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Research paper

Changes in saliva of dogs with canine leishmaniosis: A proteomic approach

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ABSTRACT

In the present study, a quantitative proteomic approach to study changes in saliva proteins associated with canine leishmaniosis (CanL) was performed. For this, canine salivary proteins were analysed and compared between dogs before (T0) and after (T1) experimental infection with *Leishmania infantum* by high-throughput label-based quantitative LC–MS/MS proteomic approach and bioinformatic analysis of the *in silico* inferred interactome protein network was created from the initial list of differential proteins. More than 2000 proteins were identified, and of the 90 differentially expressed proteins between T0 and T1, 12 were down-regulated with log2 fold change lower than −0.5849, and 19 were up-regulated with log2 fold change greater than 0.5849. This study provides evidence of changes in salivary proteome that can occur in canine leishmaniosis and revealed biological pathways in saliva modulated in canine leishmaniosis with potential for further targeted research.

1. Introduction

Vector-borne diseases are considered a major threat to human and animal health (Kuleš et al., 2016a). Among them, visceral leishmaniosis is a major global zoonosis caused by the protozoan *Leishmania infantum* (Martínez-Subiela et al., 2017) that can be potentially fatal to humans and dogs (Solano-Gallego et al., 2011), which are the main reservoir of infection (Gramiccia and Gradoni, 2005). Canine leishmaniosis (CanL) is presented almost worldwide and considered endemic in more than 70 countries, with increasing cases in non-endemic areas (Solano-Gallego et al., 2011).

CanL can have clinical presentations, ranging from subclinical to fatal illness. There are many different signs that a sick dog can manifest, being the most frequent enlargement of lymph nodes and skin lesions (Solano-Gallego et al., 2011). In any case, CanL may potentially involve any organ, tissue, or body fluid, producing a wide range of nonspecific clinical signs that make the diagnosis of CanL challenging (Solano-Gallego et al., 2011).

Saliva sampling is non-invasive, painless, easy to perform, and well tolerated by dog patients and owners compared to traditional diagnostic methods such as biopsy or vein puncture (Parra et al., 2005; Yoshizawa et al., 2013). In the last years, saliva has proved to be a promising tool in the diagnosis of human visceral leishmaniasis since saliva-based diagnostic test are demonstrated to be equally sensitive as serum-based ones, specifically a rk39 antibody detection test (Mohapatra et al., 2016; Vaish et al., 2012). In a recent report, antibodies against *L. infantum* have been detected and quantified in the saliva of infected dogs, proposing this specimen as a promising tool in the diagnosis of CanL (Cantos-Barreda et al., 2017). However, to the authors' best knowledge, there are no studies about changes of salivary proteome in canine leishmaniosis. The knowledge of these possible changes could help to better understand the physiopathology of the disease and identify biomarkers that could be of clinical use. Currently, there are established guidelines for the diagnosis of canine leishmaniosis based on clinical signs, serology, and laboratory findings (Solano-Gallego et al., 2011). However, in some cases, the diagnosis can be difficult and the interpretation controversial, needing to be adapted for each patient (i.e. dogs with no clinical signs can have high antibody titters) (Martínez-Subiela et al., 2017). In addition, the samples employed for the molecular diagnosis of canL consists in invasive methods

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Table 1
Main clinicopathological findings in dogs with clinical CanL and clinical stagesbased on LeishVet guidelines at the moment of T1 (Solano-Gallego et al., 2011).

	Gender / age in years	External examination	Laboratory abnormalities	Clinical stage (Leishvet)
1	Male/ 1	Lymphadenomegaly Cutaneous lesions (exfoliative and ulcerative dermatitis)Ocular lesions (blepharoconjunctivitis)	Hypoalbuminemia Hyperglobulinemia Increased CRP and ferritine	II a
2	Male/1	Lymphadenomegaly Cutaneous lesions (exfoliative and ulcerative dermatitis)Ocular lesions (blepharoconjunctivitis)	Hypoalbuminemia Hyperglobulinemia Increased CRP and ferritine	II a
3	Female /1	Lymphadenomegaly Ocular lesions (blepharitis)	Hyperglobulinemia Increased CRP and ferritine	II a
4	Female/ 6	Lymphadenomegaly	Normocytic hypochromic anemia Hypoalbuminemia Hyperglobulinemia Increased CRP, ferritine, ALP, AST and ALT	II a

such as bone marrow and popliteal lymph node aspirations, skin biopsies and peripheral blood (Corpas-López et al., 2016). These invasive approaches, besides requiring technical expertise, carry the risk of pain, bleeding and bacterial or fungal infections (Taslimi et al., 2017). Thus, non-invasive diagnostic procedures are of high interest nowadays, as stated for humans patients (Taslimi et al., 2017), and the efficacy of a set of non-invasive samples such as oral, conjunctival or vulvar swab and urine have been evaluated for the diagnosis and follow-up of CanL experimental infection through qPCR (Hernández et al., 2015).

Utilization of novel techniques such as "-omics" is bringing more light and comprehension in disease mechanisms, that could allow more accurate diagnostics tools (Guillemin et al., 2006). Proteomics offers an integrative view of proteomes in healthy and unhealthy animals, as well as in different physiological states, with the aim to identify candidate disease-related biomarkers in biological fluids (Li et al., 2011; Ruiz-Romero and Blanco, 2010). Thus, different studies have been published addressing 2D-PAGE serum proteomics studies in canine diseases such as babesiosis (Kuleš et al., 2014), glomerular disease (Nabity et al., 2011), and leishmaniosis (Escribano et al., 2016), allowing the identification of novel diagnostic biomarkers. Recently, the apparition of quantitative gel-free proteomics using isobaric labelling reagents such as Tandem Mass Tags (TMT®; Proteome Sciences) has emerged as a very sensitive and reproducible analytical technique (Baeumlisberger et al., 2010; Dayon et al., 2011; Giron et al., 2011). Through TMT-based techniques, multiplexed quantification and identification of proteins from up to ten different samples can be performed in the same run, since each tag corresponds to a characteristic fragment ion in the MS/MS spectrum. In a recent study addressing CanL, gel-free TMT-based quantification allowed the identification of 20 additional serum proteins differentially represented when compared to the data obtained by the 2D-PAGE analysis of the same samples (Martínez-Subiela et al., 2017). However, to the authors' best knowledge, although TMT technology has been used in canine salivary proteome study for identification of proteome changes in canine parvovirosis (Franco-Martínez et al., 2018), it has not been yet applied to study salivary proteome changes in CanL.

In the present study, we hypothesised that changes in salivary proteome can occur in dogs with clinical leishmaniosis. To test this hypothesis, saliva samples were collected from a group of dogs experimentally infected with *L. infantum* before the infection (T0) and when clinical signs of the disease were observed (T1). Salivary proteins were analysed and compared between the two groups by using TMT-based technology for potential protein biomarker identification and quantification.

2. Material and methods

2.1. Animals

A total of 4 beagle dogs from the University of Murcia (south-eastern Spain) were involved in this study. All dogs were negative to the presence of *L. infantum* (tested by ELISA and real-time PCR of lymph node and bone marrow aspirates) and *Dirofilaria immitis, Anaplasma Phagocytophylum, Borrelia Burgdorferi*, and *Ehrlichia Canis* antibodies using SNAP test Canine SNAP 4Dx (IDEXX laboratories, IDEXX Europe B.V., Hoofddorp, The Netherlands).

