven salivary proteins showed differences in their abundances in dogs with hypothyroidism compared with controls (Table 3). 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 (LDHA). On the other hand, the only upregulated protein was keratin type II cytoskeletal 4 (KRT4). No gender differences were observed in these proteins. A complete description of the proteins identified in the salivary proteomics study is presented in supplementary data (Supplementary Table 3)

GO annotation reveals that of the differentially expressed proteins, three –Coronin-1A (CORO1A), MSN, and KRT4– are cytoskeletal proteins located in different components and related to diverse cellular processes (Fig. 1). Among the others, there are two cytosolic dehydrogenase enzymes: glucose-6-phosphate 1 dehydrogenase (G6PD) and L-lactate dehydrogenase A chain (LDHA). HSPA8 is an ATP-dependent molecular chaperone and thus has ATB-binding and hydrolyzing activity, allowing it to refold misfolded proteins. Lastly, HYAL1 is a hyaluronoglucosidase, a lysosomal enzyme that breaks down hyaluronic acid, which might have a role in cell migration, adhesion, and proliferation.

When comparing the salivary proteome results in both genders, a total of nine other proteins were found to show differences in their abundance. Among these, serine protease inhibitor Kazal type-1, desmocollin-2 isoform X4, protease inhibitor, allergen Can f 4 precursor, alpha-2-macroglobulin-like protein 1 isoform X1 and ribosomal L40e family protein were found to be upregulated in females. Meanwhile, uridine phosphorylase 1, desmoglein-1 and pregnancy zone protein-like isoform X1 were found to be downregulated in females. A complete description of the results related to sex differences for salivary proteins are presented in supplementary data (Supplementary Table 4).

3.3. Serum proteomic profile

A total of six serum proteins differed in their abundances in dogs with hypothyroidism compared with controls (Table 4). From these, five proteins were downregulated and one upregulated in the serum of hypothyroid dogs. The most downregulated proteins were sex hormone-

Table 1
Clinical manifestations observed in dogs included in the hypothyroidism group.

Clinical Manifestation	Number of cases	Percentage from total
Obesity	35	83.3
Lethargy	32	76.2
Skin disorders (hair thinning or alopecia, hyperpigmentation, seborrhea)	32	62.3
Vestibular syndrome	3	7.2

Table 2
Diseases observed in dogs included in the non-thyroid group.

Clinical Manifestation	Number of cases	Percentage from total
Obesity	22	52.4
Hepatopathy	14	33.3
Leishmaniasis	9	21.4
Cushing's syndrome	6	14.3
Otitis	4	9.5
Heart disease	3	7.2
Chronic kidney disease	2	4.8
Epilepsy	2	4.8
Myopathy	2	4.8
Neoplasia	2	4.8
Irregular interoestrus interval	1	2.4

Table 3
Statistically significant expression changes of protein in 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

binding globulin (SHBG), complement component C7 (C7), and antithrombin-III (SERPINC1). On the other hand, the upregulated protein was inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4). No gender differences were observed in these proteins. A complete description of the proteins identified in the serum proteomics study is presented in supplementary data (Supplementary Table 5).

According to their GO annotation, all differentially expressed proteins in serum were extracellular/secreted proteins (Fig. 2). Two proteins are serine protease inhibitors (SERPINC1and ITIH4), while others have transporter roles: GC vitamin D-binding protein (GC) and SHBG, both with steroid-binding activity, and apolipoprotein M (APOM). GC is involved in vitamin D transmembrane transport, while SHBG is probably involved in androgen transport. APOM assembles into high-density lipoprotein particles and is thus involved in lipid transport in the blood, in particular cholesterol and phospholipids. Lastly, C7 is a constituent of the membrane attack complex that plays a role in the innate immune response.

