THE USE OF SALIVARY PROTEOME FOR UNDERSTANDING DOG OBESITY

SÓNIA FÉLIX VILAS BOAS DE LUCENA

Tese apresentada à Universidade de Évora
para obtenção do grau de Doutor em Ciências Veterinárias
Especialidade: Ciências Veterinárias

ORIENTADORES: *Elsa Cristina Carona de Sousa Lamy*
Asta Tvarijonaviciute
Fernando Manuel Salvado Capela e Silva

ÉVORA, MAIO DE 2019

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

THE USE OF SALIVARY PROTEOME FOR UNDERSTANDING DOG OBESITY

SÓNIA FÉLIX VILAS BOAS DE LUCENA

Tese apresentada à Universidade de Évora
para obtenção do grau de Doutor em Ciências Veterinárias
Especialidade: Ciências Veterinárias

ORIENTADORES: *Elsa Cristina Carona de Sousa Lamy*
Asta Tvarijonaviciute
Fernando Manuel Salvado Capela e Silva

ÉVORA, MAIO DE 2019

INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

Constituição do Júri

Presidente: Doutora Rita Maria Payan Martins Pinto Carreira, Professora Catedrática da Universidade de Évora.

Vogais:

Doutora Graça Maria Leitão Ferreira Dias, Professora Catedrática, da Universidade de Lisboa;

Doutor Francisco Manuel Lemos Amado, Professor Associado c/ Agregação, da Universidade de Aveiro;

Doutora Sílvia Martinez Subiela, Professora Titular, da Universidade de Múrcia;

Doutora Catarina Falcão Trigoso Vieira Branco Lavrador, Professora Auxiliar, da Universidade de Évora;

Doutora Asta Tvarijonaviciute, Post-doc, da Universidade de Múrcia, orientadora.

ACKNOWLEDGEMENTS

ABSTRACT

Obesity became one of the most important health issues of humans and pets. Some studies report a relationship between owner and dog obesity, so this condition should be considered within the concept of "One Health". The literature found about this issue show differences among countries in dog obesity prevalence and no reports were found for Portugal. As in humans, dog obesity has been linked to several related conditions. Saliva composition is affected by obesity and these related diseases. However, dog salivary proteome as a diagnosis fluid has been quite unexplored. The main goal of this thesis was to study obesity-related changes in dogs' salivary proteome, aiming at improving understanding of the obesity pathophysiology and/or to identify potential biomarkers. Initially, dog obesity prevalence, in Portugal, was studied, evidencing high rates. Secondly, to solve methodological difficulties in dog saliva analysis, the optimization of protocols was needed. Protein precipitation using 20% (w/v) tichloroacetic acid was the concentration method able to achieve protein profiles highly correlated with the ones from original samples. Moreover, it was observed that dog salivary proteome is influenced by breed and acid stimulation. The two final studies evidenced changes in saliva protein composition in: 1) obesity-related metabolic dysfunction (ORMD); 2) metabolic changes induced by weight loss. Comparison of salivary proteomes of dogs with and without ORMD revealed that proteins related with inflammation and prothrombotic state differ significantly between groups. Concerning experimentally-induced weight loss, changes in salivary proteins were observed, which were correlated with the percentage of weight loss. Several of these proteins are related with immune system/inflammation, oxidative stress and glucose metabolism. The correlation of some salivary proteins with serum triglycerides and cholesterol levels were observed. This thesis reinforces the relevance of salivary proteome in obesity study and the potential use of saliva in veterinary health.

Key-words: Dog, saliva, proteome, obesity, One Health

A UTILIZAÇÃO DO PROTEOMA SALIVAR PARA COMPREENDER A OBESIDADE CANINA

RESUMO:

A obesidade tornou-se um dos maiores tópicos da saúde humana e animal. Dada a relação comprovada entre a obesidade canina e humana, esta deve ser abordada no âmbito do conceito "Uma Só Saúde". Na literatura, a prevalência da obesidade canina apresenta diferenças entre países não existindo informação para Portugal. A obesidade canina e humana estão associadas a diversas condições. A composição da saliva é afectada pela obesidade e doenças associadas. Em cães, o proteoma salivar está pouco explorado como meio de diagnóstico. O principal objectivo desta tese foi estudar alterações no proteoma salivar associadas à obesidade canina, no sentido de melhorar a compreensão da sua patofisiologia e/ou identificar potenciais biomarcadores. Inicialmente estudou-se a prevalência da obesidade canina em Portugal, obtendo-se valores elevados. Para resolver dificuldades metodológicas relativas à análise da saliva de cão foi necessário optimizar protocolos. A precipitação proteica utilizando ácido tricloroacético (20%; w/v) foi o método de concentração que permitiu perfis proteicos altamente correlacionados com os das amostras originais. A raça e a estimulação com ácido foram factores que influenciaram o proteoma salivar. Finalmente foram observadas alterações na composição proteica da saliva: 1) na disfunção metabólica associada à obesidade (ORMD); 2) em alterações metabólicas induzidas pela perda de peso. A comparação do proteoma salivar revelou existirem diferenças significativas em cães com e sem ORMD, em proteínas relacionadas com estados inflamatórios e protrombóticos. Relativamente ao estudo da perda de peso induzida experimentalmente, observaram-se alterações nas proteínas salivares, as quais foram correlacionadas com a percentagem da perda de peso. Algumas destas proteínas estão associadas ao sistema imunitário, inflamação, *stress* oxidativo e metabolismo da glucose. Foi verificada correlação entre algumas proteínas salivares e os níveis séricos de triglicerídeos e colesterol. Esta tese reforça a relevância do proteoma salivar no estudo da obesidade canina e o seu potencial uso na saúde veterinária.

Palavras-chave: Cão, saliva, proteoma, obesidade, Uma só Saúde

INDEX OF CONTENTS

I – INDEX OF FIGURES

II – INDEX OF TABLES

III – LIST OF ABBREVIATIONS

AB - ammonium bicarbonate
ACN - acetonitrile
ANGPTL5- angiopoietin like 5
BF - body fat
BFI - body fat index
BCS - body condition score
BChE - butyrylcholinesterase
BLAST - Basic Local Alignment Search Tool
BMI - Body Mass Index
BPIFA1 - Bactericidal permeability increasing fold containing family A1
BPIFA2 - Bactericidal/permeability increasing fold containing family A2
BSA – bovine serum albumin
BVRB - Biliverdin reductase B
BW - body weight
CAVI – Carbonic anhydrase VI
CAI - Carbonic anhydrase I
CAII - Carbonic anhydrase II
CBB - Coomassie Brilliant Blue partial
CE - capillary electrophoresis
CHAPS - 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CRP - C-reactive protein
DEXA- dual-energy X-ray absorptiometry
2-DE - two-dimensional electrophoresis 1D-LC - One-dimension liquid chromatography
2D-LC - two-dimensional liquid chromatography
DM - Diabetes Mellitus
DNA - deoxyribonucleic acid
DTT – dithiothreitol
FA - formic acid
FCI – Federation Cynologique Internationale
FDR – false discovery rate
GO - Gene Ontology
HDL - High Density Lipoprotein –Cholesterol
HCl – chloridric acid
IAA – iodoacetamida
IDA - information-dependent acquisition
IEF - isoelectric focusing
IFN-γ - interferon-γ
Ig - immunoglobulin
IgA – immunoglobulin A
IgG – immunoglobulin G
IgE – immunoglobulin E
IGF-1 - insulin-like growth factor-1
IL6 - interleukin-6
IPG – immobilized pH gradient
KLK - Kallikreins
LC-MS - liquid chromatography coupled to mass spectrometry

LC-MS/MS - liquid chromatography tandem mass spectrometry
LDL-cholesterol - low-density lipoprotein-cholesterol
LTA$_4$H - Leukotriene A(4) hydrolase MALDI-TOF/MS - matrix-assisted laser desorption ionization-time of flight mass spectrometry
MCS - muscle condition score T4 - thyroxine
MetS - metabolic syndrome
MRI - magnetic resonance imaging
mRNA – messenger ribonucleic acid MS - mass spectrometry
NAC - N-acetyl cysteine
NCBI – National Centre of Biotechnology Information
ORMD - Obesity-related metabolic dysfunction
PANTHER - protein annotation through evolutionary relationship
PCR - Polymerase Chain Reaction
pI - isoelectric points
PK – prekallikrein, kallikrein precursor
PLS-DA - least squares discriminant analysis
PMF - Peptide mass fingerprinting
PRPs - proline-rich proteins T3 - triiodothyronine
RBP4 - Retinol binding protein 4
rpm – revolutions per minute
SBP/SAP - systolic blood pressure/ systolic arterial pressure
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD - standard deviations
SELDI-TOF/MS - surface enhanced laser desorption/ionization time-of-flight mass spectrometry
sIgA - secretory IgA
SWATH-MS - Sequential Window Acquisition of All Theoretical Mass Spectra
TCA - trichloroacetic acid
T2DM/T2D - type 2 diabetes mellitus/ type 2 diabetes
TNF-α - Tumour necrosis factor alpha
VDBP - Gc vitamin D-binding protein
VIP variable importance in the projection
WHO - World Health Organization
WSAVA - World Small Animal Veterinary Association

CHAPTER I
GENERAL INTRODUCTION

1. DOG OBESITY

As in humans, obesity is a growing problem worldwide in companion animals, particularly in cats and dogs (1,2). Obesity can be defined as excessive accumulation of adipose tissue in the body causing serious effects on the individual's health and not only shortens the expected lifespan of the affected animals, but also reduces their health-related quality of life (1). Excess body weight is defined as a body composition where the levels of body fat exceed those considered optimal for good health (1). Values ranging from 15 to 30 per cent are considered by several authors as being the 'optimal' per cent body fat (BF) in dogs (3,4).

The onset and progression of obesity translate into changes in body composition. Adipose tissue is a key factor in regulating whole body lipid flow, thereby modulating lipid homeostasis. Therefore, to evaluate overweight/obesity, body conformation and body composition need to be assessed. The Body Condition Scores (BCSs), developed for both dogs and cats, are widely used as morphometric method for assessing the body composition in these animals (2,5). The BCSs include a number of categories, ranging from 'emaciated' to 'severely obese', based on a subjective semi-quantitative method for assessment of specific features. These features include visual inspection of the animal shape viewed from above, and palpable characteristics of the ribs that correlate with subcutaneous and abdominal fat, and superficial musculature (e.g., ribcage, dorsal spinous processes, and waist) (6). BCSs charts can be used for defining these features and can be expressed on a 5-point, 7-point or 9-point scale (2,4,7). BCSs expressed on a 5-point scale were previously used by several authors and correlate well with more advanced measurements of the body fat amount (2,7,8). In a 5-point scale, 1-2 defines underweight dogs, 3 define normal weight dogs and 4-5 defines overweight dogs (Figure 1) (9,10). However, quantification of obesity in dogs is difficult due to the great differences in body type according to breed, age and sex (11,12) and, for this reason, numerous methods were developed for objective evaluation of body composition in this species (2,5). In clinical practice, besides BCS, muscle condition score (MCS) chart are commonly used, as well, to determine the degree of obesity (12). In the same way, a body fat index (BFI) chart focused on the estimation of body fat percentage in overweight and obese dogs was recently developed (6). These charts are based on animals' observation and palpation. However, determination of the degree of obesity by these methods can vary among different evaluators and due to the experience and subjectivity of them in each case. Thus obtained data can present bias.

SELECT A BODY CONDITION SCORE FOR YOUR PET

①	②	③	④	⑤
VERY THIN	**UNDERWEIGHT**	**IDEAL**	**OVERWEIGHT**	**OBESE**
RIBS – Easily felt with no fat cover TAIL BASE – Bones are raised, no fat cover SIDE VIEW – Severe abdominal tuck OVERHEAD VIEW – Accentuated hourglass shape	RIBS – Easily felt with little fat cover TAIL BASE – Bones are raised with slight fat cover SIDE VIEW – Abdominal tuck OVERHEAD VIEW – Marked hourglass shape	RIBS – Easily felt with slight fat cover TAIL BASE – Smooth contour with slight fat cover SIDE VIEW – Abdominal tuck OVERHEAD VIEW – Well proportioned waist	RIBS – Difficult to feel under moderate fat cover TAIL BASE – Some thickening, bones palpable under moderate fat cover SIDE VIEW – No abdominal tuck OVERHEAD VIEW – Back is slightly broadened at waist	RIBS – Difficult to feel under thick fat cover TAIL BASE – Thickened and difficult to feel under thick fat cover SIDE VIEW – No waist, fat hangs from abdomen OVERHEAD VIEW – Back is markedly broadened

Ribs – easily felt with no fat cover **Tail base** – bones are raised, no fat cover **Side view** – severe abdominal tuck **Overhead view** – accentuated hourglass	**Ribs** – easily felt with no fat cover **Tail base** – bones are raised with slight fat cover **Side view** – abdominal tuck **Overhead view** – middle hourglass shape	**Ribs** – easily felt with slight fat cover **Tail base** – smooth contour with slight fat cover **Side view** – abdominal tuck **Overhead view** – well proportionated waist	**Ribs** – difficult to feel under moderate fat cover **Tail base** – some thickming, bones palpable under moderate fat cover **Side view** – no abdominal tuck **Overhead view** – back is slightly broadened at waist	**Ribs** – difficult to feel under tick fat cover **Tail base** – thickened and difficult to feel under tick fat cover **Side view** – no waist, fat bangs from abdomen **Overhead view** – back is markedly broadened

Figure 1: Body condition score (BCSs) expressed on a validated 5-point scale (https://occupaws.org).

The body fat index (BFI) was created based on evaluations and descriptions of various aspects or regions of dog's body (body shape as viewed from the side, above and from behind and also prominence, ease of palpation, and fat covering of the ribs and of the tail base) (6). Dogs were categorized according to dual-energy X-ray absorptiometry (DEXA) - measured body fat percentage creating BFI scores (26 to 35%, BFI 30; 36 to 45%, BFI 40; 46 to 55%, BFI 50; 56 to 65%, BFI 60) (Figure 2).

Besides morphometric methods and body weight there are others techniques available to measure body composition such as chemical analysis, densitometry, total body water measurement, absorptiometry including DEXA, ultrasonography, electrical conductance, and advanced imaging techniques (computed tomography and magnetic resonance imaging, MRI) (2,13–15). However, in the clinical setting, there is a need for quick, inexpensive, and non-invasive methods of body composition measurement (2).

Ribs	Ribs	Ribs	Ribs	Ribs	Ribs
Slightly prominent. Easily felt. Thin fat cover.	Slightly to not prominent. Can be felt. Moderate fat cover.	Not prominent. Very difficult to felt. Tick fat cover.	Not prominent. Extremely difficult to feel. Very tick fat cover.	Not prominent. Impossible to feel. Extremely tick fat cover.	Unidentifiable. Impossible to feel. Extremely tick fat cover.
Shape from above Well proportionated lumbar waist.	**Shape from above** Detectable lumbar waist.	**Shape from above** Loss of lumbar waist, broadened back.	**Shape from above** Markedly broadened back.	**Shape from above** Extremely broadened back.	**Shape from above** Extremely broadened back, bulging mid-section.
Shape from the side Abdominal tuck present.	**Shape from the side** Slight abdominal tuck.	**Shape from the side** Flat to bulging abdominal.	**Shape from the side** Marked abdominal bulge.	**Shape from the side** Severe abdominal bulge.	**Shape from the side** Very severe abdominal bulge.
Shape from behind Clear muscle definition, smooth contour.	**Shape from behind** Losing muscle definition, rounded appearance.	**Shape from behind** Rounded to square appearance.	**Shape from behind** Square appearance.	**Shape from behind** Square appearance.	**Shape from behind** Irregular or upside down pear shape.
Tail base bones Slightly prominent. Easily felt.	**Tail base bones** Slightly to not prominent. Can be felt.	**Tail base bones** Not prominent. Very difficult to feel.	**Tail base bones** Not prominent. Extremely difficult to feel.	**Tail base bones** Not prominent. Impossible to feel.	**Tail base bones** Unidentifiable.
Tail base fat Thin fat cover	**Tail base fat** Moderate fat cover	**Tail base fat** May have a small fat dimple.	**Tail base fat** Very tick fat cover. Fat dimple or fold present.	**Tail base fat** Extremely tick fat cover. Large fat dimple or fat fold.	**Tail base fat** Extremely tick fat cover. Large fat folds or pads.

Figure 2: Body fat index (BFI) based on body fat percentage of dogs (http://www.fifthavenuevetclinic.ca).

1.1. Prevalence

In the western world, obesity has been considered, probably, the most important health issue of humans and pets, with a dramatic increase of human obesity along with obesity among companion animals (16). Several authors reported that 34–59% of dogs, between 5 and 10 years of age, was overweight (9,10,17,18). A recent study developed in European countries reported differences between countries with prevalence between 22 and 56% of obese dogs (19). In these industrialized countries, amongst others, obesity is the most common form of nutritional imbalance of companion animals (20).

1.2. Causes and risks

A positive mismatch between energy intake and energy expenditure is the main reason for obesity development in dogs (20). Obesity is a serious metabolic and hormonal disease, that leads to reduced lifespan and health-related quality of life (21–24).

Various "risk factors" have been associated with obesity in dogs, among which neutering and inactivity (18). If the animal is neutered, then its food intake should be reduced and regular exercise and other physical activities should be increased to prevent the animal becoming overweight. A number of potential risk factors, for the development of this condition, may be relevant including genetics, sex status, exercise, and owner characteristics and behaviours. Risk factors reported as mainly leading to be an obese dog are shown in Table 1.

Table 1: Risk factors of dogs' obesity.