2.2. Study design

Pre-infection specimens (T0) were obtained immediately before the experimental infection through intravenous injection with 1×10^6 stationary-phase infective *L. infantum* (MCAN/BR/00/BA262) promatigotes. *L. infantum* parasites (amastigotes form) were isolated from spleen of an experimentally infected hamster and cultured in Schneider's *Drosophila* medium (Biowest) supplemented with 10% of

inactivated fetal bovine serum (iFBS) and 50 µg/ml gentamicin at 26 °C. Promastigotes derived from the splenic amastigotes were cultured in modified RPMI-1640 medium supplemented with 20% of iFBS, and 50 µg/ml gentamicin at 26 °C. Parasite shape and motility was examined by microscopic examination. With the aim of avoiding re-infections by *L. infantum* during the study, all dogs were externally dewormed with an anti-sandfly activity insecticide (permethrin 500 mg/ml and imidacloprid 100 mg/ml, Advantix pipettes, Bayer®) every three weeks and internally dewormed (50 mg praziquantel, 50 mg pyrantel and 150 mg febantel; Prazitel tablets, Ecuphar®) every three months, in accordance with manufacturer's instructions. In order to reduce possible variations due to the use of drugs, deworming was performed at least three weeks before each sampling time.

Post-infection specimens (T1) were collected subsequently to the onset of haematological and/or clinical signs compatible with CanL, which were observed between 6–8 months post infection and are shown in Table 1. At T1, all dogs were classified as "Moderate disease, Stage IIa" according to the LeishVet guidelines (Solano-Gallego et al., 2011). This stage includes medium to high positive antibody levels and clinicopathological abnormalities that may include mild non-regenerative anemia, hypoalbuminemia, hyperglobulinemia, and a normal renal profile with creatinine < 1.4 mg/dl and non-proteinuric profile with UPC < 0.5. Among the external clinical signs, dogs in clinical stage II may present peripheral lymphadenomegaly, cutaneous lesions, anorexia, weight loss, fever, or epistaxis –among other signs-, and present good to guarded prognosis.

L. *infantum* infection was confirmed by a commercial ELISA test (Leiscan® Leishmania ELISA Test, Esteve Veterinaria, Laboratorios Dr. Esteve SA, Barcelona, Spain) and time resolved-immunofluorometric assays (TR-IFMAs).

2.3. Saliva sampling

Saliva specimens were collected as described previously (Contreras-Aguilar et al., 2017). Briefly, a small sponge was placed around the mouth until it was thoroughly moist and then placed in collection devices (Salivette, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany), centrifuged (3000*g* for 10 min at 4 °C), and the supernatant was stored at −80 °C until analysis (Contreras-Aguilar et al., 2017).

All the procedures were approved by University of Murcia Animal Ethics Committee (protocol number: 276/2016) and the Regional government of Murcia (identification code number: A13151002), and were performed in compliance with the law RD53/2013 about animal experimentation in Spain.

2.4. Proteomic study of saliva samples from CanL infected dogs and LC–MS/MS analysis

Protein concentration was determined by Bradford assay and 100 µg of proteins from each sample was precipitated by adding six volumes of ice-cold acetone (VWR, Pennsylvania, USA) and storing at −20 °C overnight. The precipitate was collected by centrifugation and subsequently resuspended in 100 µl of 100 mM TEAB (pH 8.5) containing 1% SDS and protein concentration was determined by Bradford assay.

From each sample, 35 µg of acetone-precipitated proteins were subjected to reduction, alkylation, digestion and labelled using 6-plex Tandem Mass Tag reagents according to manufacturer's instructions (Thermo Scientific) with some modification, as described elsewhere (Franco-Martínez et al., 2018; Martínez-Subiela et al., 2017). Briefly, 35 g of each sample and internal standards were reduced with 200 mM DTT (Sigma-Aldrich), alkylated with 375 mM iodoacetamide (Sigma-Aldrich) and precipitated with ice-cold acetone overnight. The next day, the samples were centrifuged and acetone was decanted. Then, the pellets were resuspended with 50 µL of 100 mM TEAB buffer and digested with trypsin (Promega) overnight at 37 °C. TMT labelling reagents were equilibrated at room temperature, resuspended in anhydrous acetonitrile (Thermo Scientific, LC–MS grade) and added to each sample. Labelling reaction was incubated for 1 h at room temperature and then quenched by adding 5% hydroxylamine (Thermo Scientific) for 15 min. Samples were then combined at equal amounts and 5 µg of each mixed sample set was placed in a new tube, vacuum-dried and stored at −20 °C before further LC–MS/MS analysis.

The LC–MS/MS analysis was performed on Dionex Ultimate 3000 RSLS nanoflow system (Dionex, Camberley, UK) and Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) as described elsewhere (Franco-Martínez et al., 2018).

2.5. Statistical analysis

In order to compare the abundances of peptides detected in proteomic analysis between T0 and T1, data were normalized by logarithmic transformation and Student's *t*-test (one-tailed, paired) was performed. In all cases, values of $P < 0.05$ were considered to be significant. Fold changes (FC) have been calculated as follows FC = log2 (Group 1 / Group 2). Statistics was performed using RStudio (v1.0.143) ("RStudio Team. RStudio: Integrated Development Environment for R [Internet]. Boston, MA: RStudio, Inc.; 2015. Available from: http://www.rstudio.com/," 2015).

2.6. Bioinformatics and GO pathways

A Heatmap were generated using the web tool for visualizing multivariate data (ClustVis) (Metsalu and Vilo, 2015) in order to evaluate similarities and differences in the salivary proteomic profile of each animal and time point. The data obtained in the proteomic study were used for the Gene Ontology (GO) analysis. Canine genes encoding proteins differentially expressed were converted to their human orthologs using the Ensembl orthologs database and its BioMart tool for data mining (www.ensembl.org). Obtained genes were used to determine the GO terms over-represented after the development of CanL, by the utilization of the Cytoscape (v3.6.1) plug-in ClueGO (v2.5.0) (Bindea et al., 2009; Shannon et al., 2003) on the *Homo sapiens* GO-biological process (22/01/2018). GO terms over-represented in T1 were submitted to analysis by REVIGO (allowed similarity = 0.7, SimRel) to remove redundant GO terms and groups related GO terms based on their functional description (Supek et al., 2011). Finally, pathways interactomes were designed in Cytoscape using the radial layout.

3. Results

3.1. High resolution quantitative proteomic analysis

The high-resolution quantitative proteomic analysis allowed the identification with high confidence of 2218 canine proteins from eight non-depleted canine saliva samples. For the comparison of T0 and T1, 47 unique proteins were differentially expressed (Table 2 and Fig. 1).

Of the 47 differentially expressed proteins between T0 and T1, 12 were down-regulated with log2 fold change lower than −0.5849, and 19 were up-regulated showing a log2 fold change greater than 0.5849. When isoforms were grouped, the proteins that were more down-regulated corresponded to thymosin beta-10 and beta-4, haemoglobin, carbonic anhydrase 2, apolipoprotein A-I, and glucose-6-phospate isomerase. On the other hand, the isoforms of most up-regulated proteins corresponded to immunoglobulins, olfactomedin-4, unnamed protein product, ceruloplasmin-like, allergen Fel d-4-like, L-amino -acid oxidase-like protein, Apo glutamine synthetase, and glutamine synthetase.

3.2. Bioinformatics

These 47 differentially expressed proteins were used as a starting point for creating protein interacting networks using Cytoscape/ClusPedia application and enriched according to data interaction deposited

Table 2
Proteins in saliva with significantly differentially abundances between before (T0) and after (T1) the development of clinical signs of CanL identified using TMT-based proteomic analysis.