When comparing the serum proteome results in both genders, a total of eight other proteins were found to show differences in their abundance. Among these, alpha-2-macroglobulin, carboxypeptidase N subunit 2, sulfhydryl oxidase 1 isoform X1, apolipoprotein A-IV and complement component C8 beta chain were found to be upregulated in females. Meanwhile, serum albumin-like, inter-alpha-trypsin inhibitor heavy chain H1 and IgA heavy chain constant region were found to be downregulated in females. A complete description of the results related to sex differences for serum proteins is presented in supplementary data (Supplementary Table 6).

3.4. Changes in salivary lactate dehydrogenase in dogs with hypothyroidism, non-hypothyroid diseases and controls

Salivary concentrations of LDH were significantly lower in dogs with hypothyroidism (median; min – max; 55.35 IU/L; 4.70–115.60)

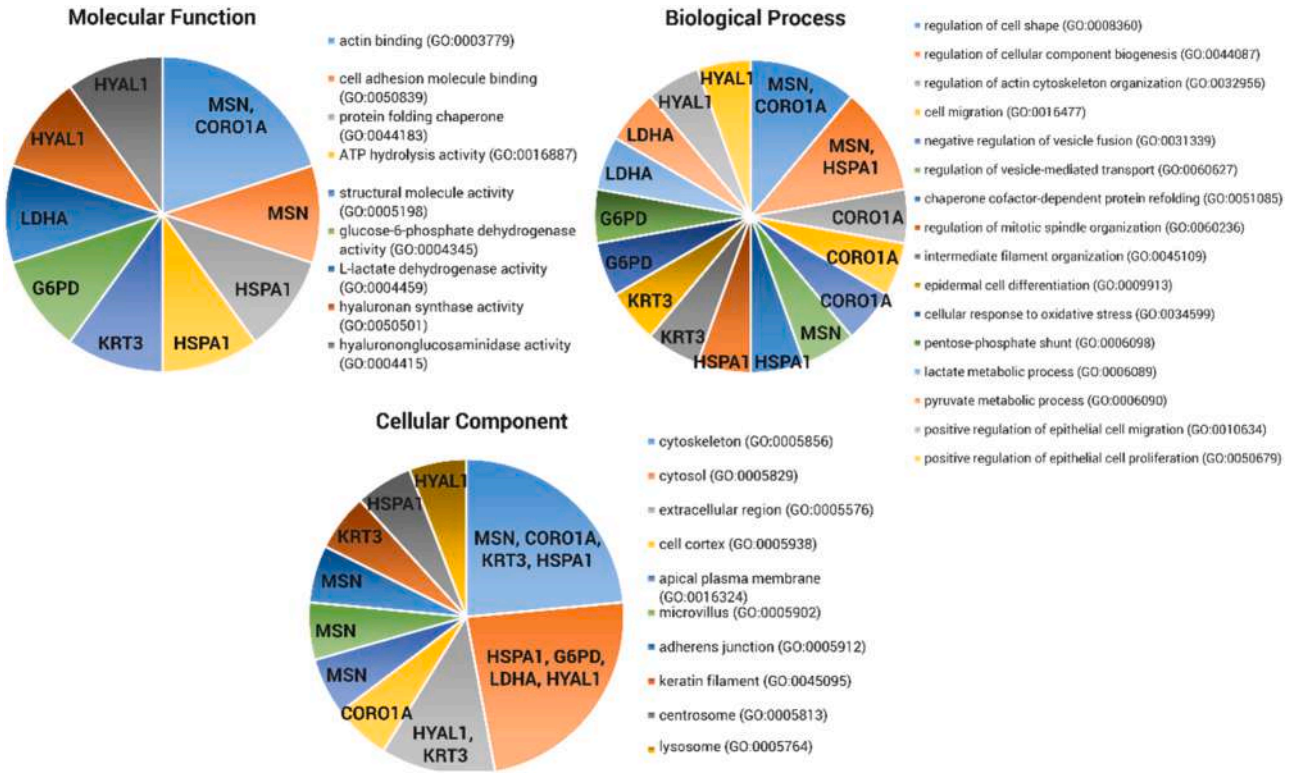

Fig. 1. Pie charts showing GO terms from the three ontologies (molecular function, biological process and cellular component) associated to differentially expressed proteins in the saliva of dogs with hypothyroidism. GO terms in the legend follow a clockwise order. GO annotation for each gene was obtained 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.