Risk factors	Details (higher prevalence groups)	References
Genetics/ Breed	Breed: Labrador Retriever, Cairn Terrier, Cavalier King Charles Spaniel, Scottish Terrier, Cocker Spaniel, Shetland Sheep dog, Dalmatians, Dachshunds, Basset Hound, Pembroke Welsh Corgi, Rottweiler, Beagle, Collie, Bernese mountain, Chow chow and "mixed" breeds - Genes related to fat metabolism due to breed selection process	(2,9,15,25–28)
Age, years	> 7 (middle age) and owners age >50	(2,9,10,17,26,29,30)
Gender/ sex status	Females	(2,10,17,25–27,30–32).
	Neutering - Reduced concentrations of androgens and oestrogens: decreased metabolic rate, increased food intake (satiety factors in the central nervous system) and reduced physical activity (breeding, including not searching for a mate)	
Iatrogenic	Pharmaceutical - Drug-induced polyphagia caused by glucocorticoids and anticonvulsant drugs	(2)
Endocrine and others diseases	Hypothyroidism (rarely) - Decreases metabolism and activity levels Hyperadrenocortism - Cortisol-driven increase in appetite Osteoarthritis - Restricting the animal's willingness or ability to move Pancreatitis	(2,10,33–35)
Dietary factors	Greater number of meals and snacks, table scraps (monthly fed), snacks and treats (monthly, weekly and daily fed), inexpensive food, high-fat diets, fresh meat, non-commercial food. No difference in the type of dog food (prepared pet food or homemade diets).	(2,17,29–32,36–38)
Lifestyle	Occasional or lack of exercise.	(2,30,32,36,38)
Household	Single-dog households (households with more residents and more play, either between dogs or between dog and human appear to present less risk); rural and semirural areas - free available food supply and self-exercising especially on farms, less structured exercise.	(10,27,32,38)
Humans characteristics and behaviours	Obese owners, lower income level, inaccurate assessment of dogs' body condition score, body weight and wet pet food labels, low interest in pet nutrition, inexistence of health consciousness, longer duration observing the dog eating and dog's presence when its owners prepare or eat their own meal, dogs sleeping more often in owner's bed, owners speaking more often with the dog, owners were less afraid of contracting diseases from their dogs, less interest in exercise, work or protection by the dog, less importance of health care.	(1,2,17,36,39–44)

1.3.   Obesity-related diseases

The list of conditions associated with obesity is increasing. New research identifies the relationships between pro inflammatory adipokines usually increased in obese and several disorders (1,2) (table 2). Obesity in dogs is associated with oxidative stress, prothrombotic state, insulin resistance and increased susceptibility to inflammation (2,18).

Table 2: Diseases reported to be associated with obesity in dogs.

Obesity-related diseases	Details	References
Endocrine diseases	Diabetes Mellitus (DM) - Dogs' DM resembling human type I DM Hypothyroidism and thyroid function - Higher concentrations of both total thyroxine (T4) and total triiodothyronine (T3) Hyperadrenocorticism	(2,10,24,45)
Metabolic diseases	Hyperlipidemia and dyslipidemia - Increased cholesterol, triglycerides, phospholipids Insulin resistance Glucose intolerance Obesity-related metabolic dysfunction (ORMD)	(2,8,23,24,39,40,46–59)
Orthopaedic disorders	Hip dysplasia Osteoarthritis Fractures	(2,22)
Cardiorespiratory disease	Tracheal collapse Brachycephalic airway obstruction syndrome Laryngeal paralysis Hypertension Cardiac rhythm alterations - Increased left ventricular volume, blood pressure, and plasma volume	(60–62)
Urinary tract and reproductive disorders	Dystocia - Excess adipose tissue in and around the birth canal Incontinent - Caudal displacement of the bladder Renal dysfunction Calcium oxalate urolithiasis	(2,27,63–66)
Neoplasia	Mammary carcinoma Transitional cell carcinoma of the bladder	(67,68)
Other disorders	Dermatologic disorders Decreased immune function - Infections development Heat intolerance Anaesthetic complications	(1,2,9)

1.4.   Canine obesity: a One Health approach

Over the last two decades, the One Health concept has been gaining attention because the growing awareness of the need for collaboration between the fields of human and veterinary medicine (1,69). Six out of every 10 infectious diseases in humans are spread from animals (Centres for Disease Control and Prevention 2018; https://www.cdc.gov/onehealth/index.html accessed 28.08.2018) and for that reason initially, preventing the spread of disease from farm animals and wild animals into human populations was the main focus of this concept (70). However, there are other One Health activities under comparative and translational medicine, that do not involve zoonotic diseases, in some cases highlighting the role of companion animals in this

approach (1,69). Thus, in 2010, the World Small Animal Veterinary Association (WSAVA) established a One Health committee with the concern of 'positioning small companion animals in the global One Health framework' (16). This committee has so far focused on the spread of zoonotic diseases from dogs, cats and other pet animals to humans. Nonetheless, two other fields of work were added, such as, comparative medicine and the human-animal bond, both human-centred, reporting benefits to human health and wellbeing from association with their pet animals (16). The human-centred "One Health" approach, in which the study of animal health is seen merely as a means to improving human health, is questionable. Furthermore, through collaboration between researchers in human and veterinary medicine, optimal health for both humans and animals can be achieved, and that this should be seen as central to the One Health concept, considering One Health as a two-way engagement (1,69). Furthermore, differences and similarities in obesity-related comorbidities between people and pets afford opportunities for interdisciplinary research collaborations between human and veterinary health care specialists (71,72).

a) Dog obesity *vs.* human obesity – animal-human bond

Obesity is a multifactorial problem in both dogs and humans, and some of the factors are similar between these species (1). According to Day (16,73), in many countries their incidence and growth are due to aspects related to shared lifestyles, between owners and pets. Thus, in the One Health approach, in order to understand canine obesity it is of importance to learn human obesity related items, including the owners' social status and the relationships that owners have with their dogs (1). Moreover, the study of the effects of overweight and obesity in dogs can contribute to understanding obesity-related health consequences in humans. Overall, this engagement can benefit both humans and dogs. The need for collaboration between veterinary and humans health care specialists reinforces the relevance and adequacy of veterinary communication skills among veterinary practitioners in communicating with clients (74). Taking all together, educating and assigning roles to all members of the health care team would improve staff engagement and consistency to achieve effectiveness of nutritional counselling for dogs' and humans' preventive care improving both patient health (75). In that context communication skills to assess the client's ability to change and implement a weight loss plan at the right time in the right way and to achieve better adherence to weight loss treatment plans will be very important.

In humans, as it was discussed previously in dogs, obesity is associated with health impairment being the main driver of the increased incidence of type 2 diabetes, cardiovascular disease, several cancers (breast, ovarian and prostate), asthmatic disease, sleep apnoea, osteoarthritis, and infertility, and a reduced life expectancy of 5 to 10 years, depending on severity (76). These health problems are not only of a physical nature, but also severe psychological and social problems, including low self-esteem, depression, stigmatisation and suicide may arise from being obese (1). In children, obesity increases the risk for obesity-associated health problems such as fatty liver, hypertension, hyperlipidaemia, type 2 diabetes and orthopaedic problems (77).

The nature of obesity-related health risks is similar in all populations; although, the specific level of risk at a given level of obesity may be different depending on gender, race, and societal conditions (78,79). Obesity prevalence has been reported being increased with age and trends has been similar for men and women (80,81). Although, for influence gender factor taking alone worldwide overweight/obesity prevalence has been significantly higher among women compared to men in almost the studied countries (World Health Organization, WHO, 2015) (82). Obesity in humans and its prevalence has been linked to low socioeconomic status and poverty (83), which has been related to the so called ''obesogenic environment'' of current society promoting a combination between an excess of caloric and unhealthy food consumption and inadequate physical activity (80). Although potentially variable for different races and ethnics, it has been reported a link between obesity and environmental conditions, as well (84).

There are several options for assessing body composition and, thus a degree of obesity in humans. All of them present limitations, thus they should be used with caution, and the emerging methods although they present an accurate assessment need to be validated (85). A commonly parameter used for assessing body fat it's the Body Mass Index (BMI). Self-reported body weight and height derived BMI [BMI = weight (kg)/height2 (m2)] is a useful, simple, low cost and non-invasive assessment tool to monitor the prevalence of overweight/obesity. According to the actual guidelines, healthy-weight individuals have a BMI in the range of 18.8 to 24.9 kg/m2, while overweight is defined as a BMI between 25 and 29.9 kg/m2 and obesity as a BMI of >30 kg/m2 (84).

Several studies have found a relationship between obesity in dogs and their owners, reporting that if an owner weighs too much it is more likely that his dog will also be overweight or obese (25,36,86). A similar phenomenon is found in the relationships between humans (87). Factors such as social, demographic, physiological as well as possible psychological mechanisms have been considered to increase the risk of an owner having an overweight dog pet (1).

Companion animals have some advantages over other animal models of obesity, such as rodents, including the outbred genetic background and relatively long lifespan of pets and the fact that they share common lifestyles with their owners (71). On the other hand several of the factors responsible for obesity development in one species are also responsible for obesity development in the other. Thus, there is great potential in employing a 'One Health' framework to provide novel solutions for the prevention and treatment of this condition in people and their pets (71), being that The World Small Animal Veterinary Association One Health Committee has proposed three areas of focus, the first two of which are particularly applicable to the undertaking of confronting obesity in people and animals (71).

Pet obesity has been related to what happens in children, once both share similar environments and are dependent on their caregivers for care and feeding; moreover, the

causes of pet and child obesity are strikingly similar (88,89). These findings suggest similar environmental influences on obesity, particularly the 'family food environment', which include family practices and attitudes towards food and feeding habits that may impact on childhood and dog obesity risk. Dogs tend to adjust their energy intake in response to diets of differing energy density; however, energy intake declines once energy content is diluted enough (e.g. with added dietary fibre), but feeding highly palatable and energy-dense diets can lead to overeating and obesity (90). The increasing prevalence of obesity in people has coincided with an increase in food portion sizes, and both children and adults often respond to larger portions of palatable food by eating more (91). Indulgent parenting has also been linked to increased obesity and parents may use food (high in sugar, snacks and fast food) as rewards or treats to control child behaviour (92), similar to a practice often used in dogs by dogs' owners and trainers (2,36,43,89).

Lack of exercise not only predisposes to higher levels of obesity in humans and dogs, but also exacerbates health problems linked to being overweight (2). When it comes to people exercising their dogs, the benefits are clearly mutual (1). A positive correlation between having a dog and getting exercise from walking has been reported in adults, adolescents and children, which in turn is likely to have an effect on the prevention of a number of human lifestyle-related diseases (93–96). Concerning dog's obesity, studies report that those who are overweight are significantly less likely to be walked daily, and their walks tend to be shorter (97).

Low income and low education may be associated with both human obesity and obesity comorbidities (78,98–100). Studies seem to indicate a link with the owner's income: the lower monthly income of the owner, the more likely it is that both the owner and the dog weigh too much (17,36). It was also reported that the level of education of owners is a determinant in the health and welfare of their dog pets (101).

Psychological mechanisms, including "over-humanization", a behaviour of dog-owners in which a dog is a substitute for human companionship, are also responsible for dogs weight gain. Dogs are indulged as "fellow-humans," and are no longer treated as typical companion animals (1,36,102). The aforementioned authors suggested that owners of overweight dogs use food as an acceptable form of communication and interaction with their pets. These behaviours seem to be a transfer of the owners' eating habits and attitudes to health to their dogs. These owners tend to be obese themselves, to took little interest in their own health compared to owners of normal-weight dogs (1,2,36). Furthermore, in the same way that parents of overweight children consistently underestimate their children's weight (103,104), owners of overweight or obese dogs underestimate the body condition of their animals (41,42,105,106). These human characteristics and behaviours have been already previously reported in Table 1.

In one study the authors tried to assess health aspects and quality of life of dogs following weight loss (107). A clear correlation was found between a reduction in weight and fat mass and an increase in vitality, as well as a decreased signs of being

emotionally disturbed and of being in pain following a successful weight loss programme (107). Moreover, a number of undesirable behaviours have been stated more frequently by dog-owners of overweight animals (108). In this recent study, overweight dogs were significantly more likely to display a range of undesirable behaviours including abnormal eating behavior (e.g. coprophagia, stealing food and food guarding), barking, growling or snapping at people (including other dogs, strangers and familiar people) and being more fearful of the outdoors. In children, obesity has been associated with lower levels of self-esteem, higher rates of sadness, loneliness and nervousness (109). In human adults, obesity has been related with depression and anxiety, stress, stigma/reduced support, diet and exercise adherence, chronic disease, medication use, negative thoughts and low self-esteem (110).

b) Methodological issues in the study of dog obesity

In the study of dog obesity, several methodological issues emerge, which difficults a higher knowledge about the real situation and about the relationship between this condition in dogs and humans. Most animal studies are based on questionnaires (1). Cross-sectional questionnaires were used for many purposes such as: a) profiles of dog-owners participating in a pet neutering campaign (101); undesirable behaviours of overweight dogs (108); relationship between dog ownership and adults, adolescents or children's physical activity and sedentary behaviour (93–95); nutritional status (111); relationship of feeding patterns and obesity or wellbeing in dogs (36,37,43,112,113); obesity prevalence and risk factors (9,10,17,25,27,30,32,114); relationship between quality of life in obese dogs and after weight loss programme (107); relationship between inaccurate assessment of canine body condition score, bodyweight, and pet food labels (44); relationship of energy-intake and activity risk factors for owner-perceived obesity (31); relationship between veterinary practices' and owners' opinions on cause and management of obesity (115); owners' attitudes and behaviour (38,42,116); relationship between veterinarian and owner perception of dog obesity (41,105); veterinarian-client communication skills (74,117). The application of these surveys are usually made through interview or *online* distribution, or using both methods simultaneously.

Several limitations have been reported about surveys use namely: 1) susceptibility to bias on self-report of owner weight and height, as well dogs' body weight and BCS (body shape misperception), since no verification by a veterinary or other professional exists; 2) other uncontrolled data information; 3) dog-owners unwilling to participate, underreporting of cases or false information; 4) an *online* format leading to limited response options, with owners having to choose from categories or binary options (i.e. yes or no) thus without an opportunity to explore the observations in any more detail; 5) anonymous surveys, which means that further information cannot be obtained from the owners who have participated; 6) inability to enrol a diverse owner (among which owners' age, gender, race/ethnicity) and dog population (among which age, gender, sex status, breed); 7) data interpretation being performed from only one origin, with the studied participants possibly representing a particular cohort; 8) the cross-sectional

nature of the studies design, which limits interpretation about causal pathways, as well as the analyses of key factors over time; 9) convenience sampling and a relatively small sample size relating to generalizability of the studied population; 10) the existence of studies that are based on referral service and not from obesity clinics that run in general practice (9,37,41,43,95,105,107,108,112,115).

Deep and concerted research, involving large teams and multidisciplinary approaches, is needed to increase knowledge about dog obesity, in the One-Health context.

Although, several attempts to find solutions over decades, obesity remains a major global health problem for people and companion animals (71). In this context, the great potential of employing a One Health framework to provide novel solutions for obesity prevention and its treatment is unquestionably. Efforts and leadership of a committed group of like-minded individuals representing a range of scientific and medical disciplines will be needed, as well the means and opportunities to communicate and to collaborate, including resources and funding for research.

Although, obesity is a major health problem, it continues being an issue needing innovative research. Salivary Proteomics is a field under development in human and animal medicine, as it will be further discussed. As such, the study of saliva should also be explored in the context of obesity.

2. SALIVA AND SALIVARY GLANDS

2.1. Salivary glands

The mammals' salivary glands system can be divided into two distinct exocrine groups: a) the major salivary glands such as parotid, submandibular and sublingual glands, zygomatic glands in small carnivores, and molar glands in felines (Figure 3) (118–121), responsible for the majority of total fluid; and b) the minor salivary glands, dispersed through the tissues of the mucosa and oral submucosa, named according to their location (labial, lingual, buccal, and palatine glands), which produce a smaller amount of saliva (118–121).
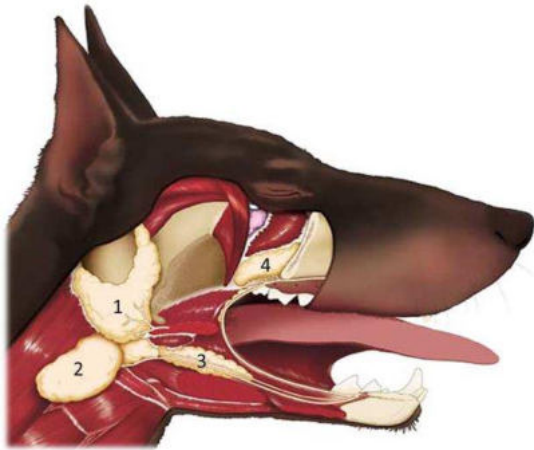
The salivary glands comprise a series of secretory units (glandular epithelium) that originate from the oral ectoderm and grow into the underlying mesoderm as large aggregates of simple branched to compound tubule-alveolar glands that are housed within the propria-submucosa of the oral cavity (118,119,121). The salivary glands consist of parenchyma comprising the secretory units with their associated ducts and stroma, which is the surrounding connective tissue that penetrates and divides the gland into lobules (122).

The salivary glands are basically composed by two saliva-producing cells types, arranged in acini (alveoli), namely mucous and serous acinar cells (seromucous/mixed secretory units are frequently observed as serous demilunes capping the mucinous acini). The ductal cells modify and transport saliva into the oral cavity (118–121). Each acini, the secretory subunits of lobules and the functional unit in salivary glands, empties into a small duct, the intercalated duct, and at least several of the intercalated ducts come together to form a striated duct (121). Within lobules, striated ducts merge and form intra-lobular ducts that join with one another to form interlobular ducts, and finally the interlobular ducts eventually empty saliva into inter-lobar ducts, which then empty the saliva into the terminal duct and then into the oral cavity (121). Additionally to the glandular and ductal cell types, there are also the myoepithelial cells, associated with the acini and the minor ducts and located between the epithelial cells and the basement membrane (118–121). Myoepithelial cells have several functions, including the propagation of nerve stimuli, formation and maintenance of the basement membrane, transport of metabolites and contraction, also playing an important role in the histogenesis of many tumours of the salivary glands (123–125).