Accession*	Description	Fold T1/T0	p value
208342206	Immunoglobulin heavy chain variable region, partial *[Canis lupus familiaris]*	1.27	0.016
208342228	Immunoglobulin heavy chain variable region, partial *[Canis lupus familiaris]*	1.25	0.031
208342279	Immunoglobulin heavy chain variable region, partial *[Canis lupus familiaris]*	1.25	0.031
164430480	Immunoglobulin lambda light chain variable region, partial *[Canis lupus familiaris]*	1.07	0.043
164430516	Immunoglobulin lambda light chain variable region, partial *[Canis lupus familiaris]*	1.06	0.031
123774	RecName: Full = Ig heavy chain V region MOO	0.88	0.038
208342048	Immunoglobulin heavy chain variable region, partial *[Canis lupus familiaris]*	0.80	0.038
545538003	PREDICTED: olfactomedin-4 *[Canis lupus familiaris]*	0.76	0.039
928124865	PREDICTED: LOW QUALITY PROTEIN: IgGFc-binding protein *[Canis lupus familiaris]*	0.73	0.044
16607663	Unnamed protein product *[Canis lupus familiaris]*	0.67	0.042
345788999	PREDICTED: ceruloplasmin-like *[Canis lupus familiaris]*	0.66	0.038
16607718	Unnamed protein product *[Canis lupus familiaris]*	0.64	0.045
16607675	Unnamed protein product *[Canis lupus familiaris]*	0.62	0.047
73971966	PREDICTED: allergen Fel d 4-like isoform X1 *[Canis lupus familiaris]*	0.62	0.002
545524998	PREDICTED: L-amino-acid oxidase-like *[Canis lupus familiaris]*	0.60	0.043
928186502	PREDICTED: uncharacterized protein LOC608320, partial *[Canis lupus familiaris]*	0.60	0.004
648216199	Glutamine synthetase isoform 1 *[Canis lupus familiaris]*	0.59	0.009
55976526	RecName: Full = Glutamine synthetase; Short = GS; AltName: Full = Glutamate decarboxylase; AltName: Full = Glutamate–ammonia ligase	0.59	0.009
158430857	Chain G, Crystal Structure Of Apo Glutamine Synthetase From Dog (*Canis Familiaris*)	0.59	0.009
74000476	PREDICTED: peptidyl-prolyl cis-trans isomerase B *[Canis lupus familiaris]*	0.57	0.042
P23284	Peptidyl-prolyl cis-trans isomerase B	0.55	0.049
P27797	Calreticulin	0.53	0.039
345787749	PREDICTED: calreticulin *[Canis lupus familiaris]*	0.53	0.039
P61626	Lysozyme c	0.53	0.049
24021298	Glutamine synthetase, partial *[Canis lupus familiaris]*	0.52	0.011
158936956	Protease inhibitor *[Canis lupus familiaris]*	0.47	0.019
164499359	Secretory leukocyte peptidase inhibitor, partial *[Canis lupus familiaris]*	0.46	0.018
345806460	PREDICTED: lymphocyte antigen 6D *[Canis lupus familiaris]*	0.45	0.013
928172856	PREDICTED: immunoglobulin lambda-like polypeptide 5 isoform X44 *[Canis lupus familiaris]*	0.45	0.005
P01766	Ig heavy chain V-III region BRO	0.45	0.049
545546410	PREDICTED: ovostatin homolog 2-like *[Canis lupus familiaris]*	0.44	0.044
Q6YHK3-2	Isoform 2 of CD109 antigen	0.42	0.011
Q6YHK3-4	Isoform 4 of CD109 antigen	0.42	0.011
Q6YHK3-1	CD109 antigen	0.42	0.011
345782613	PREDICTED: LOW QUALITY PROTEIN: extracellular matrix protein 1 *[Canis lupus familiaris]*	0.42	0.011
P35527	Keratin, type I cytoskeletal 9	0.40	0.022
Q6YHK3-3	Isoform 3 of CD109 antigen	0.30	0.050
57092971	PREDICTED: malate dehydrogenase, cytoplasmic isoform X1 *[Canis lupus familiaris]*	0.28	0.009
356461044	Peroxiredoxin-1 *[Canis lupus familiaris]*	0.26	0.023
Q06830	Peroxiredoxin-1	0.26	0.031
50950209	Desmoglein-3 precursor *[Canis lupus familiaris]*	0.22	0.032
345800677	PREDICTED: LOW QUALITY PROTEIN: alpha-enolase *[Canis lupus familiaris]*	0.21	0.042
928183828	PREDICTED: LOW QUALITY PROTEIN: ubiquitin-like modifier-activating enzyme 1 *[Canis lupus familiaris]*	0.17	0.042
P40925	Malate dehydrogenase, cytoplasmic	0.17	0.039
P40925-3	Isoform 3 of Malate dehydrogenase, cytoplasmic	0.17	0.039
P22314	Ubiquitin-like modifier-activating enzyme 1	0.15	0.048
P22314-2	Isoform 2 of Ubiquitin-like modifier-activating enzyme 1	0.15	0.048
1272412	Fibronectin, partial *[Canis lupus familiaris]*	−0.08	0.029
Q01518-1	Adenylyl cyclase-associated protein 1	−0.20	0.050
1272414	Fibronectin, partial *[Canis lupus familiaris]*	−0.20	0.038
Q01518-2	Isoform 2 of Adenylyl cyclase-associated protein 1	−0.27	0.011
558757359	Ezrin *[Canis lupus familiaris]*	−0.31	0.015
4103761	GDP dissociation inhibitor isoform 1 *[Canis lupus familiaris]*	−0.32	0.044
P15311	Ezrin	−0.35	0.008
928122728	PREDICTED: ezrin isoform X1 *[Canis lupus familiaris]*	−0.37	0.006
928167632	PREDICTED: serotransferrin *[Canis lupus familiaris]*	−0.38	0.021
14331125	Carboxylesterase D1 *[Canis lupus familiaris]*	−0.39	0.020
545524893	PREDICTED: fibrinogen beta chain *[Canis lupus familiaris]*	−0.40	0.031
545546412	PREDICTED: alpha-2-macroglobulin-like isoform X1 *[Canis lupus familiaris]*	−0.40	0.015
545546414	PREDICTED: alpha-2-macroglobulin-like isoform X2 *[Canis lupus familiaris]*	−0.40	0.015
545492085	PREDICTED: F-actin-capping protein subunit beta isoform X4 *[Canis lupus familiaris]*	−0.41	0.002
P47756-1	F-actin-capping protein subunit beta	−0.41	0.002
928128987	PREDICTED: F-actin-capping protein subunit beta isoform X2 *[Canis lupus familiaris]*	−0.41	0.002
928128985	PREDICTED: F-actin-capping protein subunit beta isoform X1 *[Canis lupus familiaris]*	−0.41	0.002
P47756-2	Isoform 2 of F-actin-capping protein subunit beta	−0.41	0.002
545492083	PREDICTED: F-actin-capping protein subunit beta isoform X3 *[Canis lupus familiaris]*	−0.41	0.002
545492087	PREDICTED: F-actin-capping protein subunit beta isoform X5 *[Canis lupus familiaris]*	−0.41	0.002
P02774-2	Isoform 2 of Vitamin D-binding protein	−0.43	0.037
P02774-3	Isoform 3 of Vitamin D-binding protein	−0.44	0.047
P02774	Vitamin D-binding protein	−0.44	0.047
545507351	PREDICTED: L-lactate dehydrogenase A chain-like *[Canis lupus familiaris]*	−0.44	0.050
345798353	PREDICTED: protein S100-P *[Canis lupus familiaris]*	−0.45	0.007