Table 4
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

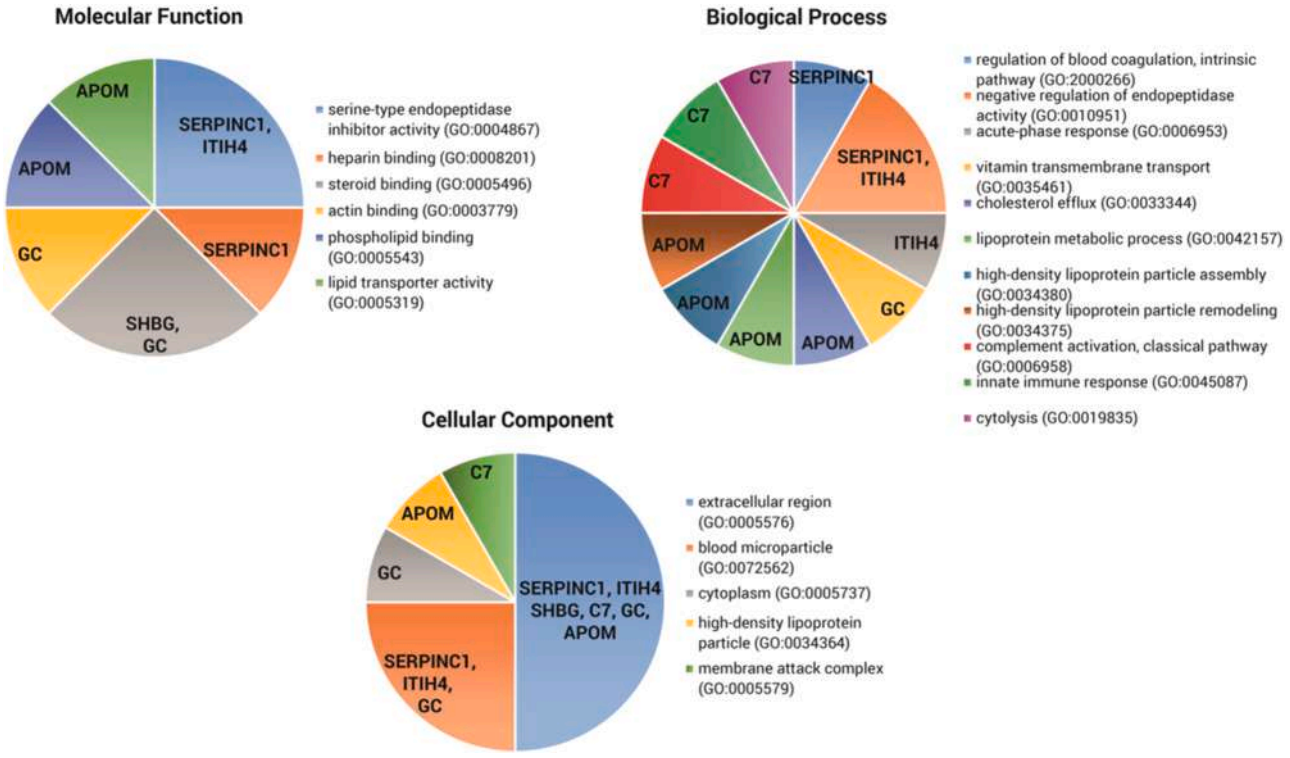

Fig. 2. Pie charts showing GO terms from the three ontologies (molecular function, biological process and cellular component) associated to differentially expressed proteins in serum. GO terms in the legend follow a counterclockwise order. GO annotation for each gene was obtained with PANTHER. For dog proteins lacking annotations, we used those of their human orthologs. For clarity, the most relevant GO terms for each protein were manually selected.

compared to both controls and non-hypothyroid-diseased dogs (204.80 IU/L; 17.2–1506 and $p == 0.0001$, and 314.50 IU/L; 4.30–2374 and $p < 0.0001$, respectively) (Fig. 3). The ROC curve analysis to discriminate dogs with hypothyroidism from dogs with non-hypthyroid diseases and healthy dogs 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 %) (Fig. 4).