The parotid glands are usually serous in domestic animals, humans and rodents; a few mucous cells or adenomerous are present in carnivores and may be mixed in young puppies and lambs (118,126,127). These glands have a greater contribution to total saliva after chewing stimulation; its secretion is serous, i.e., with high water content, including different salivary proteins, with different functions, according the species (128). The mandibular glands are usually mucous in dogs and cats, serous in rodents and mixed in horses, humans and ruminants (118,126,127). In the case of mixed glands, the distribution of serous and mucous cells is variable (118), and in the epithelium of the main duct goblet cells may occur (119). The submandibular glands contribute to a high proportion of total salivary secretion, in resting conditions, which have characteristics between parotid and sublingual secretions. The sublingual glands, which are also mixed in small carnivores (dogs), humans and horses, are predominant mucous in ruminants, swine and rodents (118,128). In addition to the typical mucous acini and demilunes the glands of dogs and cats contain clusters of serous acini with periodic acid-Schiff (PAS)-positive granules in their basal portions (119). Striated and intercalated ducts are present, but not prominent, in dogs and cats (119). The sublingual glands produce a saliva rich in mucins (118). The zygomatic glands, also known as the orbital glands, are only found in carnivores (129), and are predominantly mucous with some serous demilunes present (118,126). Despite these being frequently reported as major salivary glands, other author classifies them as minor salivary glands (119).

The minor salivary glands include a cluster of small sets of acinar structures of the same type as the larger salivary glands (118,119,121). They are located in the lamina propria-submucosa, close to the mucosa epithelium, and are named according to their location, including the labial, lingual, buccal, and palatine glands. The organization of minor salivary glands is similar to that of the major salivary glands, however simpler, not having connective tissue capsules, with each gland having a single duct which secretes, directly into the oral cavity. Saliva produced by these glands can be either serous, mucous, or mixed (122). The minor mucosal glands have a reflexively secretion and function more or less continuously during the day and at night (130,131). The minor glands may primarily play a part in protecting the oral mucosa, by secreting a highly glycosylated mucins-rich saliva, containing blood group determinants, several antimicrobial proteins and immunoglobulin probably active in tissue lubrication and bacterial aggregation (132). In the case of humans and rodents, it is known that the particular type of minor salivary glands – von Ebner glands - also secrete digestive enzymes and proteins with possible taste perception functions (132).

Finally, besides salivary glands, other sources contribute to whole saliva composition, namely serum filtrate, gingival cervicular fluid, oral microbiome, food debris, among others.

2.2. Saliva

2.2.1. Composition

Saliva is the fluid produced by salivary glands that, besides being mainly composed by water, contains also electrolytes and organic compounds, among which a unique complex mixture of glycoproteins, enzymes, hormones and growth factors (122). Saliva plays important physiological functions that will be presented below. It is a dilute aqueous fluid, presenting several constituents also present in blood, but not always in the same proportions. The substances in saliva originate primarily from salivary glands but also from blood, nasal-bronchial secretions and gingival cervicular fluid.

2.2.2. Functions

Saliva has diverse different functions that go from oral cavity lubrication to digestion, immunity, teeth protection and total maintenance of organism homeostasis (122). The representativeness that each of these functions has depends on the species. For example, in humans, saliva contains a considerable proportion of alpha-amylase and has relevance in starch digestion, but in the majority of animal species this function is negligible. Besides the reported functions, in dogs saliva also has a major role of aiding in cooling through evaporation off of the tongue (133).

Salivary proteins are greatly responsible for many of the saliva functions. Salivary amylase and salivary lipase have major roles in the digestive properties of saliva. In the case of salivary lipase, besides presenting variable levels among different animal species, is particularly present in new-borns and young animals, decreasing through life. Salivary proline-rich proteins (PRPs) are a family of salivary proteins, which also present variation among species. Acidic and glycosylated PRPs are clearly associated to oral health whereas basic PRPs are considered a defence mechanism against the effects of tannins, being particularly present in animal species with high levels of these plant-derived compounds in their diets (128). These proteins are also associated with food perception, by influencing astringency and bitterness perception (134,135). In dogs they were reported not to exist or to be present only at residual levels (128). Lactoferrin and peroxidase are involved in antimicrobial defence (136) and statherins allow saliva to keep oversaturation of calcium and phosphate salts, contributing to teeth protection.

Saliva also contains immunoglobulin (Ig), particularly IgG and secretory IgA (sIgA). This last one is produced from Ig originated in plasma cells, present in the juxtalveolar connective tissue of salivary glands, to which a secretory piece is added during the transport through the alveolar lining cells. Secretory IgA has a role in oral defence, protecting the oral mucous membrane from pathogenic microorganisms (137).

The lubricating properties of saliva are mainly conferred by a group of proteins with high molecular masses and with a considerable proportion of sugar residues (glycoproteins) – mucins. These proteins, which are components of the acquired

pellicle, present a high hydration potential, providing lubrication to the oral cavity, participating also in its defence (138).

2.2.3. Regulation

Saliva secretion is mainly under control of the autonomic nervous system (both parasympathetic and sympathetic systems) (139). Parasympathetic innervation occurs mainly through cranial nerves VII, IX and X. As such, the senses of taste, smell, and sight as well as the process of mastication all can trigger salivation. Besides nervous regulation, the presence of receptors for different hormones, together with the observation of changes in salivation induced by these, suggest that saliva may also be under hormonal regulation (140,141). Concerning nervous regulation, both branches of the autonomic nervous system excite salivary secretion: parasympathetic stimulation results in increased volume of diluted saliva, whereas sympathetic stimulation induces the release of pre-formed protein vesicles, with saliva having diminished volume and increased protein concentration.

2.2.4. Factors influencing composition and flow rate

As it was stated above, saliva is regulated by nervous and hormonal factors. As such, composition and flow rate are influenced by different conditions. Animal species is one of the factors affecting saliva secretion. For example, ruminants produce higher amounts of saliva than a dog, what is related with their high dependence of saliva for rumen buffering (142). Humans, during a 24-hour period, may produce 1-2 litres, whereas sheep can reach 4 litres and horses 38 litres. One of the constraints reported in a number of studies with dogs is the lack in achieving enough volumes of saliva (143). Our own experience shows us that this is even more relevant in small breed dogs.

Mastication, odour and type of diet also influence the amount and nature of salivation (144). In dogs, it was observed that a fresh meat diet results in viscous saliva, whereas the ingestion of dry food results in watery saliva. Moreover, saliva flow rate may be also induced by psychic stimulation, as is well known through Pavlov's experiments: the suggestion of food, through a bell that was used to sound before food presentation, can cause a conditioned reflex flow of saliva, even in the absence of food.

Besides flow rate, inorganic and organic composition of saliva also changes by influence of diverse factors (145). First of all, the rate of salivation will influence the concentration of ions, proteins and other components. If salivary flow rate is high, the velocity with which saliva passes through duct system is high and less exchange of ions occurs in ducts. In fact, the striated duct cells influence ionic and watery content, as such the basal surface of these cells constitutes a large surface area where some ions are absorbed and others excreted.

In humans, physiological and environmental factors, such as gender, age, inter-individual variability, taste stimulation, and circadian rhythms, were identified to cause differences in the human salivary protein profiles (146). However, to the best of the

author's knowledge, in dogs such influences in salivary proteome were not deeply studied, yet.

2.3. Salivary proteomics

The Proteomics concept was introduced by Marc Wilkins, in 1995, being defined as a large scale characterization of the total of proteins present in cell, tissue or organism (147). Proteomics includes the knowledge of the structure, function, modification, quantification and location of proteins, as well as the interactions among them (148).

In the particular case of saliva, proteomics proved to be of interest in many situations: for identification of salivary proteins involved in different pathologic and physiologic mechanisms (149); to identify biomarkers of different pathologies, in both humans (150) and animals (151,152); or even to understand oral processing events, namely oral food processing during mastication and in salivation (153).

Characterization and comprehension of salivary proteome became possible due to the development of diverse techniques for protein separation and identification. These may be classified in: 1) in gel-based proteomics techniques, such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis (2-DE), non-denaturing PAGE, isoelectric focusing (IEF); 2) gel-free proteomics techniques such as liquid chromatography (LC) (154). Different types of mass spectrometry have been used for salivary proteomics studies, namely liquid chromatography coupled to mass spectrometry (LC-MS) or to tandem mass spectrometry (LC-MS/MS), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS) and surface enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF/MS) (154).

Electrophoresis is a separation technique based in the migration of charged molecules under the application of an electric field. In the case of SDS-PAGE, proteins are separated in a polyacrylamide gel according the molecular masses of the proteins. One of the advantages of this technique is the possibility of separation and visualization of proteins with molecular masses between 5000 and 300000 Da, even for proteins with particular characteristics, among which acidity and hydrophobicity, being low sensitive to the presence of salts. However, this technique has the advantage to separate proteins only according to one characteristic – molecular mass – what results in several different proteins in the same band. This is a concern for protein mixtures such are the ones present in saliva (155). The same type of limitations is found for IEF, where each band can contain several different proteins, limiting the success of conclusions obtained through identifications by mass spectrometry.

2-DE is a technique introduced by O'Farrell and Klose in 1975, which has the potential of separating proteins taking simultaneous advantage of two different properties: molecular mass and charge. This technique has been widely used in salivary proteomics studies (149,156). Since proteins are separated based in two different properties, each protein spot contains a much lower amount of different proteins,

comparatively to uni-dimensional protein bands. This technique became useful in the study of samples like saliva, once it allows not only a good protein separation, but also the mapping of post-transduction modifications.

Although less used in saliva studies, capillary electrophoresis (CE) can also be used with several benefits. CE consists in protein separation through the use of capillaries, in which occurs the migration of analytes through an electrolyte solution, under the influence of an electric field. The main advantage of this technique is the need for very low amounts of total protein (sample) (157).

Despite the large usage and advantages of electrophoresis techniques in saliva, this fluid presents particularities that result in the need for other Proteomics' techniques. One of the particularities of saliva is the content in ions, with these being present in different amounts according to the animal species. For techniques such as 2-DE, a high content in ions, represents an increase in charged molecules that will affect the separation of proteins according to their intrinsic charge. Pre-separation of proteins can be achieved by the use of ultracentrifugation membranes or through the precipitation with acids (158). Nevertheless, these methods always have the problem of to result in some protein loss.

Mucins also limit the use of electrophoresis for salivary proteomics assessments (157). Besides these proteins confer high viscosity to saliva, several mucins have large molecular masses that impede them to enter the polyacrylamide gel.

It is also important to have in mind that saliva has lots of salivary proteins with reduced molecular masses (<5000 Da), which have also important functions. These peptides cannot be separated by gel electrophoresis, since they are excluded from the gels (155).

Since saliva is a sample containing a high diversity of proteins, which are present in very different proportions, the reduction of sample complexity is necessary. If not, the high abundant proteins can mask the detection of the lower abundant ones (159). Different methodologies exist for reducing sample complexity, for example the use of affinity columns (160), or the combination of different protein separation techniques [for example, ultrafiltration using membranes followed electrophoretic separation of different fractions (161)]. On the other hand, the depletion of the major proteins can result in alteration in the concentrations of the rest of saliva components, what, depending on the objectives of the study, can result in erroneous data.

To overcome some of the limitations of gel-based methods, such as gel electrophoresis, other methods for separating proteins started to emerge and used in the study of saliva. The use of liquid chromatography is one of them. One-dimension liquid chromatography (1D-LC) can separate proteins or peptides according only one of different parameters: molecular mass, isoelectric focusing or hydrophobicity. The use of two-dimensional liquid chromatography (2D-LC) performs protein or peptide separation according to two parameters: isoelectric point, in the first dimension and hydrophobicity, in the second (162,163).

2.3.1. Salivary protein identification by mass spectrometry

To identify proteins by mass spectrometry, a previous digestion of proteins into peptides is usually performed. This protein digestion is performed chemically or with a restriction enzyme that cleaves the peptide chain in known amino acid positions. One of the most used enzymes is trypsin, which cleaves the peptide chain after an arginine or a lysine residue. The only exception is when these residues are followed by proline (164). The mixture of the peptides generated by digestion is analysed by mass spectrometry (MS) and a list of masses is generated. Peptide mass fingerprinting (PMF) is one of the most used methods for protein identification using this generated list of peptides. In the case of PMF, the mass to charge (*m/z*) ratio obtained for each peptide is accurately measured and compared with all theoretical masses present in databases obtained by in silico proteolytic digestion (165). This comparison is made through the use of existent software, as for example MASCOT (166). The confidence level in protein identification is extremely dependent on the match between the mass detected and the theoretical mass (167).

Proteomic approaches that consist in the use of gel electrophoretic techniques, followed by excision of protein bands or spots and subsequent tryptic digestion and MS (GeLC-MS) analysis of these, only allow the identification of the proteins present in higher levels. On the other hand, shotgun proteomics relies on protein digestion (from the whole or a part of the sample) followed by a fractionation and allows the identification of a considerable portion of the proteome (159). In the last years, shotgun proteomics was used in saliva samples (168), with a recent publication presenting results for dog saliva (169).

2.4. Dog saliva studies

Dogs are used not only as companions but also as experimentation animals for research of several diseases, and also used in medical or animal-assisted therapy (170).

Concerning dog saliva, until now, most of the studies have been performed for assessing stress and welfare. Recently several authors reviewed and made a meta-analysis of salivary cortisol measurement in dogs, showing a considerable number of studies (171,172).

Besides salivary cortisol (173,174), salivary secretory IgA (sIgA) has been also related to stress in dogs: in adult animals, a decrease in this protein following long-term (151) and acute (152) stress has been demonstrated. However, some studies report a lack of evidence about the potential of sIgA as a salivary marker of stress in dogs (175). Salivary IgA plays an important role in mucosal immunity and is also used to assess the immunocompetence in dogs. The mRNA expression of the metabolism and oxidative stress-related genes in saliva following acute exercise stress in dogs has been studied, as well, by real-time PCR (polymerase chain reaction) (176). The absence of alpha-amylase, another stress marker, has been reported in dog saliva (177). However, the presence of salivary amylase enzymatic activity in dog saliva samples has been

supported by some authors (178). Furthermore, recently through a proteomic approach, the presence of this protein in dog saliva was reinforced by the identification of this protein, by mass spectrometry (179).

Another area whereas dog saliva study received attention was concerning its content in allergens for humans. The most well-known salivary dog allergens are *Canis familiaris* allergen 1 and 2 (Can f 1 and Can f 2, respectively). These salivary proteins belong to the lipocalin family, being reported the Can f proteins with high allergic potential (180). A study using immunostaining in a proteomic approach identified 4 novel salivary proteins potentially involved in allergy (181). In this study, IgE from allergic patients were incubated with dog saliva, after separation by SDS PAGE and two-dimensional electrophoresis (2DE). Bactericidal/permeability increasing protein family A2 (BPIFA2), mucin-5B, angiopoietin like 5 (ANGPTL5) and sIgA were identified proteins, which had the capacity of binding IgE from allergic patients, but which authors referred as potentially co-migrants with Can f proteins. Interestingly, saliva from different breeds of dogs have different allergic potential (182,183). For example, Golden Retriever was observed as a breed with a reduced amount of IgE-binding proteins in saliva (182).

Peptides such as statherins or histatins, which are constituents of human saliva, have not been found in dog saliva, so far. Curiously, these are peptides associated with wound healing and dog saliva is frequently described as having wound healing properties (184). It's possible that different types of proteins are involved in the cicatrizing characteristics of dog saliva (185). Nonetheless, caseins, which have been not reported in human saliva and which appear to be ancestral of histatins and statherins, were identified in dog saliva (179).

Measurement of creatine kinase and aspartate aminotransferase in dogs saliva and serum samples was recently performed to assess and evaluate their possible changes in situations of muscle damage (186).

Saliva proteome of dogs under healthy conditions was only recently studied. Some authors have been studying dogs saliva samples with different purposes among which to catalogue the proteins present in dogs saliva, and to verify variations within and between breeds (169,187). In humans, physiological and environmental factors, such as gender, age, inter-individual variability, taste stimulation, and circadian rhythms, were identified as influence factors in the human salivary protein profiles (146,188,189).

As well as in other species, like humans, dog saliva also presents a non-invasive alternative source of high quantities of canine genomic DNA suitable for genotyping studies and also in forensic studies for canine mRNA determination (190,191).

3. SALIVA AND OBESITY

3.1. Potential of saliva in the study of obesity

The connection between local (salivary glands, oral cavity) and systemic (blood) sources of molecules makes saliva an important fluid for search of biomarkers of diseases or to study a physiological status in particular (192). The non-invasive and simple nature of saliva collection allows for repetition and multiple collection of biological material with minimally trained personnel and in a stress-free, painless, and economically viable manner (143,155,193). As such, saliva may also be relevant for the study of obesity.

In humans, the term 'metabolic syndrome' describes a clustering of obesity (especially visceral obesity), hypertension, insulin resistance or hyperglycaemia, and hyperlipidaemia, namely increased fasting triglycerides and decreased High Density Lipoprotein Cholesterol (HDL-C) (48,52,194,195). The metabolic syndrome (MetS) itself causes no clinical signs, but comprises a set of important risk factors for diseases that cause morbidity and mortality in humans, including atherosclerosis, coronary heart disease, stroke, and type 2 diabetes (48,196,197).

The possibility of diagnoses, through saliva, of complications associated with obesity, such as insulin resistance, diabetes and metabolic syndrome, has been reported in studies with humans or rodent animal models. Insulin resistance has been linked to reduced salivary peroxidase activity and antioxidant status (198). Salivary adiponectin and its oligomeric profiles were suggested as representing a promising biological marker for the analysis of metabolic diseases (199). Moreover, the potential of saliva as a source of biomarkers of inflammation and insulin resistance, associated to obesity, was recently reviewed (150), with different authors reporting the elevation of inflammation markers, including C-reactive protein (CRP), tumour necrosis factor-α (TNF-α), insulin, ghrelin, interleukin-6 (IL6) and interferon-γ (IFN-γ), in the saliva from overweight/obese individuals. Besides these biomarkers, salivary HDL-C and several adipokines (such as adiponectin, leptin, resistin) has been reported either in adults or adolescents and children (200–202).

In summary, obesity-related changes in human salivary proteome, including changes after weight loss are shown in Table 3.

Table 3: Salivary proteins obesity-related changed in humans and animal models.