(*continued on next page*)

Table 2 (*continued*)

Accession*	Description	Fold T1/T0	p value
645985803	Chain B, Crystal Structure Of A Dog Lipocalin Allergen	−0.46	0.030
P61158	Actin-related protein 3	−0.52	0.017
345784150	PREDICTED: actin-related protein 3 *[Canis lupus familiaris]*	−0.52	0.017
Q56UQ5	TPT1-like protein	−0.52	0.047
57088159	PREDICTED: glutathione S-transferase Mu 3 *[Canis lupus familiaris]*	−0.53	0.013
345778725	PREDICTED: lactoylglutathione lyase *[Canis lupus familiaris]*	−0.53	0.030
73947982	PREDICTED: glucose-6-phosphate isomerase *[Canis lupus familiaris]*	−0.83	0.030
P68871	Hemoglobin subunit beta	−0.95	0.019
928133662	PREDICTED: apolipoprotein A-I *[Canis lupus familiaris]*	−1.03	0.039
3915607	RecName: Full = Apolipoprotein A-I	−1.04	0.045
P00918	Carbonic anhydrase 2	−1.07	0.005
103484123	Globin, partial *[Canis lupus familiaris]*	−1.16	0.018
227343817	Chain B, Crystal Structure Of Dog (*Canis Familiaris*) Hemoglobin	−1.20	0.033
399567834	Hemoglobin subunit beta-like *[Canis lupus familiaris]*	−1.20	0.029
P02042	Hemoglobin subunit delta	−1.23	0.035
73980925	PREDICTED: thymosin beta-10 *[Canis lupus familiaris]*	−1.34	0.012
545557464	PREDICTED: thymosin beta-4 *[Canis lupus familiaris]*	−1.34	0.012
345784463	PREDICTED: thymosin beta-4 *[Canis lupus familiaris]*	−1.34	0.012

* Accession number from NCBI protein database for *Canis Lupus familiaris*.

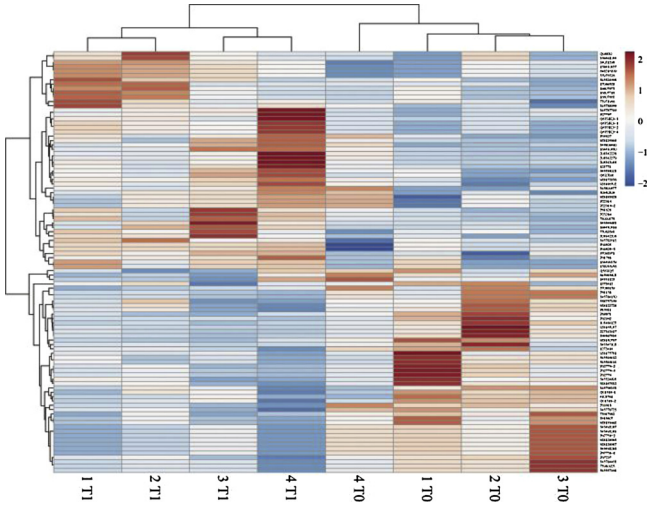

Fig. 1. Heatmap showing the relative abundance (color) and relationship (dendrogram) of salivary proteins before (T0) and after (T1) the experimental infection with L. *infantum* and the appearance of clinical signs.

in IntAct, STRING, Reactome, and Apid databases (Fig. 2). The performed analysis indicates that most of the identified proteins were present in the extracellular compartment and had crossroads between them.

Further GO analysis done with Cytoscape plugin ClueGO identified 115 GO terms differing between conditions T0 and T1, which were filtered for redundancies into 62 terms and then grouped into 18 main groups using REVIGO. The groups included pyruvate metabolism, regulated exocytosis, supramolecular fiber organization, regulation of body fluid levels, response to wounding, drug metabolisms, response to toxic substance, generation of precursor metabolites and energy, low-density lipoprotein receptor particle metabolism, antigen processing and presentation of exogenous peptide antigen, cofactor metabolism, regulation of small GTPase mediated signal transduction, carbohydrate metabolism, regulation of plasma lipoprotein particle levels, low-density lipoprotein particle clearance, receptor metabolism, negative regulation of cell killing and indolakylamine metabolism (Fig. 3).

Finally, the GO terms most represented in this study after being filtered by REVIGO and depicted by Cytoscape are shown in Fig. 4, together with proteins at the crossroad between at least 2 GO terms. When the network was analysed, carbohydrate metabolic process showed a central role in the development of the disease since it is related to the most of represented GO terms and proteins.

4. Discussion

This paper was focused on the identification of the differentially expressed proteins in saliva, as well as in describing the biological processes affected due to CanL. This high-resolution quantitative proteomic analysis performed for the first time in saliva of dogs with CanL allowed the identification of more than two thousands of salivary proteins, 47 of which showed significantly different abundance after the experimental infection with *L. infantum* and the appearance of clinical symtoms. Overall the variations in salivary proteins expression in the same dogs before and after the infection and development of canine leishmaniosis detected by high-resolution quantitative proteomics, indicate that there are physiopathological changes in saliva ocurring during the development of CanL.

The proteins that showed to be the most down-regulated after the development of clinical signs of CanL were thymosin beta-10 and beta-4, haemoglobin, carbonic anhydrase 2, apolipoprotein A-I, and glucose-6-phospate isomerase. Beta-thymosins are a versatile family of small peptides (5 kDa) related to a variety of functions such as cardiac protection, wound healing stimulation or angiogenesis (Bosello et al., 2016). In humans, TMSB10 has been reported to be overexpressed in carcinogenesis; while thymosin beta 4 increased abundance has been reported in the presence of oxidative stress due to its potential scavenger properties (De Santis et al., 2011). In mice infected with Leishmania, MALDI MS analysis of liver suggested an important role of thymosin beta-4 levels in acute infection stage and development of the disease, being proposed as a potential key biomarker of leishmaniosis (Jaegger et al., 2017). Hemoglobins are presented in almost all vertebrates' red blood cells, playing a key role in iron-containing oxygen transport, among other functions. Outside red blood cells, haemoglobin acts as antioxidant and regulates iron metabolism. Inflammation, as the caused by CanL, may cause anaemia and prevent the body from using stored iron reservoirs, causing a decrease in hemoglobin. Acordingly, blood hemoglobin was decreased in beagles after the intravenous infection with *Leishmania chagasi* (Paranhos-Silva et al., 2003). In previous studies, haemoglobin subunit beta was found to be down-regulated in serum of dogs after anti-leishmania treatment (Escribano et al., 2016; Martínez-Subiela et al., 2017). In canine saliva, carbonic anhydrases have been shown to have antimicrobial properties by binding to *Staphylococcus aureus* (Torres et al., 2018) and contributes maintaining oral pH homeostasis (Delius et al., 2017). Carbonic anhydrases inhibitors have been proposed as new targets with biomedical application, including treatments against *Leishmania* carbonic anhydrases