4. Discussion

In this study, the proteomics changes in saliva and serum of dogs with hypothyroidism are described for the first time. In saliva, proteins related to skin function, the immune system, and metabolism were found to be changed in this disease.

Concentrations of two proteins related to skin function, an isoform of hyaluronidase-1 and KRT4 changed in the saliva of dogs with hypothyroidism. Hyaluronidase-1, which was found to be decreased in our study, hydrolyzes hyaluronan, a glycosaminoglycan associated with proteoglycans and with connective tissue, tissue damage, and inflammatory response [28–30]. Thyroid hormones promote the breakdown of hyaluronic acid by stimulating the synthesis of hyaluronidase-1 and hyaluronidase-2. Therefore the lack of hyaluronidase that is produced in hypothyroidism leads to myxedema as a consequence of the accumulation of hyaluronic acid in the skin [31,32]. On the other hand, KRT4 was found to be upregulated in hypothyroid dogs. Keratins are structural proteins that assemble into intermediate filaments in epithelial tissue, and these are heavily influenced by thyroid hormones, as they play a role in barrier formation, hair growth, wound healing, and keratinocyte proliferation [30,33]. Although the function of thyroid hormones on the skin is often controversial, it has been described that these hormones induce keratin activity, contributing to epidermal regeneration, therefore an alteration in thyroid hormone synthesis can lead to an incorrect balance between skin proliferation and differentiation [34]. Further studies should be performed to evaluate if the increase in KRT4 could be related to skin disorders commonly seen in canine hypothyroidism.

Two proteins related to the immune response, coronin-1A and moesin were found to be downregulated in the saliva of dogs with hypothyroidism. Coronin-1A is related to T lymphocyte activation, migration, survival, and calcium signaling. It is highly expressed in lymphocytes and serves as a positive regulator of transforming growth factor β, helping regulate immune [35–38]. Moesin helps maintain epithelial integrity [39], and plays an important role in the regulation of immune response [40]. The downregulation of these two proteins could be associated with the dysregulation of the immune system that occurs in primary hypothyroidism in dogs [41], although further studies should

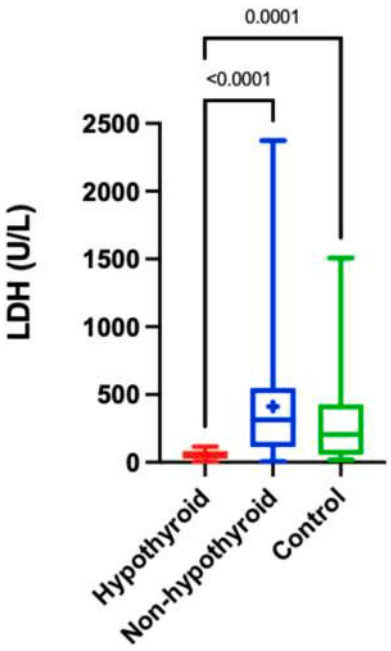

Fig. 3. Lactate dehydrogenase in dogs with hypothyroidism ($n = 42$) compared with non-hypothyroid diseased ($n = 42$) and healthy control dogs ($n = 46$). The plot shows the median (line within the box), the interquartile range (20 and 90), and the range (whiskers).