Proteins	Pathophysiological condition	Protein Function	References
High Density Lipoprotein –Cholesterol (HDL)	MetS (decreased levels) Obesity (deceased levels)	Lipoprotein transport	(203)
Adiponectin	Type 2 Diabetes (T2DM) and insulin resistance (decreased levels) Obesity (decreased levels)	Anti-inflammatory activity	(150,200,202)

Leptin	T2DM and insulin resistance (increased levels) Obesity (increased levels)	Body mass regulator and appetite regulator Energy balance	(150,200,202, 204)
Resistin	Obesity (increased levels) T2DM and insulin resistance (increased levels) Inflammation state (increased levels) MetS (increased levels)	Anti-inflammatory activity	(200,205,206)
Visfatin	Obesity (increased levels) T2DM Inflammation state	Mimics the effects of insulin	(207)
C-reactive protein (CRP)	Oral infection (increased levels) Inflammatory states (increased levels) Obesity (increased levels) T2DM (increased levels) MetS (increased levels)	Innate immune activity	(150,200,208)
Ghrelin	T2DM (increased levels) Obesity (increased levels)	Regulation of energy homeostasis – insulin release and glycemia anti-inflammatory activity	(209,210)
Insulin	T2DM (increased levels) Obesity (increased levels) MetS (increased levels)	Glucose homeostasis and lipid metabolism	(200,211)
Tumour necrosis factor alpha (TNF-α)	Obesity (increased levels) T2DM and insulin resistance (increased levels) Periodontal disease (increased levels) Inflammatory state (increased levels)	Immune activity Anti-inflammatory activity	(201,212)
Interleukin-6 (IL6)	T2DM (increased levels) Periodontal disease (increased levels) Obesity (increased levels) Inflammatory state (increased levels)	Immune activity Anti-inflammatory activity	(210,212)
Interferon-γ (IFN-γ)	Inflammatory state (increased levels) Obesity (increased levels) T2DM (increased levels)	Immune activity Anti-inflammatory activity	(210)
Alpha-amylase	Morbidly obesity/obesity (increased levels) T2DM (increased levels) Stress marker (increased levels)	Enzymatic activity Sympathetic nervous system activity	(149,213,214)
Carbonic anhydrase VI (CAVI)	Morbidly obesity (decreased levels) T2DM (increased levels)	Taste sensitivity Metabolism	(149,215)
Carbonic anhydrase I (CAI)	T2DM (decreased levels)	Metabolism	(215)
Carbonic anhydrase II (CAII)	T2DM (decreased levels)	Metabolism	(215)
Zinc-alpha-2 glycoprotein	Morbidly obesity (increased levels)	Taste sensitivity	(149,216)
Cystatin S	Morbidly obesity (increased levels)	Taste sensitivity	(149,216)

Salivary lysozyme	MetS (increased levels) Pro-inflammatory state (increased levels) Periodontitis (increased levels)	Proteolytic enzyme, anti-infective action, glucose metabolism	(217)
Cortisol	Psychological stress marker (increased levels) Obesity (increased levels) T2DM (increased levels) Low grade inflammation marker (increased levels)	Hypothalamic-pituitary-adrenal activity	(150,218,219)
Bactericidal permeability increasing fold containing family A1 (BPIFA1)	T2DM Periodontitis	Innate immune activity Anti-inflammatory activity	(220)
Cystatin A	Obesity (increased levels)	Taste sensitivity Immune activity	(149)
Cystatin B	Obesity (increased levels)	Taste sensitivity	(149)
Cystatin C	Obesity (increased levels)	Taste sensitivity Anti-inflammatory activity	(215)
Leukocyte elastase inhibitor (LEI)	T2DM	Neutrophil proteases regulator Anti-inflammatory actitvity	(215)
Neutrophil collagenase (matrix metalloproteinase, MMP-8)	T2DM (increased levels)	Nonspecific inflammatory marker	(215)
Retinol binding protein 4 (RBP4)	T2DM (increased levels) Obesity and insulin resistance	Antiapoptotic activity Signal transduction	(215)
Kallikrein 10 e 13	T2DM (decreased levels)	Extracellular matrix protein constituent	(215)
α-Defensins	Obesity (increased levels)	Innate immune activity	(221)

3.2. The study of dog obesity – proteomic approach

The pathophysiology of obesity and its progression to diabetes and others hormonal diseases in dogs share similarities and many of the detrimental effects that are associated with human obesity (222,223). Obesity has been reported to have increased in dogs in the last decade and the increase was paralleled by an increase in diabetes of similar magnitude suggesting a cause/effect relationship (222,224). However, dogs might be protected from the development of type 2 diabetes by compensating adequately for obesity-induced insulin resistance (223). Obese dogs appear to compensate for years of insulin resistance by maintaining high fasting insulin concentrations and an increased first phase insulin secretion during glucose tolerance tests (57). Nonetheless, as in humans, dogs obesity has been linked to insulin resistance as well (58,222).

In dogs, analytes such as C-reactive protein (186,225) and adiponectin (226) have been measured in saliva and were found to be suitable biomarkers of inflammation. However, saliva of dogs was not previously studied in obesity condition, whereas serum proteome

has been intensely studied and reviewed (21). Leptin, adiponectin, total cholesterol, and cortisol levels have been found to be significantly different in obese dogs from Beagles and mixed breeds (20,227). Plasma leptin concentration is a good index of body fat content in dogs, as it is in other species; making the measurement of plasma leptin useful for assessment of adiposity and obesity in dogs (7,228). However, influence factors that might affect have to be considered. Results of a study by Ishioka and colleagues (229) demonstrated that dogs' breed influences leptin concentrations, showing that within body condition score groups, Shetland sheepdogs had higher circulating leptin concentrations, whereas other breeds, such as Dachshunds, Shih Tzu, and Labrador retrievers had lower concentrations. Similar to humans, dogs have lower circulating concentrations of adiponectin with increased fat mass, and it is decreased in the obese state, suggesting that this hormone may have similar roles in the development of the metabolic changes, insulin resistance, and type 2 diabetes (230). Although, a wide variety inflammatory cytokines are produced by adipose tissue, TNFα and IL6 are the most widely studied cytokines produced by adipose tissue in any species, including dogs (21). These cytokines are actively involved in many processes, among which inflammation, obesity and metabolic syndrome, and have an important role in the development of insulin resistance and others obesity-related disorders. Detectable circulating TNF-α concentrations are found in almost 50% of dogs with naturally occurring obesity, with concentrations declining significantly after weight loss (58). In a study by Rafaj *et al*. (231) analytical changes in dogs had reflected that overweight was associated with a chronic low degree of inflammation once elevated concentrations of serum IL6 and CRP were found.

Another study has revealed that mean values of serum insulin and insulin/glucose ratio were significantly different in obese than in control dogs (54,227). Elevation of blood glucose and serum cholesterol and triglycerides concentrations has been associated with the increases in the BCS (55,232,233). Moreover, hypertriglyceridemia has been often associated with insulin resistance in certain pure breeds such as Miniature Schnauzers and Shetland Sheepdogs for example (234). Canine secondary hyperlipidaemia (hypertriglyceridemia, hypercholesterolemia) has been linked to obesity, and endocrine diseases such as DM, hypothyroidism, hyperadrenocorticism and also pancreatitis (56). Lipoproteins are believed to play important roles in energy and lipid metabolism of animals, and can reflect metabolic changes (232). In obese dogs, according to Piantedosi *et al*. (227), hyperlipidaemia was associated with the presence of liver dysfunction with significant increases of alanine aminotransferase and alkaline phosphate, compared to non-obese dogs.

A significant increase of serum butyrylcholinesterase (BChE) concentrations in Beagles was found after obesity experimentally-induced (235). Significant correlation was detected between BChE and BCS, as well as a negative correlation with serum adiponectin concentration and a positive correlation with serum lipid profile.

Because of the seriousness of obesity as a metabolic, hormonal, and inflammatory disease, prevention and management of obesity is essential (21). Recently German (236)

made a review of weight management in obese pets that report many studies concerning dogs obesity and weight loss programmes that have failed. In his work, this author presents the reasons for weight loss failures and makes some suggestions for its prevention. Although, controlled weight loss in obese dogs has been considered challenging by some authors (237), studies have demonstrated that many of the obesity-related conditions can be alleviated through weight loss (238).

Several studies had demonstrated that short term weight loss in obese dogs improves their metabolic status (lipidic profile, insulin and insulin-like growth factor-1 concentrations, and insulin: glucose ratio) (39,238–240). The higher serum triglycerides and total cholesterol that have been reported in obese dogs compared to lean seem to be reversed by weight loss (240,241).

Blanchard and colleagues (242) had suggested that a rapid weight loss following diet restriction in dogs could lead to a return of both TNF-α and insulin-like growth factor-1 (IGF-1) to normal levels, and normalizing levels of insulin resistance, as well.

In another study, serum proteome of pure breed Beagles before and after body weight (BW) loss has been studied (243) and several proteins levels were observed to significantly differ, as well: Retinol-binding protein 4, Clusterin precursor, and Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member 1. Thus, these authors had suggested that these proteins were potential biomarkers of obesity or obesity-related disorders such as metabolic syndrome, low-grade chronic inflammation or DM and insulin resistance and on the other hand useful as measurement of therapeutic effectiveness of weight loss. Furthermore, clusterin and cystatin C were suggested by the same authors to be a renal function biomarker in serum of obese dogs undergoing short term weight loss (66).

In the case of long term weight loss (244), it has been reported that when dogs are obese they presented lower plasma ghrelin and higher plasma leptin and insulin concentrations, but that weight loss resulted in an increase in plasma ghrelin concentrations and in a decrease plasma leptin and insulin concentrations. The same results after short term induced weight loss were found by other authors (238). Besides leptin concentrations, ghrelin concentrations correlate well with morphometric measures. Leptin, ghrelin, and glucagon-like protein- 1 concentrations are important biomarkers because of their known effects on appetite suppression and stimulation (238).

Metabolic syndrome, in dogs, has been also studied through proteomic approaches. In dogs, some of the criteria used in humans were adapted to define the condition of obesity-related metabolic dysfunction (ORMD) (10), once dogs develop only some of the components of the human metabolic syndrome: obesity (9), insulin resistance (49,50), increased blood pressure (61), and hyperlipidaemia (53). Based on what was stated above, dogs are considered as having ORMD if the following criteria were met: (a) body condition score (BCS) of 7-9/9 (on a 9-point scale) or of 4-5/5 (on a 5-point scale); and (b) any two of the following: 1) plasma triglycerides >200 mg/dL, > 2.3

mmol/L; 2) plasma total cholesterol > 300 mg/dL, > 7.8 mmol/L; 3) systolic blood pressure (SBP) > 160 mmHg; 4) fasting plasma glucose >100 mg/dL, > 5.6 mmol/L, or previously diagnosed diabetes mellitus (10). The measurement of HDL-cholesterol concentration was replaced by total plasma cholesterol concentration, since obese humans display increased low-density lipoprotein-cholesterol (LDL-cholesterol) but decreased HDL-cholesterol (52,194,195), whereas in obese dogs the circulating concentrations of both LDL and HDL-cholesterol are increased (235,240,245). In one study ORMD was associated with hypoadiponectinaemia and hyperinsulinaemia (10). Approximately 20 % of dogs with naturally occurring obesity were described as presenting with concurrent ORMD by those authors. Plasma proteome analysis have demonstrated that the obese dogs with ORMD have alterations in the concentrations of proteins related to lipid metabolism, immune response, and antioxidant system among which glutathione peroxidase, j-chain of immunoglobulin, apolipoprotein A1 and albumin (46).

3.3. Changes in salivary gland histology and composition associated with obesity

Although not very explored, some evidences emerge about changes in salivary glands induced by obesity or alterations associated with obesity.

Hyperleptinemia, a condition that has been associated to obesity, was studied after inducing of increased leptin levels in lean male Wistar rats to levels similar to those previously observed in obese animals (246). In the referred study it was observed an atrophy of the salivary glands, with decreased dimensions of secretory granules, which were in higher density in the parotid glands of leptin-treated animals; this suggested that lower protein synthesis activity occurs, since rough endoplasmic reticulum was observed to be less well developed.

As a result of diabetes, developing metabolic disorders and oxidative stress cause some morphological and pathophysiological changes salivary glands that was shown in some experimental diabetes models (247). These changes may include the cellular hypertrophy, and hyperplasia in parotid gland of DM (248). In the histological analysis of these glands in experimental diabetes mellitus, acinus epithelial damage, increased lipid droplets, eosinophilic stained dense chromatin cells, polymorph nuclear cell infiltration and fibrosis in the connective tissue were determined (249). Increase of lipid accumulation cells could be related with carbohydrate and/or fat metabolism and can lead to damage of the acinus epithelium that stimulates migration of inflammatory cells (249). Hypertrophic changes in parotid gland acinar cells in DM groups might be associated with alterations in glucose and lipid mechanisms that could be secondary effects of DM. Bailey *et al.* (250) had reported that bilateral growth of the salivary glands, especially the parotid gland, usually seen in obesity was linked to many diseases such as diabetes, hypertension, hyperlipidemia.

Obese subjects have been reported to exhibit a significant enlargement of parotid glands probably by an enhanced storage of adipocytes in the parotid parenchyma (251). Pro-inflammatory cytokines derived from adipocytes as well as macrophages, accumulated in adipose tissue (252) may negatively affect the function of salivary glands due to chronic low-grade inflammation in the gland (253), suggesting a hypothesis that inflammatory mediators play a role in the hypofunction of salivary glands among obese subjects. However, the negative effect of obesity on salivary glands may be related to stress hormones linked to hypothalamic–pituitary–adrenal axis (254) that may negatively influence the function of saliva glands as well (255).

From our knowledge, changes in salivary glands in obese dogs have not been reported, yet.

3.4. Changes in saliva composition and salivary secretion associated with obesity

In humans, few studies have been carried out about variations in salivary protein composition with potential for elucidating the physiological mechanisms involved in the development of obesity, or even for making inferences about individuals' susceptibility to this condition (149). One study has been performed to compare salivary proteome of obese individuals with the one of normal-weight subjects and to assess differences when individuals were subjected to weight loss due to bariatric surgery (149). Interestingly, obese women presented differences in salivary proteome, comparatively to the regular weight ones, but some of these differences were not observed in the case of women who had been subjected to bariatric surgery. Some of the proteins reported to present different levels in obese are proteins potentially related to the metabolic changes associated with obesity, or even with oral food perception, among which alpha-amylase, CAVI and Zinc-α-2 glycoprotein. These proteins were observed to be well correlate with BMI.

Studies in animal models, namely Wistar rats showed that the animals prone to obesity presented higher salivary alpha-amylase levels, at young ages, comparatively to animals resistant to the development of obesity (246). These authors suggested a possible use of saliva in signalling individuals susceptible to weight gain. In another study, above mentioned by changes in salivary glands, it was reported that hyperleptinemia decreases salivary alpha-amylase levels (140).

Modéer *et al*. (247) had demonstrated that childhood obesity is associated with reduced flow rate of stimulated whole saliva with 1 g of paraffin wax for chewing. These results were compared to normal-weight controls. The link between overweight and hyposalivation has been reported among adults, as well (256).

Taking into account the growing interest in saliva samples as a tool for diagnosis of several conditions and diseases, to study obesity-related changes in dogs' salivary proteome, with the perspective of improving understanding of the pathophysiology of obesity condition and/or for identifying novel potential biomarkers was the general goal of this work.

To achieve this, several specific purposes were carried out and outlined in the original research articles (Studies 1, 2, 3, 4 and 5) that comprise experimental work, such as:

a) To evaluate and characterize dogs obesity prevalence, in Portugal, in the context of "One Health";

b) To optimize protein precipitation/concentration method for separation by two-dimensional electrophoresis of dog salivary proteins;

c) To evaluate the effect of different factors in the salivary proteome of healthy normal weight dogs, namely acid stimulation, breed and sex;

d) To identify changes in the salivary proteome of obese dogs with ORMD that can help to understand the metabolic/pathophysiological changes related to this condition;

e) To identify changes in the salivary proteome of obese dogs undergoing weight loss in order to identify the salivary proteins that could reflect the physiological changes occurring during this condition.

List of original studies:

Study 1 - Links between human and canine obesity – a cross-sectional observational study in Portugal.

Study 2 - Comparison of protein precipitation methods for two-dimensional electrophoresis of dog salivary proteins.

Study 3 - The effect of breed, gender and acid stimulation in dog saliva proteome.

Study 4 – Comparative proteomic saliva analysis from dogs with and without obesity-related metabolic dysfunction.

Study 5 – Effect of body weight loss in the salivary proteome of obese Beagles dog.

CHAPTER II

EXPERIMENTAL WORK

LINKS BETWEEN HUMAN AND CANINE OBESITY – A CROSS-SECTIONAL OBSERVATIONAL STUDY IN PORTUGAL

PREPARED TO SUBMISSION

LINKS BETWEEN HUMAN AND CANINE OBESITY
A CROSS-SECTIONAL OBSERVATIONAL STUDY IN PORTUGAL

Sónia Lucena[1,2], Catarina Lavrador[1,2], Paulo Infante[3], Fernando Capela e Silva[1,4], Asta Tvarijonaviciute[5], Elsa Lamy[1]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM); [2]Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, Portugal; [3]Departamento de Matemática, Escola de Ciências e Tecnologia, Universidade de Évora, Portugal; [4]Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Portugal; [5]Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad Autónoma de Barcelona, Espanha.

Abstract

Human obesity prevalence has tripled in many countries of the European region since the 1980s, and the numbers of those affected continue to rise at an alarming rate. Once humans and pets share lifestyle, with studies reporting a positive relationship between owner and pet obesity, obesity should also be considered in the context of the One Health approach. The main objectives of this study were to evaluate obesity prevalence among owners and their pets in Portugal, and to identify possible relationship between them, and its main risk factors. This work was included in a multi-country cross-sectional questionnaire-based study targeted to owners of at least one dog. The results obtained to Portugal showed that men over 55 years, without any regular physical activity were more prone to be obese. In dogs, the higher probability of being obese was related to being neutered, to get sick easily, not doing regular exercise, not having a daily intake based on commercial recommendations and not doing physical activity alone or with its owner. No positive association between dogs' and owners' obesity was found. All of the obesity risks factors considered in our final model, for both dogs and their owners, have been stated by several authors with similar results in a One Health approach. However, concerning obesity relationship between dogs and owners, the type of population studied, which was mainly from regions with rural characteristics may partly explain it. Moreover, results derive mainly from an on-line survey, which have some limitations and extension of this study, based on interviews, may be performed to increase the representativeness of the results.