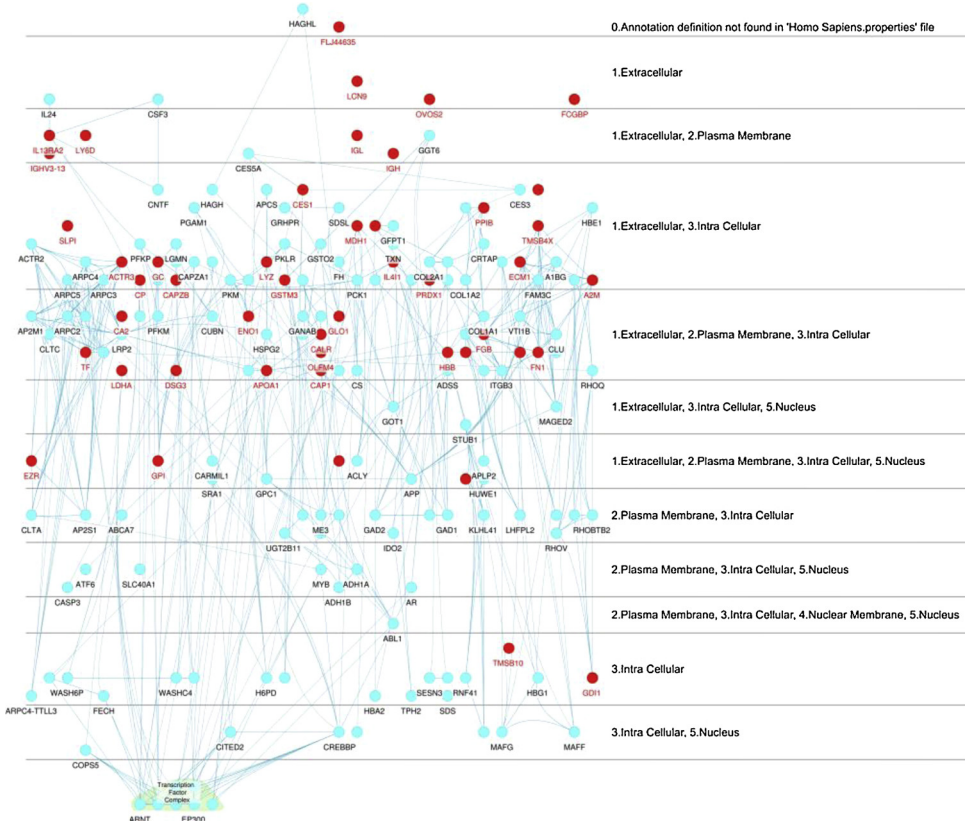

Fig. 2. Interactome of significant deregulated proteins in saliva of dogs after the development of CanL enriched with the best interactors visualized by Cytoscape/ClueGO (CEREBRAL layout), and organized by cell compartment. Deregulated proteins used to generate this network are highlighted in red.

(Vermelho et al., 2017). The low levels of carbonic anhydrase found in our study could be due to the response of the organism against the parasite by decreasing antioxidant compounds. Apolipoprotein A-I is the major protein component of high-density lipoproteins in most mammals (Huang et al., 2014) and it is considered as a negative marker of inflammation in humans (Li et al., 2011). Our results showed decreased abundance of apolipoprotein A-I in saliva after the development of CanL. Previous reports also described decreases in apolipoprotein A-I in serum of dogs with CanL (Escribano et al., 2016; Martínez-Subiela et al., 2017), canine idiopathic dilated cardiomyopathy (Bilić et al., 2018), or in saliva of dogs with canine parvovirosis (Franco-Martínez et al., 2018).

On the other hand, among the most up-regulated proteins in saliva from dogs with CanL were immunoglobulins, olfactomedin-4, and ceruloplasmin-like protein. Higher abundance of immunoglobulins in saliva were expected since increases in immunoglobulins in CanL were reported previously both in serum and in saliva (Britti et al., 2010; Cantos-Barreda et al., 2017). In this recent study, IgG2 has shown higher increases than IgA in saliva and has been proposed as a potential biomarker of diagnosis (Cantos-Barreda et al., 2017). Olfactomedin-4 is

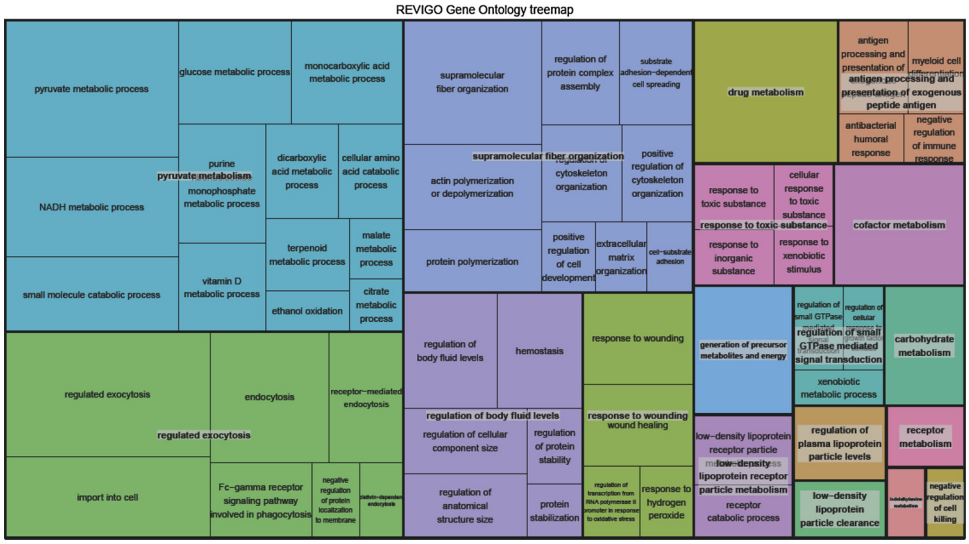

Fig. 3. Over-represented GO terms grouped by REVIGO, based on their description. Leading GO term (N = 18) for each group was defined as the one charring the highest proportion of genes with the enriched network.

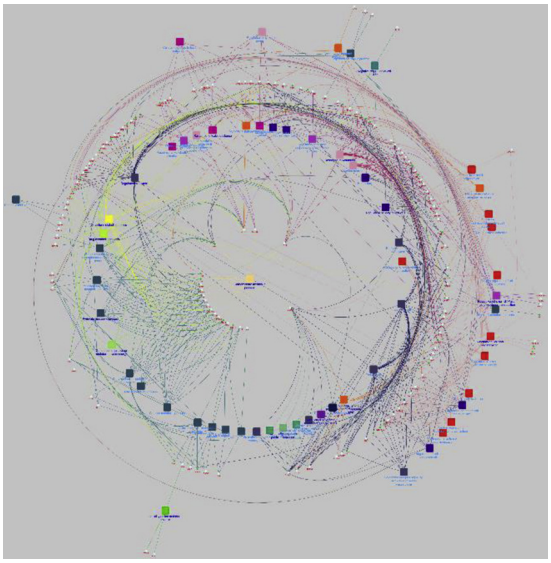

Fig. 4. *In silico* inferred interactome network of identified GO terms over-represented in canine leishmaniosis. Differentially expressed proteins interacting with at least 1 term were added. Radial layout was applied and the GO group leader terms are in dark blue text. Nodes colours represent the group of GO terms (determined by ReviGO).

a glycoprotein which is highly expressed in small intestine, colon, and prostate, being up-regulated in several digestive diseases such as *Helicobacter pylori* infections, inflammatory bowel disease or cancer (Wang et al., 2018). Although the relationship between olfactomedin-4 and CanL is unclear, CanL is known to be able to cause digestive disorders (Solano-Gallego et al., 2011), which could explain the rise of olfactomedin-4. Glutamine synthetases catalyze ATP-dependent conversion of glutamate and ammonia to glutamine, being required for endothelial cell migration during vascular development (Nilsson et al., 2009). Although there is a lack of information regarding the increase of glutamine synthetases in saliva of dogs with CanL, this pathway in the Leishmania has been proposed as possible candidate for the development of novel anti-leishmania treatments (Kumar et al., 2017). In dogs, ceruloplasmins are considered as moderate acute phase proteins, acting as major preventive antioxidants as well. In agreement with our results, ceruloplasmin was increased in serum of dogs with CanL (Burillo et al., 1994; Solter et al., 1991), although others reported serum decrease in this protein (Bildik et al., 2004).