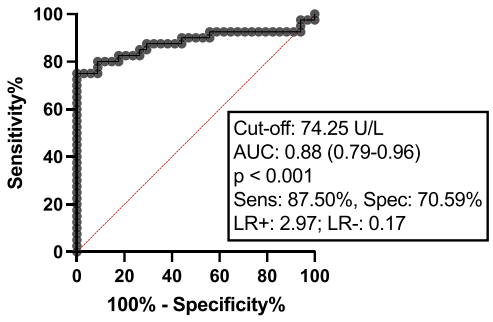

Fig. 4. Receiver operating characteristic curves of lactate dehydrogenase (LDH) for discriminating hypothyroid dogs from dogs with other diseases and healty dogs. AUC, area under the curve; CI, confidence interval; Sens, sensitivity; Spec, specificity; LR, likelihood ratio.

be made to clarify its specific role.

Two proteins related to metabolism such as HSPA8 and G6PD were also downregulated in the saliva of dogs with hypothyroidism. HSPA8 is a molecular chaperone responsible for proper protein folding and refolding of misfolded proteins and is involved in a wide range of cellular processes such as diverse cell signaling pathways and cell apoptosis [42–44]. Additionally, HSPA8 is associated with the modulation of enzymes involved in inflammation and metabolism, as well as oxidative stress [45,46]. G6PD is a key enzyme in the regulation of the pentose phosphate pathway, which is involved in the production of nicotinamide adenine dinucleotide phosphate (NADPH), this plays an important effect as NAPDH serves as a scavenger of reactive oxygen species (ROS), therefore maintaining redox homeostasis [47]. The activity of G6PD has been described to be lowered in rats with hypothyroidism, as well as in neonatal congenital hypothyroidism in humans, additionally, an increase in G6PD activity has also been reported when administrating thyroid hormones [48–53]. We hypothesize the downregulation of G6PD could be associated with a lower metabolic rate and an increase in oxidative stress, although further studies should be made to elucidate the direct effects of thyroid hormones on this enzyme.

In saliva, LDH was found to be downregulated in the proteome of hypothyroid dogs and these findings were confirmed in the validation study with a larger population of dogs. LDH is recognized as one of the most common enzymes in nature [54], and it serves as a key enzyme for glycolytic metabolism as it allows the organism to maintain itself under anaerobic conditions in the form of lactate and be excreted as pyruvate when oxygen is available [55]. The decrease in saliva found in our report is in contrast with the increases described in the serum of different animal species in different diseases, such as hepatitis, cirrhosis and liver necrosis, mastitis, lung damage, and metabolic acidosis, among others [56]. In addition, in humans, LDH is reported to be increased in the serum of patients with hypothyroidism [57–59]. Nonetheless, a previous report identified a decrease in salivary LDH due to a modification in the thiol group found in the LDH enzyme that leads to an enzyme catalytic dysfunction effect, and the authors associate this effect with the oxidative stress caused by cigarette smoke [60]. Based on this, it could be hypothesized that the decrease found in our study could be associated with changes in the oxidative status previously reported in hypothyroid dogs [22]. However, further studies using a larger population should be made to confirm the decreases in salivary LDH found in our report and elucidate the causes and mechanisms leading to this. LDH presented values of AUC > 0.8 at ROC curve analysis and therefore a potential ability to distinguish between dogs with hypothyroidism and dogs with other diseases and clinical signs compatible with hypothyroidism or healthy dogs. However, it is important to point out that this data was obtained using a small population. In addition, LDH levels can increase in gingivitis [61], and although no dog with gingivitis was included in this study, the presence of gingivitis could be a confounding factor for the interpretation of salivary LDH values.

In serum, the SHBG and GC were found to be downregulated. Both proteins are synthesized in the liver and are influenced by thyroid hormones [62–65]. Moreover, GC was reported to be decreased in patients with Hashimoto's thyroiditis, associating its lower levels to the diminished activity of thyroid hormones [66]. We hypothesize these two proteins were found to be downregulated in dogs with hypothyroidism due to a lower amount of circulating thyroid hormones affecting their synthesis within the liver.

Two proteins that showed changes in the serum of dogs with hypothyroidism, the ITIH4 and the APOM, are associated with lipid metabolism [67–69]. The downregulation of APOM could be associated with a decreased lipid metabolism [69,70], whereas the upregulation of ITIH4 could be associated with the hypercholesterolemia occurring in canine hypothyroidism [67,71].