Key-words: Human, dog, obesity, questionnaire, One Health

1. Introduction

Over the last decade, there has been a growing awareness for collaboration between human and veterinary knowledge focused on comparative studies and human-animal bond. Initially, efforts were based on preventing the spread of disease from farm and wild animals into human populations (1). More recently, the spread of zoonotic diseases from dogs, cats and other pet animals to humans started to be considered (1). Even so, it's well known that there are several benefits to human health and wellbeing from association with pets (2).

According to Day (2010) (2), probably the most important health issue of humans and pets, in Western countries, is the shared epidemic of obesity that is often directly related to aspects of this shared lifestyle (2). Taking into account human obesity, probably this affirmation can be extended to Europe, as well, since its prevalence has tripled in many countries of the World Health Organization (WHO) European Region since the 1980s and the numbers of those affected continue to rise at an alarming rate (www.euro.who.int/ acceded in July, 22, 2018). Portugal did officially recognised obesity as a chronic disease in 2004, but remains the only country in Europe to do so (3).

The One Health concept recognizes that the health of people is connected to the health of animals and to the environment. Concerning obesity, a two-way engagement should be consider: not only the study of the effects of overweight in dogs can contribute to understanding human health but also to consider how human obesity origin may help to comprehend weight problems in these animals (1). In fact, obesity is a multifactorial problem, and some of the "risk factors" may be similar between humans and pets, such as: iatrogenic factors, endocrine diseases, age, genetics, gender (sex status), dietary factors and lifestyle (1,4,5). Besides these, owner characteristics and behaviours were suggested to influence pet obesity, as well. Obesity, in both companion animals and humans, is a result of excess of energy input (intake) over energy expenditure (6). The owner is considered as being responsible for dog's obesity, at some extent, once he has control over food selection, feed management and exercise regime, among others factors.

Obesity both in humans and dogs increases the risk of endocrine, orthopaedic, dermatological, respiratory and cardiovascular diseases, several types of cancer, hyperlipidaemia, infertility, among others (7,8). These conditions shorten expected lifespan and reduce health-related quality of life. In Portugal, a country with an

increasing prevalence of obesity in humans (9), the prevalence of pet obesity is not well known, neither the major factors contributing to it.

Body Mass Index (BMI), based on individual's weight and height, is widely used as a practical method of assessment human's body condition (underweight, normal weight and pre-obese/obese). In dogs, the Body Condition Scores (BCSs) developed for them, are widely used as the morphometric methods for assessing their relative body condition using a 5-point scale (8). The BCSs include 5 categories, ranging from 'emaciated' to 'severely obese', based on subjective assessment of specific features. These measures correlate well with more advanced measurements of the amount of body fat (8). Another index, used to evaluate dog obesity is Body Fat Index (BFI), which is assessed through evaluations and descriptions of various aspects or regions of dog's body (body shape as viewed from the side, above and from behind and also prominence, ease of palpation, and fat covering of the ribs and of the tail base) creating BFI scores (26 to 35%, BFI 30; 36 to 45%, BFI 40; 46 to 55%, BFI 50; 56 to 65%, BFI 60), with good correlation results according to DEXA-measured body fat percentage (10). Considering BFI values ranging from 15 to 30 per cent are considered by several authors as being the 'optimal' per cent body fat in dogs (11,12).

An association between dogs and owners obesity has been reported by several authors (2,13–15). To the author`s knowledge, in Portugal, there is no data about the relationship between obesity in the canine owners population and their pets.

The objectives of this study were to evaluate the current obesity prevalence among owners and their pets in Portugal, and to identify possible relationship between them, as well as the main factors affecting dog and/or owner obesity, namely social, environmental, economic, and nutritional and lifestyle. Complementary, the perceptions of the owners about obesity and risk factors were assessed, as well as their perception about possible solutions to stop obesity increase.

2. Materials and methods

This work was included in a multi-country (11 European countries) cross-sectional questionnaire-based study targeted to owners of at least one dog (16). The inclusion criteria included being adult (≥18 years) and hang out with at least one dog.

The study was approved by the Ethical committee of the University of Murcia, Spain (1374/2016) which was the responsible Institute.

2.1. Questionnaire design

The questionnaires focussed four key issues: owner's data (including height and weight, food habits and life style), dog´s data (including body condition and management), owner-pet relationship and obesity background (Supplementary material 1). The questionnaire included 25 closed-ended and 5 opened-ended questions related to owners and 17 closed-ended and 5 opened-ended questions related to dog´s data. Concerning to the two last issues of the questionnaire, human-animal bond and obesity background, the questionnaire included 10 closed-ended and 2 opened-ended questions and 2 closed-ended and 3 opened-ended questions, respectively. The last three closed-ended questions were related to the level of satisfaction for filling out this questionnaire and the first ones were related to owner and dog demographics and how the owner got the knowledge of questionnaire existence. The main questions were related to the feeding, exercising, weight monitoring routines of the dog and owner's habits and attitudes towards feeding and physical activity. Owners were asked to assess the body condition score (BCS) of their dog using a five-point scale charts (underweight to obese, 1-5) and to assess the body fat by using a body fat index (BFI) charts (normal to severely obese, 20% - 70% fat content) (17).

2.2. Questionnaire distribution

Recruitment of dog owners was conducted via questionnaires distribution through dog veterinary clinics and Hospital Universities, and *online*. The study was conducted over a 4-month period (January to May 2017).

2.3. Statistical analysis

A quantitative and qualitative analysis of questionnaire responses was used not only to determine the routine care and human and dog obesity background but also to determine the owner's life style, health conditions and feeding habits.

Owners were grouped into 3 groups according their BMI – underweight (BMI ≤ 18), normal weight (18 < BMI > 25), and overweight/obese (BMI ≥ 25), and dogs were grouped according to BCS to lean (BCS, 1 and 2), normal weight (BCS, 3), and overweight (BCS, 4 and 5), and according to BFI to normal weight (BFI, 20-30%) and obese (BFI, >30%). However, in order to evaluate the risk factors related/triggering to dog's obesity, dogs were grouped into non-obese (BCS, 1, 2 and 3) and obese (BCS, 4 and 5).

Descriptive statistical analysis included the calculation of means and standard deviations (SDs) of continuous variables and the proportion of observations in each

level of categorical variables. Three multivariable logistic regression models were used to determine risk factors associated with BMI of owners, and BCS and BFI of pets, but firstly cross tabs analysis were performed. To identify the factors that increase the likelihood of a dog or its owner be obese, logistic regression models were fitted (18). In order to fit the model, the following strategy was followed, as recommended by Hosmer *et al*., (18) but with adaptations given the number of events: a) for the initial model, all the variables that were found to be significant in the univariate phase ($p<0,10$) were selected; b) from this model were eliminated successively, and in descending order of p values, all non-significant variables ($p>0,05$); c) it was verified if any of the variables that were not included in the initial model are shown to be significant in the presence of those in the model, in which case they were added to the model; d) the interactions that made sense in the context of the study were tested ($p<0,05$); e) a residual analysis was done by covariate patterns to search for influential observations or outliers.

The significance of variables and interactions was tested using the likelihood ratio test. When each variable was excluded, it was observed the impact it had on the estimates of the remaining coefficients. The goodness of fit was done using Hosmer's and Lemeshow's goodness-of-fit test and Cessie Van Houwellingengof test, and the discriminative ability of the models was evaluated by the area under the ROC curve (AUC).

Statistical significance was considered for $p<0.05$. All statistical analysis procedures were achieved using the SPSS 23.0 software package (SPSS Inc., Chicago, USA).

In order to process responses related to the obesity background, a qualitative analysis was performed referring the three most often indicated reasons for increasing obesity rates, three recommendations to stop this increase, and the main thoughts of the actors about collaboration of human and veterinary healthcare professionals to combat obesity in both human and dog populations.

3. Results

3.1. Descriptive analysis

3.1.1. Response rates

A total of 324 surveys were received, among which 308 (95%) were from *online* distribution (Supplementary material 1). Out of those, 312 surveys were eligible for inclusion, with 12 being excluded because: a) the respondent's age information was

missing (n=1); b) existence of duplicated responses due to informatics errors (n=10); or c) incomplete responses (n=1).

According to answers, a large proportion of participants became aware of the study through social media (30.6%) and by the disseminating activities of the researchers involved in the study (26.5%). The rest of the responses were obtained through sharing among friends, family members or colleagues (24.9%), other ways (11.9%), and collaborations with or through veterinary clinics and school training (6%).

3.1.2. Weight group distributions

Among the respondents, BMI (calculations from self-reported weight and height data) indicated that 3.2% of the owners were underweight, 74.9% were normal weight, and 21.8% were overweight/obese. In dogs, according to the BCS assessed by the owners, the proportion of overweight/obesity (BCS>3) was 30.9%, while according to the BFI chart the proportion of overweight/obesity (>30%) was only 24.8%. Overweight/obese dogs (BCS>3; BFI>30) were associated with non-obese owners (BMI<25); p=0.003. Detailed descriptive data of the participants and their dogs is available in Table 1.

Table 1 Detailed descriptive data of the participants and their dogs.

Owner Data	Total number	%
Woman/Man	260/52	83.3/16.7
Age, years	18-72	
18-40	234	75.7
41-55	57	18.4
>55	19	6.1
BMI		
Underweight (BMI=<18,5)	10	3.2
Normal weight (18,5<BMI<25)	233	74.9
Overweight (25=>BMI<30)	49	15.7
Obese (BMI=>30)	19	6.1
Number of family members living with pet	2-3 (1-8)	
Educational level of the person responding to questionnaire		
Primary	3	1.0
Secondary/High School	54	17.3
University degree	255	81.7
Employment		
Student	108	34.6
Employed/Retired	286	59.6
Unemployed	18	5.8
Monthly Family income		
=< 500 €	83	27.1
500 – 1000 €	111	36.3
> 1000 €	112	36.6
Disease, Yes/No	46/266	78.9/21.1
Treatment, Yes/No	70/243	22.4/77.6

Dog data	Total number	%
Gender, Female/Male	154/157	49.5/50.5
Age, years	1-22	
< 1	49	15.8
1-7	169	54.5
>7	92	29.7
Breed (5 breeds most represented)	Labrador Retriever (n=34), German shepherd (n=11), Golden Retriever (n=9), Jack Russell Terrier (n=9), Yorkshire terrier (n=9).	
BCS		
1	2	0.6
2	27	8.7
3	186	59.6
4	81	26.1
5	15	4.8
BFI		
20%	130	41.8
30%	104	33.4
40%	44	14.1
50%	24	7.7
60%	9	2.9
70%	0	0
Housing:		
Accesses to a backyard	210	67.5
Without backyard	101	32.5
Reproductive status:		
Intact	161	51.6
Neutered	151	48.4
Disease, Yes/No	56/256	18.0/82.0
Visits to vet during last year		
because of health problems	0-48	
0	21	7
1	91	30.5
2	65	21.8
>2	121	40.6

3.1.3. Dogs and owners characteristics

Breed- Mixed breeds accounted for 56% of dogs included in the survey (Supplementary material 1). The 5 breeds highly represented are shown in table 1. Neither pure breeds nor mixed breeds did appear to be significantly associated with any estimated BCS or BFI profiles.

Gender and sex status -The overall dogs' gender distribution was 104 entire male (33.5%), 57 entire female (18.4%), 53 neutered male (17.1%) and 96 (31%) neutered female (Supplementary material 2). There was an increased risk of dogs obesity to be related with neutered animals, either considering dog obesity through estimated BCS or BFI profiles ($p<0.001$).

In owner, gender also influenced the propensity to obesity, with an increased risk of owners obesity related to being a man ($p<0.001$).

Age - In total, 49 dogs were 1 year (15.8%), 169 dogs were between 1 and 7 years (54.8%) and 92 dogs (29.7%) were over 7 years (Supplementary material 2). There was an increased risk of dogs obesity related to older dogs (>7 years), for both estimated BCS and BFI profile ($p<0.001$) and an association between non-obese dogs and younger dogs (<1 year) for BFI profile ($p<0.001$). Owners were more prone to be obese if they were older (>41 years, 24.5%; $p<0.001$).

Owners employment and monthly income - There was an increased risk of owners obesity (BMI>25) associated of being unemployed ($p<0.001$).

Feeding habits - The vast majority of dogs fed a commercial (72.6%) or mix (25.4%) diet and only 6 dogs fed a home-made diet exclusively (2%); with the majority of owners stating that dogs are fed twice per day (62.4%), under a fix schedule (71.7%) (Supplementary material 2). There were no significant associations between obesity (estimated through BCS or BFI) and type, frequency or schedule of feeding per day. However, dogs were prone to be normal weight (BCS≤3; BFI≤30) if owners usually followed commercial recommendations for daily intake of diet ($p=0.0124$). Furthermore, dogs whose owners reported that usually fed them with treats were significantly more likely to be non-obese rather than obese. This association was obtained when dog condition was estimated either according BCSs ($p=0.039$) or BFI ($p=0.023$) profiles. Type of dogs' diet and treats offer were associated with monthly income of the owner. Owners that had an increase monthly income were more likely to fed their dogs with commercial diet ($p=0.030$) and less likely to give them treats ($p=0.0005$).

Concerning owners' dietary habits, in total, 243 individuals usually have breakfast (78.1%); among which 59 were obese (19%). On the other hand, 68 owners do not take breakfast usually (21.9%) and among those 9 (2.9%) were obese (Supplementary material 2). As such, there was an increased risk of owners obesity (BMI>25) associated with owners that usually have breakfast ($p=0.042$). The breakfast intake was also associated with monthly income, when increased income level of owner higher intake breakfast ($p=0.014$). The vast majority of owners stated that eat healthy (79.7%) and that they had self-discipline (62.7%) to have a healthy diet (Supplementary material 2).

Exercise - The majority of dogs have regular exercise practice like walking (58.1%) and running (31%), among which 87 had ≤ 30 minutes (28%) and 168 had >30 minutes (54%) of practice per day (Supplementary material 2). A decreased risk of dogs' obesity associated both with "walking" and "running" practice was observed, considering both estimated BCS and BFI profiles ($p<0.001$).

The majority of owners had a positive attitude towards physical activity (85.9%) and had regular exercise (60.8%), but only 127 of them (40.8%) stated self-discipline to do it (Supplementary material 2). Concerning the regular exercise of owners with pets, 115 do it sometimes (36.9%) while 37 do it always (11.9%). 162 owners (52.1%) stated that dogs did not have any influence on their regular exercise practice. Dogs were more prone to be normal weight if owners had a positive attitude towards physical activity (BCS profile, $p=0.027$), do regular exercise with their pets (for dog obesity estimated both according to BCS and BFI profiles; $p<0.001$ and $p=0.033$, respectively) and considered that having a dog influenced his/her regular exercise practice (for dog obesity estimated both according to BCS and BFI profiles; $p=0.014$ and $p=0.019$, respectively). There was no relationship between monthly income and exercise.

Diseases - Orthopaedic (n=17), allergies (n=9), urinary (n=9), cardiovascular (n=4) and endocrine (n=2) disorders, as well breast tumour (n=2) and benign prostatic hyperplasia (n=3) were the main dogs diseases reported by owners.

Respiratory (n=18), auto-immune (n=12), orthopaedic (n=11), allergies (n=8), cardiovascular (n=8), endocrine (n=5) and psychiatric (n=5) disorders were the main owners' diseases. Forty two owners stated that their pets became ill easily (13.5%), among those almost half of them (6.1%) had obese dogs (Supplementary material 2). Dogs, which owners stated to became ill easily, were more likely to be obese using both BCS and BFI charts ($p=0.032$ and $p=0.035$, respectively). There were no significant associations between obesity and the presence of a specific chronic diseases or its treatment, neither for dogs nor humans.

Owner awareness – Three hundred two and 304 owners considered obesity as human and dog's disease (97.7% and 97.1%, respectively). Owner awareness of the risks of obesity disease was not significantly associated with dogs' obesity or overweight status. Owners most often mentioned lifestyle (a sedentary way of life), food related factors (eating fast food, high content of fats and sugar, additives, processed food), psychological factors (stress, depression, demotivation, laziness, bad self-discipline and

over-humanization of pet dogs) and modern way of life (every day rush, limited time for self-attendance and animal care) as reasons for obesity development.

The main recommendations proposed by the owners to stop the increase of obesity in humans and dogs were summarized into three topics:

a) Socio-economic: increased social education/awareness/knowledge about obesity risks and the associations between diet, exercise and health for humans and dogs. Better health care assistance for weight loss and maintenance for humans and dogs and better psychiatric monitoring.

b) Lifestyle: reinforce/implement physical exercise in the urban life (at work, in school, during leisure time, in daily life, and make room for dogs in city life).

c) Food: easily accessible healthy and balanced food for humans and dogs.

Finally, a high proportion (85.9%) of owners stated that cooperation between human and veterinary health care professionals is important because it would ensure comprehensive education in society about the importance of healthy eating and physical activity for people and their pets for the prevention of obesity. Several of those participants stated that specialists from both disciplines should concentrate more on obesity counselling and mentioned the importance of One Health approach.

3.2. Multivariable analysis

3.2.1. Factors associated with owners overweight/obesity

The adjusted multivariate logistic model (Table 2) fits well the data (Hosmer and Lemeshowgof test: p=0.411; Cessie Van Howellingengof test: p=0.376) and has a good discriminative capacity (AUC of the ROC curve = 0.743). Based on the Odds Ratio (Figure 1) we can conclude that:

1) An owner with a non-obese dog is almost 3 times more likely to be obese than an owner with an obese dog;

2) A male owner is almost 3 times more likely to be obese than a female owner;

3) An owner that does not do any regular physical activity is 2 times more likely to be obese than an owner that walks, run or play sports;

4) An owner that has 41 to 55 years old is 3.6 times more likely to be obese than an owner with less than 41 years old;

5) An owner that has more than 55 years old is about 10 times more likely to be obese than an owner with less than 41 years old.