In addition to these proteins, other proteins were also significantly differentially expressed in saliva after the experimental infection and development of CanL when compared to pre-infection specimens, but their fold change were smaller than ± 0.5849. However, we would like to point out some of these proteins due to their importance in this disease. For example, vitamin D binding proteins decreases in saliva after the development of CanL. This protein is a passive carrier and active facilitator of the cellular uptake of vitamin D and its metabolites, belonging to the albumin superfamily of binding proteins (Chun et al., 2008). The decrease in vitamin D binding protein observed is in agreement with other reports in dogs with inflammatory and necrotic disease such as ehrlichiosis or babesiosis, making this decrease indicative of poorer patient outcome (Escribano et al., 2017; Kuleš et al., 2016b, 2014). Additionally, increases in vitamin D binding protein have been observed in serum after an effective leishmaniosis treatment (Escribano et al., 2016). Similarly, the decreases in salivary serotransferrin after the apparition of clinical signs observed in the present study are compatible with the decrease described in serum in dogs with CanL (Burillo et al., 1994; Martínez-Subiela et al., 2017; Silvestrini et al., 2014).

By analysing the over-represented cellular pathways defined by differentially expressed proteins, it could be observed that they are involved in different physiopathological pathways such as antioxidant activity, binding, catalytic activity, structural molecule activity and transporter activity. Of those, the majority of the differentially expressed genes belong to proteins related to catalytic activity (42.9%) and binding (35.7%). These results are similar to our previous study regarding differentially expressed proteins in saliva of healthy dogs versus dogs with canine parvovirosis, in which more of the 85% of the genes differentially expressed belonged to catalytic activity and binding (Franco-Martínez et al., 2018).

The bioinformatics analysis of the *in silico* inferred proteins network allowed the generation of more information from the experimental results, including data of intracellular pathways. This information revealed the impact of CanL on proteins related to processes such as pyruvate metabolism, regulated exocytosis, supramolecular fiber organization and regulation of body fluid levels, among others. Bioinformatics allowed several pathways and proteins to be highlighted. In this sense, the network created by comparison of T0 and T1 revealed that carbohydrate metabolic process could play an important role in the development of the disease since it is a central node which is related to multiple GO terms and proteins. In the network created, this central node is directly related to 20 proteins, 5 of which (glucose-6-phosphate isomerase (GPI), lactoylglutathione lyase (GLO1), L-lactate dehydrogenase (LDHA), alpha-enolase (ENO1), and malate dehydrogenase (MDH1)) were differentially expressed in the present study. GPI, GLO1, and LDHA were shown to be down-regulated in saliva after the development of CanL, while ENO1 and MDH1 were shown to be up-regulated. Therefore, according to our results and in agreement with previous reports in serum (Saunders et al., 2010), these proteins included in carbohydrate metabolic process could play important roles in the development of CanL and should be considered for further studies.

Due to ethical reasons, the sample size is relatively small, although is higher than the minimum of three biological replicates recommended for proteomic studies (Westermeier et al., 2008). This sample size could have influenced on the potential to identify proteins that are significantly modulated in concentration after the infection with *L. infantum* due to the biological inter-individual variations. Although each dog has its own control sample before the experimental infection, in order to minimize inter-individual variation, ideally, an additional control group with the same conditions of the experimental group but injected with saline solution could have been used in order to confirm that the changes observed would be related to the development of CanL and not as an effect of age or other factors. However, the use of this control group will have a higher component of inter-individual variation. In addition, no significant changes in hematological and biochemical analytes in dogs in the interval of ages of our study have been reported previously (Brenten et al., 2016; Harper et al., 2003), therefore, it could be postulated that age is not a major source of variation of analytes during our study. Finally, treatments were made always at the same time (3 weeks) before samplings in order to minimize the possible variability of their effect. To minimize the inter-individual variation in our study, only proteins identified in all eight samples were selected for subsequent statistical evaluation and bioinformatics analyses.

For clinical interpretation, it should be taken into consideration that the salivary proteome could differ depending on the clinical stage of the sickness and also between experimental or natural infections of *L. infantum*. Thus, further studies of the salivary proteome using high-resolution quantitative proteomic analysis such as TMT-based ones, in dogs naturally infected at different clinical stages according to the Leishvet guidelines and at different time points will answer these questions. In addition, further large-scale studies would be required to evaluate whether these proteins could be included in routine clinical practice as non-invasive biomarkers of diagnosis or response to treatment in canine leishmaniosis.

5. Conclusions

TMT-based proteomic approach allowed the identification of proteins in saliva that change in concentrations in of *Leishmania infantum* infected dogs. Proteins such as immunoglobulins, olfactomedin-4, and ceruloplasmin-like protein were up-regulated in saliva after the development of CanL, while others such as thymosin beta-10 and beta-4, haemoglobin, carbonic anhydrase 2, apolipoprotein A-I, and glucose-6-phosphate isomerase were down-regulated. Some of these proteins such as thymosin beta-10 and beta-4, apolipoprotein A-1, olfactomedin-4, or ceruloplasmin –among others- have been firstly identified in saliva of dogs with CanL in the present study. Finally, bioinformatic analysis revealed that proteins related to carbohydrate metabolic process could play important roles in the development of CanL.

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References

Baeumlisberger, D., Arrey, T.N., Rietschel, B., Rohmer, M., Papasotiriou, D.G., Mueller, B., Beckhaus, T., Karas, M., 2010. Labeling elastase digests with TMT: informational gain by identification of poorly detectable peptides with MALDI-TOF/TOF mass spectrometry. Proteomics 10, 3905–3909. https://doi.org/10.1002/pmic.201000288.

Bildik, A., Kargın, F., Seyrek, K., Pasa, S., Özensoy, S., 2004. Oxidative stress and non-enzymatic antioxidative status in dogs with visceral Leishmaniasis. Res. Vet. Sci. 77, 63–66. https://doi.org/10.1016/J.RVSC.2004.01.005.

Bilić, P., Guillemin, N., Kovačević, A., Beer Ljubić, B., Jović, I., Galan, A., Eckersall, P.D., Burchmore, R., Mrljak, V., 2018. Serum proteome profiling in canine idiopathic dilated cardiomyopathy using TMT-based quantitative proteomics approach. J. Proteomics 179, 110–121. https://doi.org/10.1016/j.jprot.2018.03.007.

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.H., Pagès, F., Trajanoski, Z., Galon, J., 2009. ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics 25, 1091–1093. https://doi.org/10.1093/bioinformatics/btp101.

Bosello, S., Peluso, G., Iavarone, F., Tolusso, B., Messana, I., Faa, G., Castagnola, M., Ferraccioli, G., 2016. Thymosin β 4 and β 10 in Sjögren's syndrome: saliva proteomics and minor salivary glands expression. Arthritis Res. Ther. 18. https://doi.org/10.1186/s13075-016-1134-7.

Brenten, T., Morris, P.J., Salt, C., Raila, J., Kohn, B., Schweigert, F.J., Zentek, J., 2016. Age-associated and breed-associated variations in haematological and biochemical variables in young labrador retriever and miniature schnauzer dogs. Vet. Rec. Open 3, e000166. https://doi.org/10.1136/vetreco-2015-000166.

Britti, D., Gaspari, M., Massimini, G., Casalinuovo, F., Morittu, V.M., Cuda, G., 2010. Proteomic analysis in canine leishmaniasis. Vet. Res. Commun. 91–96. https://doi.org/10.1007/s11259-010-9410-5.