Another downregulated protein in serum was SERPINC1, a key glycoprotein for the regulation of the coagulation process [72]. Hypothyroidism is associated with a hypercoagulable state, which as a consequence, represents a risk factor for thromboembolism in plasma. This change is in line with what was described in humans, where a decrease of SERPINC1 was reported in hypothyroid patients [73], and could be associated with a hypercoagulable state found in this disease.

C7 was also found to be downregulated in the serum of hypothyroid dogs. C7 is a key molecule of the complement system [74] and its decrease is often associated with an increased risk of infection and autoimmune disease [75,76], as the complement system leads to the production of innate immunity defense molecules [77]. In humans, this protein was found to be upregulated in plasma after levothyroxine replacement therapy, associating this increase with an increased hepatic activity [19].

In this study, the results obtained from the salivary and serum proteome were different between the two samples types. This has been reported in other diseases in dogs [13,78], and also in other species such as horses [79] and cows [80]. This suggest that diseases can cause different changes in proteins in saliva and serum, probably due to different pathophysiological mecanisms. On the other hand, most of the proteins found in dogs with hypothyroidism were downregulated. This

could be associated with the effects of the disease in slowing metabolism and disrupting protein synthesis.

In the proteins that demonstrated significant differences between healthy and hypothyroid dogs, no differences were observed due to sex. However, other nine proteins in saliva and eight proteins in serum showed sex differences. This would indicate that, although it does not affect the proteins that changed in hypothyroidism in our study, the hormonal influence could affect the salivary and serum composition of dogs.

This study has some limitations: dogs of different breeds and ages were included to simulate a situation seen in daily clinical routine. Regarding gender, we investigated the effect of sex-differences in our population of dogs to guarantee if the significant differences of the proteins reported were influenced by gender, but further studies should be performed to investigate the possible influence of the other factors in the proteomic results. In addition, thyroglobulin antibodies were not evaluated in any of the cases included in this report, however the dogs of our study could be considered to be at the overt stage of the disease based on the clinical signs, and serum T4 and TSH concentrations [23, 41]. In any case, it would be of interest to evaluate the proteomic changes occurring in other stages of hypothyroidism previously described such as subclinical or silent hypothyroidism and antibody-positive subclinical hypothyroidism that can be detected by the measurement of thyroglobulin antibodies [41]. In addition, our results should be corroborated using a larger population of dogs, and ideally, further additional studies on the possible use of the proteins showing changes in our report as biomarkers of disease severity or treatment monitoring should be undertaken.

5. Conclusions

Hypothyroidism induces different changes in the serum and salivary protein profile of dogs. Salivary changes would reflect a lower metabolism rate, a situation of oxidative stress, and immunologic and dermatologic alterations. meanwhile, changes in serum could reflect a reduced protein synthesis, coagulation, and hypercholesterolemia. In particular, LDH activity was found to be reduced in the saliva of dogs with hypothyroidism when compared with non-thyroid diseased dogs and healthy dogs. Further studies should be made to confirm the findings of this report and evaluate if the protein that changed in this study in saliva and serum could be potential biomarkers of this disease.

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CRediT authorship contribution statement

L.G. González-Arostegui: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **A. Muñoz-Prieto:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Visualization, Writing – original draft, Writing – review & editing. **C.P. Rubio:** Methodology, Supervision, Validation, Writing – review & editing. **J.J. Cerón:** Conceptualization, Methodology, Project administration, Supervision, Writing – review & editing. **L. Bernal:** Data curation, Methodology. **I. Rubić:** Data curation, Formal analysis, Software. **V. Mrljak:** Data curation, Formal analysis, Software. **J.C. González-Sánchez:** Data curation, Formal analysis, Software, Visualization, Writing – review & editing. **A. Tvarijonaviciute:** Conceptualization, Investigation, Methodology, Project administration, Writing – review & editing.

Declaration of Competing Interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.domaniend.2023.106825.

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