According to this model, we can conclude that the profile that maximizes the probability of an owner being obese is that of a man over 55 years old, that does not do any regular physical activity and that have a non-obese dog. The estimated probability for this profile to be obese is 88.2%, with a 95% confidence interval equal to 75.3% - 100.0%.

Table 2 Estimated coefficients $(\hat{\beta})$ of the logistic regression model for owner obesity (BMI underweight or normal weight *vs*. BMI overweight), standard deviations$(\hat{\sigma}_{\hat{\beta}})$, *p*-values (Wald), odds ratio (OR) and 95% confidence intervals based on profile likelihood.

| Covariate	$\hat{\beta}$	$\hat{\sigma}_{\hat{\beta}}$	*p*-value	OR	CI$_{95\%}$ (OR)
Gender					
Female (Reference)					
Male	1.076	0.364	0.003	2.93	(1.43; 5.98)
Age					
< 41 (Reference)					
41-55	1.288	0.353	<0.001	3.62	(1.81; 7.25)
>55	2.329	0.536	<0.001	10.18	(3.64; 30.57)
Regular Practice					
Yes (Reference)					
No	0.710	0.313	0.023	2.03	(1.11; 3.19)
BFI					
>30% (Reference)					
<=30%	1.066	0.431	0.013	2.90	(1.31; 7.26)
Intercept	-3.163	0.461	<0.001		
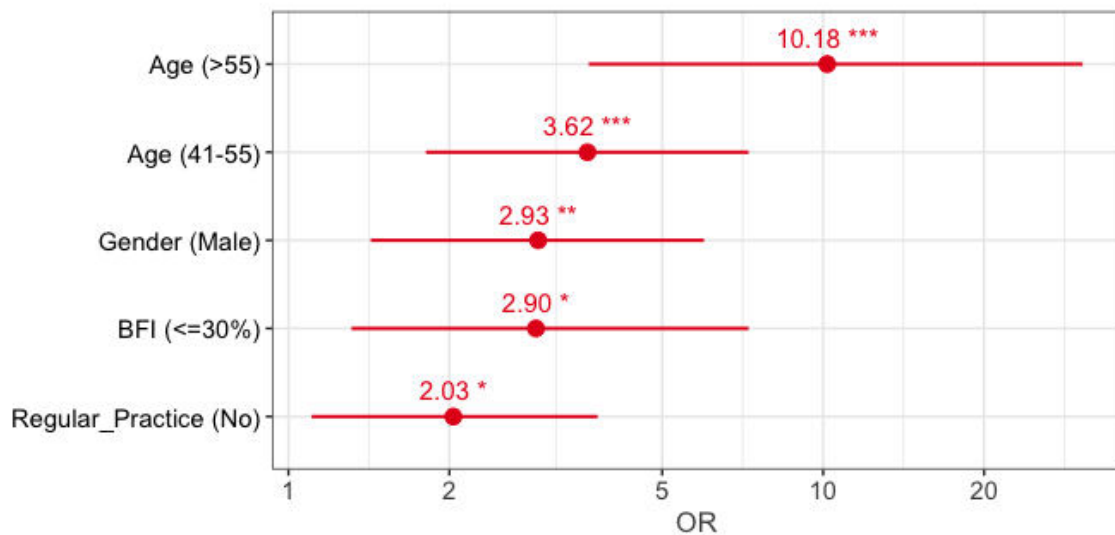
Figure 1 Odds Ratio and 95% confidence intervals based on profile likelihood, obtained from the multivariate logistic regression model for owner obesity (BMI underweight or normal weight *vs*. BMI overweight) - *sig at 5%, **sig at 1%, ***sig at 0.1%.

3.2.2. Factors associated with dogs overweight/obesity according to condition estimated considering BCS

The adjusted multivariate logistic model (Table 3) fits well the data (Hosmer and Lemeshowgof test: p=0.724; Cessie Van Howellingengof test: p=0.900) and has a good discriminative capacity (AUC of the ROC curve = 0.754).

Based on the Odds Ratio (Figure 2) we can conclude that:

1) A dog with a non-obese owner is 2 times more likely to be obese than a dog with an obese owner;

2) A neutered dog is about 3 times more likely to be obese than an intact dog;

3) A dog that easily get sick is 2.3 times more likely to be obese than a dog that do not get sick easily;

4) A dog that has a daily intake based on commercial recommendations is 2 times less likely to be obese than a dog that has a daily intake not based on commercial recommendations;

5) A dog that does not do any physical activity with its owner is 4 times more likely to be obese than a dog that runs with its owner;

6) A dog that does not do regular exercise is 3 times more likely to be obese than a dog that does do sometimes or always regular exercise.

According to this model, we can conclude that the profile that maximizes the probability of a dog being obese is that of a neutered dog, that gets ill easily, that does not do regular exercise, that doesn't have a daily intake based on commercial recommendations, and that does not practice physical activity like running with its non-obese owner. The estimated probability for this profile is 87.9%, with a 95% confidence interval equal to 76.1% - 99.7%.

Table 3 Estimated coefficients $\left(\hat{\beta}\right)$ of the logistic regression model for dog obesity (BCS underweight or normal weight *vs*. BCS overweight), standard deviations$\left(\hat{\sigma}_{\hat{\beta}}\right)$, *p*-values (Wald; [a]- likelihood ratio), odds ratio (OR) and 95% confidence intervals based on profile likelihood.

Covariate	$\hat{\beta}$	$\hat{\sigma}_{\hat{\beta}}$	p-value	OR	$CI_{95\%}$ (OR)
Status					
Intact (Reference)					
Neutered	1.065	0.292	<0.001	2.90	(1.65; 5.20)
Easily Sick					
No (Reference)					
Yes	0.829	0.391	0.034	2.29	(1.07; 4.96)
Activity					
None (Reference)					
Walking	-0.457	0.452	0.312	0.63	(0.26; 1.53)

Running	-1.392	0.517	0.007	0.25	(0.09; 0.67)
Diet Quantity					
Veterinary recommendations or *ad libitum* (Reference)					
Commercial recommendations	-0.694	0.288	0.016	0.50	(0.28; 0.87)
Regular Exercise					
No (Reference)					
Yes	-1.043	0.309	<0.001	0.35	(0.19; 0.64)
BMI					
Obese (reference)					
Non-Obese	0.718	0.372	0.053 (0.047[a])	2.05	(1.01; 4.38)
Intercept	-0.630	0.534	0.238		
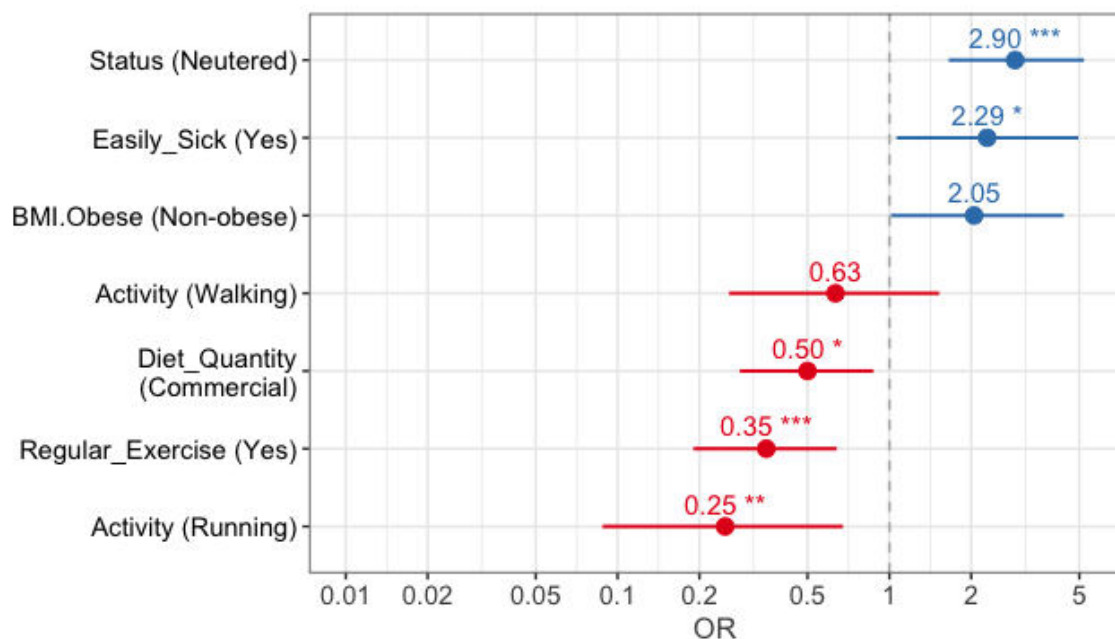
Figure 2 Odds Ratio and 95% confidence intervals based on profile likelihood, obtained from the multivariate logistic regression model for dog obesity (BCS underweight or normal weight *vs.* BCS overweight) - *sig at 5%, **sig at 1%, ***sig at 0.1%.

3.2.3. Factors associated with dogs overweight/obesity according to estimated BFI

The adjusted multivariate logistic model (Table 4) fits well the data (Hosmer and Lemeshowgof test: $p=0.595$; Cessie Van Howellingengof test: $p=0.787$) and has a good discriminative capacity (AUC of the ROC curve = 0.775). Based on the Odds Ratio (Fig. 3) we can conclude that:

1) A dog with a non-obese owner is 4 times more likely to be obese than a dog with an obese owner;

2) A neutered dog is 3 times more likely to be obese than a intact dog;

3) A dog that has a daily intake based on commercial recommendations is 2 times less likely to be obese than a dog that has a daily intake not based on commercial recommendations;

4) A dog that does not do any physical activity with its owner is 3 times more likely to be obese than a dog that walks with its owner and is about 14 times more likely to be obese than a dog that run with its owner.

According to this model, we can conclude that the profile that maximizes the probability of a dog being obese is that of a neutered dog, that doesn't have a daily intake based on commercial recommendations, that does not do practice physical activity like running with its owner and that have a non-obese owner. The estimated probability for this profile is 81.0%, with a 95% confidence interval equal to 66.5% - 95.5%.

Table 4 Estimated coefficients $(\hat{\beta})$ of the logistic regression model for dog obesity (BFI≤30% vs. BFI>30%), standard deviations $(\hat{\sigma}_{\hat{\beta}})$, p-values (Wald), odds ratio (OR) and 95% confidence intervals based on profile likelihood.

Covariate	$\hat{\beta}$	$\hat{\sigma}_{\hat{\beta}}$	*p-value*	OR	$CI_{95\%}$ (OR)
Status					
Intact (Reference)					
Neutered	1.117	0.321	<0.001	3.05	(1.65; 5.84)
Activity					
None (Reference)					
Walking	-1.130	0.442	0.011	0.32	(0.13; 0.76)
Running	-2.590	0.551	<0.001	0.07	(0.02; 0.21)
Diet Quantity					
Veterinary recommendations or *ad libitum* (Reference)					
Commercial recommendations	-1.132	0.321	<0.001	0.32	(0.17; 0.60)
BMI					
Obese (Reference)					
Non-Obese	1.375	0.455	0.003	3.96	(1.71; 10.33)
Intercept	-1.039	0.578	0.072		
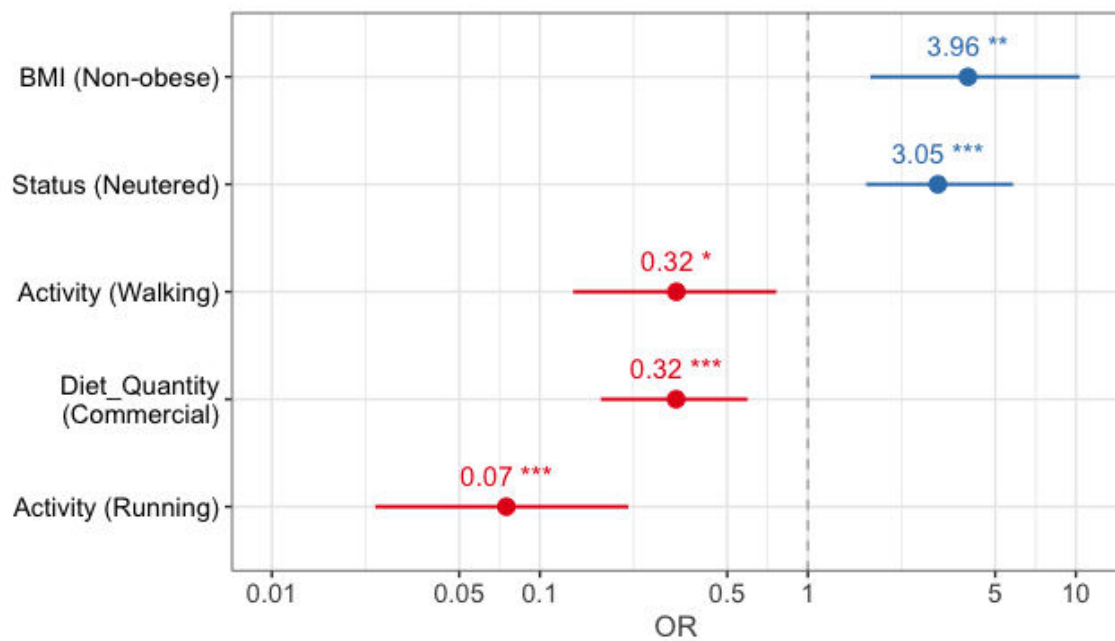
Figure 3 Odds Ratio and 95% confidence intervals based on profile likelihood, obtained from the multivariate logistic regression model for dog obesity (BFI≤30% vs. BFI>30%) - *sig at 5%, **sig at 1%, ***sig at 0.1%.

4. Discussion

In the present study, 21.9% of owners were overweight/obese, which is a little less than what has been estimated for the global population (nearly 30% with BMI=>30), (19) and lower to the more recent reports for the Portuguese population (9,20). Similarly to the reported study, men are the gender presenting higher overweight/obesity prevalence. Obesity in humans and its prevalence has been linked to low socioeconomic status and poverty, and differs substantially by gender and age (21). We did also observe an association between unemployed people and higher prevalence of obesity, which is according to findings from multiple countries (22), a significant higher proportion of obese among individuals over 55 years, was also found, in accordance with reports that obesity is increased with age (23,24).

The studies from several countries were unanimous on findings about income level, concluding that energy-dense foods and lower-quality diets composed of refined grains, added sugars, or fats are cheaper per calorie than are the healthy nutrient-dense foods (25). Furthermore, more energy-dense diets, that often devoid vegetables and fruit tended to be selected across different countries by lower-income people (26). Less-healthy food choices such as increased consumption of soft drink, snack foods and more frequent eating at fast-food (associated with inadequate consumption of vegetables and

fruits) have been related to the lack of nutrition knowledge, local attitudes, or cultural norms (23,26).

Among feeding habits, breakfast has been considered to be the most important meal of the day and a link between breakfast skipping (or the type of breakfast consumed) and overweight/obesity has been reported, both among young adults (<40 years) and older people (>50 years) (27,28). Nevertheless, to study the association of breakfast consumption with overweight/obesity prevalence, types of meal should be considered as an important factor (29). In the present study, breakfast consumption was associated with obesity in humans. It was observed that almost a quarter of the participants skipped breakfast. However, this occurred in a young population, mainly aging less than 40 years and constituted by many students. The fact of the type of food eaten in this meal being not considered does not allow us to conclude why people taking breakfast have higher BMI. Moreover, the present results were obtained through a cross-sectional questionnaire study and this result may only reflect trends of studied population.

In owners, a positive association between obesity and the absence of regular physical activity was found. This is in line with the known recommendations about physical activity, where it is stated that it is important in preventing weight gain and maintaining weight loss (19).

In the case of dogs, overweight/obesity prevalence obtained either by estimated BCS or BFI, was in line with values reported worldwide (19.7-59.3%) (30–34). Although a slightly disparity of obesity prevalence was obtained through BCS and BFI measurements (higher prevalence with BCS *vs*. BFI), the influence risk factors of dogs' obesity in our final model were the same using both metrics. The different results for obesity assessed through BCS or BFI may be related with owners misperception of their dogs' body shape, among which underestimation of BCS was the most common form of misperception, especially in overweight or obese adult dogs, as reported by several authors (14,30,31,35–38). Furthermore, for overweight and obese dogs, owner misperception persists, whether or not a BCS chart is used (39).

Human-related (i.e. pet owner) risk factors for canine obesity include, among others, owner household income and exercise habits (13,31). The present results, that showed a positive association between higher dog-owner income level, dogs' food choices (commercial diet) and treats not giving decisions, are in accordance with previous reported (13,32). It makes sense that people with higher monthly income have better life conditions, higher level of education thus, more opportunities and knowledge of obesity

risks and its prevention. Furthermore, a complex relationship between obesity and low social status in humans has been found in this work, as previously reported in other studies (40–43).

Although low expensive dog foods are of importance for people with lower income level and non-commercial source of diet, including home-made food or table scraps, greatly associated with obesity, due to its high-fat content (32,44–46), the vast majority of dogs (72.6%) in this study were fed with commercial diets. Furthermore, dogs whose owners followed commercial recommendations were less prone to be obese. Our results suggest that a feeding control with commercial diet is needed, since home-made diet is often highly palatable and energy-dense which can also lead to overeating and obesity (47). Curiously, in the present study, dogs whose owners gave them treats were less prone to be obese than the ones whose owners didn't. Some controversy exists, in the literature, concerning the effects of treats for body weight. Some authors associated commercial treats for dogs with obesity development (13,44,45), whereas others reported that treats high in crude fibre were inversely associated with dog's risk for obesity, once crude fibre is significantly correlated with a decreased incidence of obesity (48). In the present questionnaire the type of treats were not considered and the term 'treats' are often view by pet owners within a purely nutritional context, thus treat giving is commonplace in feeding regimes for the majority of owners (49). In this context, food has been considered an acceptable form of communication and interaction among owners and their pets (1,49). It is also possible to hypothesize that this relationship between treats and lower BCS may be done to treats being given during games or other types of play that implicates dog physical activity.