Burillo, F.L., Perez, F.M.G., Liesa, J.P., Fabian, M.C.A., 1994. Iron status and anemia in canine leishmaniasis. Rev. Med. Vet. (Toulouse) 145, 171–176.

Cantos-Barreda, A., Escribano, D., Bernal, L.J., Cerón, J.J., Martínez-Subiela, S., 2017. Quantification of anti-Leishmania antibodies in saliva of dogs. Vet. Parasitol. 242, 54–58. https://doi.org/10.1016/j.vetpar.2017.05.017.

Chun, R.F., Adams, J.S., Hewison, M., 2008. Back to the future: a new look at "old" vitamin D. J. Endocrinol. https://doi.org/10.1677/JOE-08-0170.

Contreras-Aguilar, M.D., Tecles, F., Martínez-Subiela, S., Escribano, D., Bernal, L.J., Cerón, J.J., 2017. Detection and measurement of alpha-amylase in canine saliva and changes after an experimentally induced sympathetic activation. BMC Vet. Res. 13, 266. https://doi.org/10.1186/s12917-017-1191-4.

Corpas-López, V., Merino-Espinosa, G., Acedo-Sánchez, C., Díaz-Sáez, V., Morillas-Márquez, F., Martín-Sánchez, J., 2016. Hair parasite load as a new biomarker for monitoring treatment response in canine leishmaniasis. Vet. Parasitol. 223, 20–25. https://doi.org/10.1016/j.vetpar.2016.04.001.

Dayon, L., Turck, N., García-Berrocoso, T., Walter, N., Burkhard, P.R., Vilalta, A., Sahuquillo, J., Montaner, J., Sanchez, J.-C., 2011. Brain extracellular fluid protein changes in acute stroke patients. J. Proteome Res. 10, 1043–1051. https://doi.org/10.1021/pr101123t.

De Santis, M., Inzitari, R., Bosello, S.L., Peluso, G., Fanali, C., Iavarone, F., Zizzo, G., Bocci, M., Cabras, T., Messana, I., Fuso, L., Varone, F., Pagliari, G., Castagnola, M., Ferraccioli, G., 2011. β-thymosins and interstitial lung disease: study of a scleroderma cohort with a one-year follow-up. Respir. Res. 12. https://doi.org/10.1186/1465-9921-12-22.

Delius, J., Trautmann, S., Médard, G., Kuster, B., Hannig, M., Hofmann, T., 2017. Label-free quantitative proteome analysis of the surface-bound salivary pellicle. Colloids Surf. B Biointerfaces 152, 68–76. https://doi.org/10.1016/j.colsurfb.2017.01.005.

Escribano, D., Cihan, H., Martínez-Subiela, S., Levent, P., Kocaturk, M., Aytug, N., Cerón, J.J., Tvarijonaviciute, A., Yilmaz, Z., 2017. Changes in serum proteins in dogs with Ehrlichia canis infection. Microb. Pathog. 113, 34–39. https://doi.org/10.1016/j.micpath.2017.10.024.

Escribano, D., Tvarijonaviciute, A., Kocaturk, M., Cerón, J.J., Pardo-Marín, L., Torrecillas, A., Yilmaz, Z., Martínez-Subiela, S., 2016. Serum apolipoprotein-A1 as a possible biomarker for monitoring treatment of canine leishmaniosis. Comp. Immunol. Microbiol. Infect. Dis. 49, 82–87. https://doi.org/10.1016/j.cimid.2016.10.002.

Franco-Martínez, L., Tvarijonaviciute, A., Horvatić, A., Guillemin, N., Cerón, J.J., Escribano, D., Eckersall, D., Kocatürk, M., Yilmaz, Z., Lamy, E., Martínez-Subiela, S., Mrljak, V., 2018. Changes in salivary analytes in canine parvovirus: a high-resolution quantitative proteomic study. Comp. Immunol. Microbiol. Infect. Dis. 60, 1–10. https://doi.org/10.1016/J.CIMID.2018.09.011.

Giron, P., Dayon, L., Turck, N., Hoogland, C., Sanchez, J.-C., 2011. Quantitative analysis of human cerebrospinal fluid proteins using a combination of cysteine tagging and amine-reactive isobaric labeling. J. Proteome Res. 10, 249–258. https://doi.org/10.1021/pr100535f.

Gramiccia, M., Gradoni, L., 2005. The current status of zoonotic leishmaniases and approaches to disease control. Int. J. Parasitol. 35, 1169–1180. https://doi.org/10.1016/j.ijpara.2005.07.001.

Guillemin, N., Horvati, A., Kuleš, J., Galan, A., Mrljak, V., Bhide, M., 2006. Omics approaches to probe markers of disease resistance in animal sciences. Mol. BioSyst. 12, 2036–2046. https://doi.org/10.1039/c6mb00220j.

Harper, E.J., Hackett, R.M., Wilkinson, J., Heaton, P.R., 2003. Age-related variations in hematologic and plasma biochemical test results in Beagles and Labrador Retrievers. J. Am. Vet. Med. Assoc. 223, 1436–1442.

Hernández, L., Montoya, A., Checa, R., Dado, D., Gálvez, R., Otranto, D., Latrofa, M.S., Baneth, G., Miró, G., 2015. Course of experimental infection of canine leishmaniosis: follow-up and utility of noninvasive diagnostic techniques. Vet. Parasitol. 207, 149–155. https://doi.org/10.1016/j.vetpar.2014.10.035.

Huang, Y., DiDonato, J.A., Levison, B.S., Schmitt, D., Li, L., Wu, Y., Buffa, J., Kim, T., Gerstenecker, G.S., Gu, X., Kadiyala, C.S., Wang, Z., Culley, M.K., Hazen, J.E., Didonato, A.J., Fu, X., Berisha, S.Z., Peng, D., Nguyen, T.T., Liang, S., Chuang, C.-C., Cho, L., Plow, E.F., Fox, P.L., Gogonea, V., Tang, W.H.W., Parks, J.S., Fisher, E.A., Smith, J.D., Hazen, S.L., 2014. An abundant dysfunctional apolipoprotein A1 in human atheroma. Nat. Med. 20, 193–203. https://doi.org/10.1038/nm.3459.

Jaegger, C.F., Negrão, F., Assis, D.M., Belaz, K.R.A., Angolini, C.F.F., Fernandes, A.M.A.P., Santos, V.G., Pimentel, A., Abánades, D.R., Giorgio, S., Eberlin, M.N., Rocha, D.F.O., 2017. MALDI MS imaging investigation of the host response to visceral leishmaniasis. Mol. Biosyst. 13, 1946–1953. https://doi.org/10.1039/c7mb00306d.

Kuleš, J., de Torre-Minguela, C., Barić Rafaj, R., Gotić, J., Nižić, P., Ceron, J.J., Mrljak, V., 2016a. Plasma biomarkers of SIRS and MODS associated with canine babesiosis. Res. Vet. Sci. 105, 222–228. https://doi.org/10.1016/j.rvsc.2016.02.011.

Kuleš, Josipa, Horvatić, A., Guillemin, N., Galan, A., Mrljak, V., Bhide, M., 2016b. New approaches and omics tools for mining of vaccine candidates against vector-borne diseases. Mol. Biosyst. 12, 2680–2694. https://doi.org/10.1039/C6MB00268D.