Considering our results for exercise habits, in accordance with previous reported and scientific data, a positive association between reduced daily dog exercise with its owner and obesity was verified which makes sense once less energy expenditure is obtained (50), as been referred above. In other studies, not only reduced daily exercise has been associated with obesity but also an association with shorter walks (21,31,45).

Owners perception of dog pets getting easily sick was associated with more prone to become obese, what reinforces not only the association between obesity and obesity-related diseases and the clear correlation between weight and fat mass loss and increased vitality, fewer signs of being emotionally disturbed and of being in pain (4).

Other obesity risks comprise age, gender and neutered status. In the present work, older dogs (>7years) and the neutered ones were more prone to be overweight/obese than the

young ones and intact ones, respectively, and no trends for gender was found. These results are according to previous observations in dogs, in which significantly association between overweight/obesity, increased age (>6) and neutered status were reported by several authors (8,21,31,51,52). Obesity risk associated with neutering is due to a combination of decreased energy requirements after neutering and an increase in food consumption (51). Those authors also found a positive association between overweight and being female. Nonetheless, others had reported that overweight/obesity status was only positively associated with neutered status, but not with sex (53).

In the majority of the studies performed so far, a positive association amongst dogs and pet-owners has been found (1,8,46). However, interestingly, in the present study, an opposite association was found. This fact could be at least in part explained relating to descriptive data of owners, such as: a) the majority lived in a rural zone or had a backyard which leads to a more independent way of dog living and exercising, b) the majority had a high level of education, possibly leading to higher awareness related to obesity risks factors and obesity-related diseases, c) the majority had a high conscious level once many participants revealed a good perception of obesity as a disease and life style and feeding habits importance in obesity prevention. Taking all together, the authors may hypothesize that the existence of considerable information related to human obesity, from different sources, including media, and the lower amount of information disseminated about canine obesity harmfulness, results in less care by the owners concerning dogs' obesity.

Furthermore, the present study has some limitations that may also explain this surprising result, namely the general limitations of *online* cross-sectional questionnaire studies and owners misperception of dogs' obesity. As such, these results should be considered for the studied population, which may not reflect all the Portuguese population. Further studies should be performed to elucidate some of the associations found so far.

5. Conclusions

All of the obesity risk factors considered in our final model, for both dogs and their owners, has been stated by several authors with similar results in a One Health approach. Concerning obesity relationship between dogs and owners, owners' data and limitations of the study may explain it. Owners' data such as household type and its geographical localization, level of education and good perception of feeding habits and

life style as influence obesity factors may explain that opposite result. Moreover, limitations of the study like a non-control of data once the results was mainly from an *online* cross-sectional questionnaire study and owners misperception of dogs' obesity may also have an influence on the results.

Obesity risk factors of our final model include owner's age, gender and level of physical activity and dog's neutered status, level of exercise, daily intake recommendations and owners perception of dogs' getting sick easily.

6. Acknowledgments

7. Funding

8. References

1. Sandøe P, Palmer C, Corr S, Astrup A, Bjørnvad CR. Canine and feline obesity: a One Health perspective. Vet Rec. 2014 Dec 20;175(24):610–6.

2. Day MJ. One Health: the small animal dimension. Vet Rec. 2010 Nov 27;167(22):847–9.

3. The Lancet Diabetes & Endocrinology. Should we officially recognise obesity as a disease? Lancet Diabetes Endocrinol. 2017 Jul;5(7):483.

4. German AJ, Holden SL, Morris PJ, Biourge V. Long-term follow-up after weight management in obese dogs: The role of diet in preventing regain. Vet J. 2012 Apr;192(1):65–70.

5. German AJ, Holden SL, Bissot T, Morris PJ, Biourge V. A high protein high fibre diet improves weight loss in obese dogs. Vet J. 2010 Mar;183(3):294–7.

6. Bland IM, Guthrie-Jones A, Taylor RD, Hill J. Dog obesity: Veterinary practices' and owners' opinions on cause and management. Prev Vet Med. 2010 May 1;94(3–4):310–5.

7. Bauer UE, Briss PA, Goodman RA, Bowman BA. Prevention of chronic disease in the 21st century: elimination of the leading preventable causes of premature death and disability in the USA. The Lancet. 2014 Jul 5;384(9937):45–52.

8. German AJ. The growing problem of obesity in dogs and cats. J Nutr. 2006;136(7):1940S–1946S.

9. Carmo I do, Dos Santos O, Camolas J, Vieira J, Carreira M, Medina L, et al. Prevalence of obesity in Portugal. Obes Rev. 2006;7(3):233–237.

10. Witzel AL, Kirk CA, Henry GA, Toll PW, Brejda JJ, Paetau-Robinson I. Use of a novel morphometric method and body fat index system for estimation of body composition in overweight and obese dogs. J Am Vet Med Assoc. 2014;244(11):1279–1284.

11. Bjornvad CR, Nielsen DH, Armstrong PJ, McEvoy F, Hoelmkjaer KM, Jensen KS, et al. Evaluation of a nine-point body condition scoring system in physically inactive pet cats. Am J Vet Res. 2011 Apr;72(4):433–7.

12. Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novotny BJ, editors. Small Animal Clinical Nutrition, 5th Edition. 5th edition. Topeka, Kansas: Mark Morris Institute; 2010. 1314 p.

13. Kienzle E, Bergler R, Mandernach A. A Comparison of the Feeding Behavior and the Human–Animal Relationship in Owners of Normal and Obese Dogs. J Nutr. 1998 Jan 12;128(12):2779S-2782S.

14. Colliard L, Ancel J, Benet J-J, Paragon B-M, Blanchard G. Risk factors for obesity in dogs in France. J Nutr. 2006;136(7):1951S–1954S.

15. Nijland ML, Stam F, Seidell JC. Overweight in dogs, but not in cats, is related to overweight in their owners. Public Health Nutr. 2010 Jan;13(01):102–106.

16. Muñoz-Prieto A, Nielsen LR, Dąbrowski R, Bjørnvad CR, Söder J, Lamy E, et al. European dog owner perceptions of obesity and factors associated with human and canine obesity. Sci Rep. 2018 Sep 6;8(1):13353.

17. Edney AT, Smith PM. Study of obesity in dogs visiting veterinary practices in the United Kingdom. Vet Rec. 1986 Apr 5;118(14):391–6.

18. Jr DWH, Lemeshow S, Sturdivant RX. Applied Logistic Regression. John Wiley & Sons; 2013. 528 p.

19. Day MJ. One Health Approach to Preventing Obesity in People and Their Pets. J Comp Pathol. 2017 May;156(4):293–5.

20. Oliveira A, Araújo J, Severo M, Correia D, Ramos E, Torres D, et al. Prevalence of general and abdominal obesity in Portugal: comprehensive results from the National Food, nutrition and physical activity survey 2015-2016. BMC Public Health. 2018 May 11;18(1):614.

21. Bomberg E, Birch L, Endenburg N, German AJ, Neilson J, Seligman H, et al. The Financial Costs, Behaviour and Psychology of Obesity: A One Health Analysis. J Comp Pathol. 2017 May;156(4):310–25.

22. Darmon N, Drewnowski A. Contribution of food prices and diet cost to socioeconomic disparities in diet quality and health: a systematic review and analysis. Nutr Rev. 2015 Oct 1;73(10):643–60.

23. Wang Y, Beydoun MA. The Obesity Epidemic in the United States—Gender, Age, Socioeconomic, Racial/Ethnic, and Geographic Characteristics: A Systematic Review and Meta-Regression Analysis. Epidemiol Rev. 2007 Jan 1;29(1):6–28.

24. Hedley AA, Ogden CL, Johnson CL, Carroll MD, Curtin LR, Flegal KM. Prevalence of Overweight and Obesity Among US Children, Adolescents, and Adults, 1999-2002. JAMA. 2004 Jun 16;291(23):2847–50.

25. Maillot M, Darmon N, Darmon M, Lafay L, Drewnowski A. Nutrient-Dense Food Groups Have High Energy Costs: An Econometric Approach to Nutrient Profiling. J Nutr. 2007 Jul 1;137(7):1815–20.

26. Darmon N, Drewnowski A. Does social class predict diet quality? Am J Clin Nutr. 2008 May 1;87(5):1107–17.

27. Deshmukh-Taskar P, Nicklas TA, Radcliffe JD, O'Neil CE, Liu Y. The relationship of breakfast skipping and type of breakfast consumed with overweight/obesity, abdominal obesity, other cardiometabolic risk factors and the metabolic syndrome in young adults. The National Health and Nutrition Examination Survey (NHANES): 1999-2006. Public Health Nutr. 2013 Nov;16(11):2073–82.

28. Mostad IL, Langaas M, Grill V. Central obesity is associated with lower intake of whole-grain bread and less frequent breakfast and lunch: results from the HUNT study, an adult all-population survey. Appl Physiol Nutr Metab. 2014 Feb 12;39(7):819–28.

29. Song WO, Chun OK, Obayashi S, Cho S, Chung CE. Is Consumption of Breakfast Associated with Body Mass Index in US Adults? J Am Diet Assoc. 2005 Sep 1;105(9):1373–82.

30. McGreevy PD, Thomson PC, Pride C, Fawcett A, Grassi T, Jones B. Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. Vet Rec-Engl Ed. 2005;156(22):695–701.

31. Courcier EA, Thomson RM, Mellor DJ, Yam PS. An epidemiological study of environmental factors associated with canine obesity. J Small Anim Pract. 2010 Jul 1;51(7):362–7.

32. Mao J, Xia Z, Chen J, Yu J. Prevalence and risk factors for canine obesity surveyed in veterinary practices in Beijing, China. Prev Vet Med. 2013 Nov 1;112(3–4):438–42.

33. Lund EM, Armstrong PJ, Kirk CA, Klausner JS. Prevalence and risk factors for obesity in adult dogs from private US veterinary practices. Int J Appl Res Vet Med. 2006;4(2):177.

34. Corbee RJ. Obesity in show dogs. J Anim Physiol Anim Nutr. 2013 Oct 1;97(5):904–10.

35. Rohlf VI, Toukhsati S, Coleman GJ, Bennett PC. Dog Obesity: Can Dog Caregivers' (Owners') Feeding and Exercise Intentions and Behaviors Be Predicted From Attitudes? J Appl Anim Welf Sci. 2010 Jun 21;13(3):213–36.

36. White GA, Hobson-West P, Cobb K, Craigon J, Hammond R, Millar KM. Canine obesity: is there a difference between veterinarian and owner perception? J Small Anim Pract. 2011 Dec;52(12):622–6.

37. Holmes KL, Morris PJ, Abdulla Z, Hackett R, Rawlings JM. Risk factors associated with excess body weight in dogs in the UK. J Anim Physiol Anim Nutr. 2007 Apr 1;91(3–4):166–7.

38. Yam PS, Naughton G, Butowski CF, Root AL. Inaccurate Assessment of Canine Body Condition Score, Bodyweight, and Pet Food Labels: A Potential Cause of Inaccurate Feeding. Vet Sci. 2017 Jun 9;4(4):30.

39. Eastland-Jones RC, German AJ, Holden SL, Biourge V, Pickavance LC. Owner misperception of canine body condition persists despite use of a body condition score chart. J Nutr Sci [Internet]. 2014 Oct 8;3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4473163/

40. McLaren L. Socioeconomic Status and Obesity. Epidemiol Rev. 2007 Jan 1;29(1):29–48.

41. Bove CF, Olson CM. Obesity in low-income rural women: qualitative insights about physical activity and eating patterns. Women Health. 2006;44(1):57–78.

42. Paeratakul S, Lovejoy JC, Ryan DH, Bray GA. The relation of gender, race and socioeconomic status to obesity and obesity comorbidities in a sample of US adults. Int J Obes Relat Metab Disord J Int Assoc Study Obes. 2002 Sep;26(9):1205–10.

43. Robert SA, Reither EN. A multilevel analysis of race, community disadvantage, and body mass index among adults in the US. Soc Sci Med. 2004 Dec;59(12):2421–34.

44. Sallander M, Hagberg M, Hedhammar Å, Rundgren M, Lindberg JE. Energy-intake and activity risk factors for owner-perceived obesity in a defined population of Swedish dogs. Prev Vet Med. 2010 Aug;96(1–2):132–41.

45. Bland IM, Guthrie-Jones A, Taylor RD, Hill J. Dog obesity: Owner attitudes and behaviour. Prev Vet Med. 2009 Dec;92(4):333–40.

46. Laflamme DP. Understanding and Managing Obesity in Dogs and Cats. Vet Clin North Am Small Anim Pract. 2006 Nov;36(6):1283–95.

47. Weber M, Bissot T, Servet E, Sergheraert R, Biourge V, German AJ. A High-Protein, High-Fiber Diet Designed for Weight Loss Improves Satiety in Dogs. J Vet Intern Med. 21(6):1203–8.

48. Heuberger R, Wakshlag J. The relationship of feeding patterns and obesity in dogs: Feeding patterns and obesity in dogs. J Anim Physiol Anim Nutr. 2011 Feb;95(1):98–105.

49. White GA, Ward L, Pink C, Craigon J, Millar KM. "Who's been a good dog?" – Owner perceptions and motivations for treat giving. Prev Vet Med. 2016 Sep;132:14–9.

50. Cutt H, Giles-Corti B, Knuiman M, Timperio A, Bull F. Understanding Dog Owners' Increased Levels of Physical Activity: Results From RESIDE. Am J Public Health. 2008 Jan 1;98(1):66–9.

51. Chandler M, Cunningham S, Lund EM, Khanna C, Naramore R, Patel A, et al. Obesity and Associated Comorbidities in People and Companion Animals: A One Health Perspective. J Comp Pathol. 2017 May;156(4):296–309.

52. German AJ. Obesity prevention and weight maintenance after loss. Vet Clin North Am Small Anim Pract. 2016;46(5):913–929.

53. German AJ, Blackwell E, Evans M, Westgarth C. Overweight dogs are more likely to display undesirable behaviours: results of a large online survey of dog owners in the UK. J Nutr Sci [Internet]. 2017 ed;6. Available from: https://www.cambridge.org/core/journals/journal-of-nutritional-science/article/overweight-dogs-are-more-likely-to-display-undesirable-behaviours-results-of-a-large-online-survey-of-dog-owners-in-the-uk/933AD361252C0F31F33ECC4D52412D41

COMPARISON OF PROTEIN PRECIPITATION METHODS FOR TWO-DIMENSIONAL ELECTROPHORESIS OF DOG SALIVARY PROTEINS

Comparison of protein precipitation methods for two-dimensional electrophoresis of dog salivary proteins

Sónia Lucena [1,2], Maria Carreira [3], Lénia Rodrigues [1], Fernando Capela e Silva [1,4], Asta Tvarijonaviciute [5], Elsa Lamy [1]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), 7002-554, Portugal, [2] Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, 7002-554, Portugal, [3] Departamento de Química, Universidade de Évora, 7000-671, Portugal, [4] Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, 7002-554, Portugal, [5] Interlab UMU, Departamento de Medicina y Cirugía Animal, Facultad de Veterinaria, Universidad de Murcia, 30100, Spain .

ABSTRACT

Despite saliva being one fluid with growing interest as a source of biomarkers, both in humans and animal models, few studies have been reported that use proteomic approaches for canine saliva analyses. Two-dimensional electrophoresis (2-DE) is considerably used in biomarker research and its use for dog saliva study may add relevant knowledge about pathology/physiology. The quality of the results obtained using 2-DE greatly depends on sample preparation. Different protein precipitation methods are frequently used for removing interfering compounds and concentrating samples, but their efficiency varies according to sample characteristics. For dog saliva samples no information was found about the best precipitant and precipitation method for electrophoretic protein profiling.

In this study, six different protein precipitation methods were compared. Precipitation of dog salivary proteins with 20% (w/v) trichloroacetic acid (TCA) resulted in lower protein recovery rate than other methods tested, but allowed protein profiles highly correlated with the ones from original samples. Moreover, this protocol resulted in good protein separation in 2-DE, with the visualization of spots from salivary proteins not observed when samples were treated using other methods. Based on this, we propose the use of TCA for dog saliva whenever precipitation is needed for protein profile analysis.

Keywords: dog, saliva, proteins precipitation, two-dimensional electrophoresis

1. Introduction

Saliva is a clear fluid mainly composed of electrolytes, immunoglobulins, proteins and enzymes [1]. Whole saliva is mainly a mixture of the secretions from the major and minor salivary glands, and oral mucosa, periodontium and oral microflora, which also contribute to its final content. Therefore, whole saliva represents a complex balance among local and systemic sources. Mostly in humans, this has been allowing for the application of saliva in the diagnosis not only for salivary gland disorders but also for oral diseases and systemic conditions such as, among others [2-7]: periodontitis and dental caries; type-2-diabetes mellitus;

obesity; several infections; cancer in a number of tissues; human immunodeficiency virus (HIV).

The interest in the characterization of biological fluids protein composition has increased in the last few years for both scientific and veterinary routine advancement [8]. The non-invasive and simple nature of saliva collection allows for repetition and multiple collection of biological material with minimally trained personnel and in a stress-free, painless, and economically viable manner [1,9]. However, saliva of different animal species, including the dog, is still little studied. Thus, protocol optimization for saliva analysis is firstly required.

Two-dimensional gel electrophoresis (2-DE) is one of the

*Corresponding author: Elsa Lamy, Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, Núcleo da Mitra, Apartado 94 7002-554 Évora, Portugal. E-mail Address: ecsl@uevora.pt

most popular techniques for the global analysis and initial profiling of salivary proteome for both humans and animals. 2-DE simultaneously separates proteins according to their isoelectric points (pI) and molecular masses, enabling the visualisation and identification of several thousand proteins on a single gel. One of the greatest advantages of 2-DE in salivary protein study relates to its capacity of separating proteins with different post-translational modifications (PTMs), allowing their separate quantification.