Kuleš, J., Mrljak, V., Barić Rafaj, R., Selanec, J., Burchmore, R., Eckersall, P.D., 2014. Identification of serum biomarkers in dogs naturally infected with Babesia canis canis using a proteomic approach. BMC Vet. Res. 10, 111. https://doi.org/10.1186/1746-6148-10-111.

Kumar, V., Yadav, S., Soumya, N., Kumar, R., Babu, N.K., Singh, S., 2017. Biochemical and inhibition studies of glutamine synthetase from Leishmania donovani. Microb. Pathog. 107, 164–174. https://doi.org/10.1016/J.MICPATH.2017.03.024.

Li, H., Li, C., Wu, H., Zhang, T., Wang, J., Wang, S., Chang, J., 2011. Identification of Apo-A1 as a biomarker for early diagnosis of bladder transitional cell carcinoma. Proteome Sci. 9, 21. https://doi.org/10.1186/1477-5956-9-21.

Martínez-Subiela, S., Ceron, J.J., Yilmaz, Z., Martinez-Subiela, S., Horvatic, A., Escribano, D., Pardo-Marin, L., Kocaturk, M., Mrljak, V., Burchmore, R., Ceron, J.J., Yilmaz, Z., 2017. Identification of novel biomarkers for treatment monitoring in canine leishmaniosis by high-resolution quantitative proteomic analysis. Vet. Immunol. Immunopathol. 191, 60–67. https://doi.org/10.1016/j.cimid.2016.10.002.

Metsalu, T., Vilo, J., 2015. ClustVis: a web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. Nucleic Acids Res. 43, W566–W570. https://doi.org/10.1093/nar/gkv468.

Mohapatra, S., Samantaray, J.C., Ghosh, A., 2016. A comparative study of serum, urine and saliva using rk39 strip for the diagnosis of visceral leishmaniasis. J. Arthropod. Dis. 10, 87–91.

Nabity, M.B., Lees, G.E., Dangott, L.J., Cianciolo, R., Suchodolski, J.S., Steiner, J.M., 2011. Proteomic analysis of urine from male dogs during early stages of tubulointerstitial injury in a canine model of progressive glomerular disease. Vet. Clin. Pathol. 40, 222–236. https://doi.org/10.1111/j.1939-165X.2011.00307.x.

Nilsson, M.T., Krajewski, W.W., Yellagunda, S., Prabhumurthy, S., Chamarahally, G.N., Siddamadappa, C., Srinivasa, B.R., Yahiaoui, S., Larhed, M., Karlén, A., Jones, T.A., Mowbray, S.L., 2009. Structural basis for the inhibition of Mycobacterium tuberculosis glutamine synthetase by novel ATP-competitive inhibitors. J. Mol. Biol. https://doi.org/10.1016/j.jmb.2009.08.028.

Paranhos-Silva, M., Oliveira, G.G.S., Reis, E.A., de Menezes, R.M.C., Fernandes, O., Sherlock, Í., Gomes, R.B.B., Pontes-de-Carvalho, L.C., dos-Santos, W.L.C., 2003. A

follow-up of Beagle dogs intradermally infected with Leishmania chagasi in the presence or absence of sand fly saliva. Vet. Parasitol. 114, 97–111. https://doi.org/10.1016/S0304-4017(03)00132-8.

Parra, M.D., Tecles, F., Martínez-Subiela, S., Cerón, J.J., 2005. C-reactive protein measurement in canine saliva. J. Vet. Diagn. Invest. 17, 139–144. https://doi.org/10.1177/104063870501700207.

RStudio Team, 2015. RStudio: Integrated Development Environment for R [Internet]. Available from:. RStudio, Inc., Boston, MA. http://www.rstudio.com/.

Ruiz-Romero, C., Blanco, F.J., 2010. Proteomics role in the search for improved diagnosis, prognosis and treatment of osteoarthritis. Osteoarthr. Cartil. 18, 500–509. https://doi.org/10.1016/j.joca.2009.11.012.

Saunders, E.C., De Souza, D.P., Naderer, T., Sernee, M.F., Ralton, J.E., Doyle, M.A., MacRae, J.I., Chambers, J.L., Heng, J., Nahid, A., Likic, V.A., McConville, M.J., 2010. Central carbon metabolism of Leishmania parasites. Parasitology. https://doi.org/10.1017/S0031182010000077.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software Environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504. https://doi.org/10.1101/gr.1239303.

Silvestrini, P., Zoia, A., Planellas, M., Roura, X., Pastor, J., Cerón, J.J., Caldin, M., 2014. Iron status and C-reactive protein in canine leishmaniasis. J. Small Anim. Pract. 55, 95–101. https://doi.org/10.1111/jsap.12172.

Solano-Gallego, L., Miró, G., Koutinas, A., Cardoso, L., Pennisi, M., Ferrer, L., Bourdeau, P., Oliva, G., Baneth, G., 2011. LeishVet guidelines for the practical management of canine leishmaniosis. Parasit. Vectors 4, 86. https://doi.org/10.1186/1756-3305-4-86.

Solter, P.F., Hoffmann, W.E., Hungerford, L.L., Siegel, J.P., St Denis, S.H., Dorner, J.L., 1991. Haptoglobin and ceruloplasmin as determinants of inflammation in dogs. Am. J. Vet. Res. https://doi.org/10.1016/S0168-1699(99)00043-5.

Supek, F., Bošnjak, M., Škunca, N., Šmuc, T., 2011. Revigo summarizes and visualizes long lists of gene ontology terms. PLoS One 6. https://doi.org/10.1371/journal.pone.0021800.

Taslimi, Y., Sadeghipour, P., Habibzadeh, S., Mashayekhi, V., Mortazavi, H., Müller, I., Lane, M.E., Kropf, P., Rafati, S., 2017. A novel non-invasive diagnostic sampling technique for cutaneous leishmaniasis. PLoS Negl. Trop. Dis. 11, e0005750. https://doi.org/10.1371/journal.pntd.0005750.

Torres, S.M.F., Furrow, E., Souza, C.P., Granick, J.L., de Jong, E.P., Griffin, T.J., Wang, X., 2018. Salivary proteomics of healthy dogs: an in depth catalog. PLoS One 13, e0191307. https://doi.org/10.1371/journal.pone.0191307.

Vaish, M., Chakravarty, J., Sundar, S., Singh, O.P., 2012. rK39 antigen for the diagnosis of visceral leishmaniasis by using human saliva. Am. J. Trop. Med. Hyg. 86, 598–600. https://doi.org/10.4269/ajtmh.2012.11-0127.

Vermelho, A.B., Capaci, G.R., Rodrigues, I.A., Cardoso, V.S., Mazotto, A.M., Supuran, C.T., 2017. Carbonic anhydrases from Trypanosoma and Leishmania as anti-protozoan drug targets. Bioorg. Med. Chem. 25, 1543–1555. https://doi.org/10.1016/j.bmc.2017.01.034.

Wang, X.-Y., Chen, S.-H., Zhang, Y.-N., Xu, C.-F., 2018. Olfactomedin-4 in digestive diseases: a mini-review. World J. Gastroenterol. 24, 1881–1887. https://doi.org/10.3748/wjg.v24.i17.1881.

Westermeier, R., Naven, T., Höpker, H.-R., Wiley InterScience (Online service), 2008. Proteomics in Practice: A Guide to Successful Experimental Design. Wiley-VCH.

Yoshizawa, J.M., Schafer, C.A., Schafer, J.J., Farrell, J.J., Paster, B.J., Wong, D.T.W., 2013. Salivary biomarkers: toward future clinical and diagnostic utilities. Clin. Microbiol. Rev. 26, 781–791. https://doi.org/10.1128/CMR.00021-13.