One of the constraints of using 2-DE in animal saliva samples is the difficulty to obtain saliva volumes sufficient to achieve the relatively high amount of proteins needed for performance of this technique. In dogs, in order to obtain high volumes of saliva may be not easy: thus different authors reported methods for stimulating saliva production, based on use of citric acid [10,11], soaking cotton rolls [12], or use of beef-flavoured cotton ropes [13]. However, these techniques could interfere with the salivary proteome and result in erroneous results and conclusions.

In 2-DE, sample preparation is an essential step for success. A high amount of charged molecules in solution will affect isoelectric focusing (IEF). In certain animal species, such as ruminants, this may be particularly relevant, since these animals have a high content of bicarbonate and phosphate ions in their saliva [14]. Moreover, charged detergents, lipids, phenolics and nucleic acids also need to be removed prior to IEF. Precipitation is frequently used to concentrate proteins from diluted samples and also to remove compounds that interfere with IEF [15,16]. Various methods for protein precipitation are applied, which rely on different chemical principles, and have different effectiveness according to the characteristics of samples. For human saliva, there are studies evaluating the efficiency and reliability of different protein precipitation protocols [17]. However, for dog saliva, which is different in composition from human saliva as based on biochemical studies [18-20], no such information was reported by now.

The objective of the present study was to compare and select the protein precipitant and precipitation method which will allow protein profiles with the highest similarity to original sample protein profile and, at the same time, efficient and reliable 2-DE analysis. For this, six of the protein precipitation methods already tested in human saliva [17] were compared using canine saliva samples.

2. Materials and Methods

2.1. Saliva collection

Saliva from seven healthy intact adult dogs (age range 2-8 years) of different breeds was collected: Portuguese Podengo (n=2), Border Collie (n=1), Bull Terrier (n=1), Boxer (n=1), Australian Cattle Dog (n=1) and mixed breed (n=1). Animals from different breeds and ages were used to constitute a pool representative of "general" dog saliva, instead of using saliva from only a particular breed and/or

age. Saliva samples were taken by the same examiners using a cotton cylinder (Salivette®) [14]. The salivettes were inserted into the oral cavity of the dogs, under the tongue and for chew, until completely soaked with saliva [14]. Dogs did not eat for at least 12 hours prior to the salivary sampling but water were provided ad libitum. After collection, the salivettes were immediately placed in test tubes on ice, until laboratory arrival. The saliva was extracted from the cotton roll by centrifugation at 4°C, at 2650 g, for 5 min. Samples were mixed in one pool, which was aliquoted and stored at -20°C until further analysis, less than two months in all cases.

2.2. Determination of total protein concentration

The total protein concentration of the dog saliva pool was determined using Bradford method protein assay [17] with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA). Standards and samples were run in triplicate, in 96 wells microplates. Absorbance was read at 600 nm in a microplate reader (Glomax, Promega).

2.3. Protein precipitation

Five different precipitant solutions and six precipitation methods were employed, which were already tested in human saliva [17]: method A (trichloroacetic acid-TCA only), method B (TCA/acetone), method C (TCA/acetone/dithiothreitol-DTT), method D (acetone only), method E (ethanol only) and method F (TCA/acetone fast method). Each of the methods was performed in triplicate.

TCA precipitation (method A)

For precipitation with TCA saliva sample was mixed with 20% w/v TCA (1:1). The mixture was vortexed to mix thoroughly and allowed to precipitate overnight at -20°C. This was followed by centrifugation at 23876 g, at 4°C for 30 min. The supernatant was discarded and the pellet obtained was washed twice with 200 µL of cold acetone. For each wash, the pellet suspended was sonicated for 5 min or more until the whole pellet was fully broken to form a suspension (maximum 20 min). This suspension was then incubated at -20°C for 20 min and subsequently centrifuged at 4°C for 5 min at 23876 g. This wash procedure was repeated twice. At the end, the acetone-containing supernatant was decanted and the pellet obtained was dried at air to remove any residual acetone. The pellets prepared were stored at -20°C until further use.

TCA/Acetone precipitation (method B)

The procedures were performed as described for method A, except that the solution used for protein precipitation was 1 ml of TCA 20% (w/v) plus 9 mL of acetone (90% v/v).

TCA/Acetone/DTT precipitation (method C)

The procedures were performed as described for method A, except that the solution was constituted by TCA 20% (v/v), acetone 90% (v/v) and 20 mM DTT (2.5 mL of TCA+acetone and 0.0075 g of DTT); and that the first wash was done using acetone 90% (v/v) and 20 mM DTT and the second using acetone 80% (v/v) and 10 mM DTT.

Acetone precipitation (method D)

In this method, saliva was mixed with 90% (v/v) acetone at a proportion 1:3. The mixture was incubated overnight at -20°C followed by centrifugation at 4°C, for 30 min at 23876 g . The supernatant was discarded and the precipitated was dried at air and frozen until further analysis.

Ethanol precipitation (method E)

The method was carried out similar to method D except that precipitation was done with absolute ethanol.

TCA/Acetone precipitation (fast assay) (method F)

The method was carried out as described for method B, but instead incubation being overnight, it was performed during 1h at 4° C. The rest of procedures were similar.

2.4. Determination of the protein concentration after protein precipitation methods

A volume of saliva corresponding to 25 µg of total protein was precipitated following the methods described above. The precipitates obtained were re-suspended in 25 µL 2-DE rehydration buffer [7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 60 mM DTT], since this is the buffer used in 2-DE. In order to avoid that compounds interfere with Bradford, the solution was diluted with 400 µL ultrapure water. Total protein concentration was determined following Bradford method as described in the section 2.2.

2.5. SDS PAGE and two-dimensional electrophoresis

Proteins were separated by SDS-PAGE electrophoresis in 14% acrylamide gels in a mini-protean apparatus (BioRad) [21]. A volume corresponding to 12 µg of total protein was precipitated by each of the methods described before. The precipitates were re-suspended in sample buffer [Tris–HCl 0.125 M pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoetanol, 20% (v/v) glycerol traces of bromophenol blue], heated at 95°C for 5 minutes and run at a constant voltage of 140V until the dye front reach the end of the gel. A volume of saliva correspondent to 12µg of total protein that was not previously precipitated was run for control. Gels were fixed in 40% methanol, 20% acetic acid, for one hour, stained with Coomassie Brilliant Blue (CBB) G-250 (0.125% CBB G-250,

20% ethanol) for two hours and destained in several washes with distilled water. The procedures were done in triplicate. Analysis was performed with GelAnalyzer software (http://www.gelanalyzer.com/).

For 2-DE, volumes of saliva correspondent to 150 µg of total protein were precipitated by the methods evaluated in this study, with exception of method F. The precipitates were mixed with 125 µL rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer, 60 mM DTT and traces of bromophenol blue], sonicated until total re-suspension and incubated during 1h at room temperature, being subsequently centrifuged for 5 min at 9390 g. IPG strips (7 cm, pH 3-10 NL; GE, Healthcare) were passively re-hydrated overnight with this solution. Focusing was performed in a Multiphor II (GE, Healthcare) for approximately 16kVh, at 20°C. Focused strips were equilibrated in two steps of 15 min each with equilibration buffer [50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol and 2 % (w/v) SDS], with the addition of 1% (w/v) DTT and 65 mM iodoacetamide in the first and second steps, respectively. After equilibration the strips were applied in the top of a SDS-PAGE gel 14% acrylamide and run at 150V constant voltage in a mini-protean system (BioRad). Staining with CBB-G250 and destaining were done through the same protocol described for SDS-PAGE gels. Gel images were acquired using a scanning Molecular Dynamics densitometer with internal calibration and LabScan software (GE, Healthcare), and images were analysed using ImageMaster 2D Platinum v7 software. Spot editing was performed manually and the match was done automatically and corrected manually.

2.6. Protein identification

Spots of interest were manually excised from gels and digested with trypsin following the protocol described before [21]. MALDI TOF-TOF mass spectrometry was used for protein identifications and this technique was performed by an external laboratory (MS Lab, ITQB-UNL; Portugal). Samples were diluted and concentrated using a reversal phase column (R2 pores-Applied Biosystems) and eluted with matrix α-cyano-4-hydroxycinnamic acid (CHCA; Fluka) 5mg/mL in 50% (v/v) CAN and 5% (v/v) formic acid. MS and MS/MS data were acquired in positive reflector mode through MALDI TOF/TOF (4800 Plus AB SCIEX) and through software 4000 Series Explorer, version 3.5.3.3 (Applied Biosystems). The mass of monoisotopic peptides was determined using the algorithm SNAP 2 in the analysis software (Bruker Daltonics) version 3.4. The external calibration was executed using CalMix5 (Protea). The 30 precursor ions most intense of the MS spectra were selected for analysis by MS/MS.

The monoisotopic masses of the peptides were used to search for the protein identification through the use of Protein Pilot v 4.5 (ABSciex) software with the Mascot search engine (MOWSE algorithm). Swiss Prot database,
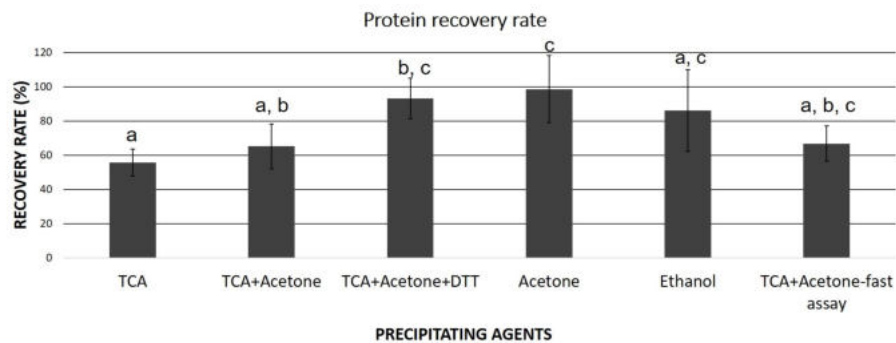
Figure 1. Protein recovery rates obtained for each of the precipitation methods studied. Different letters represent statistically significant differences among methods for p<0.05.

restricted to Canidae canidae (32092 sequences; 16404078 residues), was used for all the searches. A minimum mass accuracy of 50 ppm and a mass tolerance of 0.3 Da, two missed cleavages in peptide masses, carbamidomethylation of Cys and oxidation of Met, as fixed and variable amino acid modifications, respectively, were considered. Criteria used to accept the identification were homology scores higher than 58 and, at least, one fragmented peptide with individual significant score (P<0.05), in Mascot.

2.7. Statistical analysis

Data for protein recovery rate, percentage volume of SDS-PAGE bands and 2-DE spots were tested for normality and homocedasticity using Kolmogorov-Smirnov and Levene tests, respectively. One-way ANOVA was used for protein recovery rate, with Tukey test used for comparison of the different methods. The relationship between SDS-PAGE profiles (% volume of bands) from each precipitation method and the original sample was evaluated by calculating Pearson coefficient. Statistical significance was considered for p<0.05. All statistical analysis procedures were achieved using the SPSS 21.0 software package (SPSS Inc., Chicago, USA).

3. Results

3.1. Protein recovery rate

The different precipitation methods resulted in statistically

significant different rates of total protein recovery. The methods with higher losses of protein were TCA (method A) and TCA+acetone (methods B and F). On the other hand, the precipitation with acetone (method D) was the one for which higher protein recovery was achieved (Figure 1).

3.2. SDS-PAGE protein profiles

By relating the percentage of volume of the different profiles, strong positive correlations were obtained between original sample and methods A (r=0.717; p=0.003), B and F (r= 0.751; p=0.001, for overnight and r=0.715; p=0.003 for 1h incubation) and C (r=0.711; p=0.002). On the other hand, the precipitation methods with acetone (D) and with ethanol (E) resulted in profiles not correlated with control (r=0.389; p=0.151 and r=0.339; p=0.216, respectively) (Figures 2 and 3).

When comparing the different 16 protein bands consistently present in SDS-PAGE gels, the profile obtained without sample protein precipitation differs from profiles using protein precipitation methods for 3 protein bands: B, H and N. The differences are mainly in relation to the profiles obtained through precipitation with acetone or ethanol (Table 1).

3.3. Two-dimensional electrophoretic protein profiles

2-DE was run only for precipitated samples, since the volume of original sample needed exceeded the maximum of 25 μL allowed for the dry-gel strips used. Figure 4 shows the

Table 1. Expression levels (% volume) of the protein bands mainly affected by protein precipitation protocols. A – TCA; B – TCA/acetone; C - TCA/acetone/DTT; D – acetone; E – ethanol; F – TCA + acetone fast assay.

Band	TCA (method A)	TCA+acet (method B)	TCA+acet+DTT (method C)	Acetone (method D)	Ethanol (method E)	TCA+acet fast assay (method F)	Control	P value
B	4.54 ± 1.17	6.54 ± 0.32	9.62 ± 1.05	2.40 ± 0.00*	3.57 ± 0.86*	6.79 ± 0.17	6.90 ± 0.65	0.010
H	7.50 ± 0.45*	5.62 ± 0.31*	1.97 ± 0.00	5.39 ± 0.61*	3.96 ± 1.13	5.31 ± 0.70*	1.97 ± 0.00	0.014
N	8.19 ± 0.82	8.50 ± 0.38	2.43 ± 0.00	12.04 ± 3.25*	12.04 ± 1.26*	9.49 ± 2.09*	3.24 + 0.82	0.016

* Differences comparatively to control group (P<0.05)
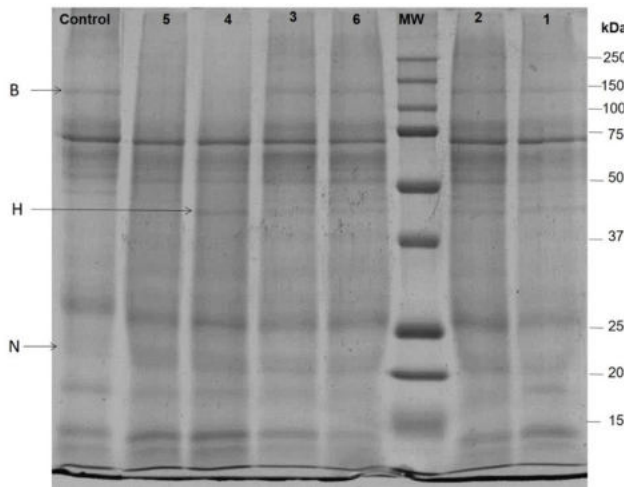
Figure 2. Representative SDS-PAGE profiles for each of the precipitation methods tested. Numbers in each lane indicate the precipitation method used (1 – TCA; 2 – TCA/acetone; 3- TCA/acetone/DTT; 4 – acetone; 5 – ethanol; 6 – TCA + acetone fast assay); MW – molecular mass marker. Precipitation methods 5 and 4 were the ones presenting higher differences from control protein profiles.

representative 2-DE of dog saliva, after each of the precipitation methods under study.

The highest number of resolved spots were obtained for the precipitation with TCA (83 spots), decreasing for the methods acetone (69 spots), ethanol (64 spots), TCA+acetone+DTT (59 spots) and TCA+acetone (55 spots).

The profile in which protein spots were less well resolved was the one obtained for precipitation with absolute ethanol (method E). On the other hand, when precipitations were performed with methods A and D, the profiles did appear enriched in proteins from the alkaline range of the pH gradient. Even in the other pH gradient regions, these two profiles were the ones allowing better focusing of the proteins, with well resolved protein spots. It is particularly interesting the group of protein spots from the alkaline region of the gel that were only observed in the gels obtained following these two precipitation methods (methods A and D - Figure 4).

The six spots observed only in the precipitation methods

TCA (A) and acetone (D) were identified by mass spectrometry (Table 2).

These spots correspond to proteins of keratin family or to serine-type endopeptidase inhibitor family. Although some of them are present in databases as uncharacterized proteins, one has been already identified as a submandibular gland secreted protein.

4. Discussion

The present study compared six different protein precipitation methods for the gel-based proteomic analysis of dog saliva. It characterizes the precipitation efficiency on the amount of total protein that can be loosed at the end, as well as on a protein-specific level. Although there are studies in which different protocols for preparation of human saliva samples for 2-DE were compared [17], the protein composition of dog saliva is not well known. As such, there are no guarantees that the most appropriate protocols for preparing human saliva samples are also the ideal ones for the preparation of dog saliva samples.

The proteome is a dynamic structure, so the sample itself and the conditions of its storage for research directly affect the result of proteomic analysis [22]. Moreover, the ideal sample preparation procedure should reproducibly capture the most comprehensive repertoire of proteins without any artefactual modification, proteins loss, degradation or non-proteinaceous contamination [14,15,20-26]. Besides the need for removing sample components that may interfere with isoelectric focusing, saliva analysis by electrophoresis requires a concentration step, due to the dilute nature of these samples, where protein precipitation methods can be useful.

Precipitation methods based in acetone and/or TCA were used by several authors in several animal tissues like ovaries, pericardium, brain, muscle and animal fluids like saliva, cerebrospinal fluid and blood [14,15,20-26]. One of the limitations of precipitation protocols is the protein loss, usually resulting from a poor re-suspension of the precipitates [27]. Among the protein precipitation methods tested in the present work, it was the acetone precipitation

Table 2. MS Identification of differentially expressed salivary proteins.

Spot	Protein	Uniprot entry reference	ID score	Estimated/ Theoretical MW (kDa)	Estimated/ Theoretical pI	Function/Family
1	Keratin, type I cytoskeletal 10	Q6EIZ0	207	14.0/57.7	8.1/5.1	Structural molecular activity
2	Keratin, type II cytoskeletal 8	F1PW98	111	16.0/55.0	8.1/5.7	Structural molecular activity/ Keratin family
3	Double-headed protease inhibitor, submandibular gland	P01002	96	16.0/12.8	7.9/8.3	serine-type endopeptidase inhibitor
4	Uncharacterized protein	L7N097	168	15.5/51.9	6.0/6.0	Structural molecular activity/Keratin family
5	Uncharacterized protein	F1PR78	121	14.0/12.7	5.0/8.4	serine-type endopeptidase inhibitor
6	Keratin, type I cytoskeletal 10	Q6EIZ0	192	14.0/57.7	7.9/5.0	Structural molecular activity
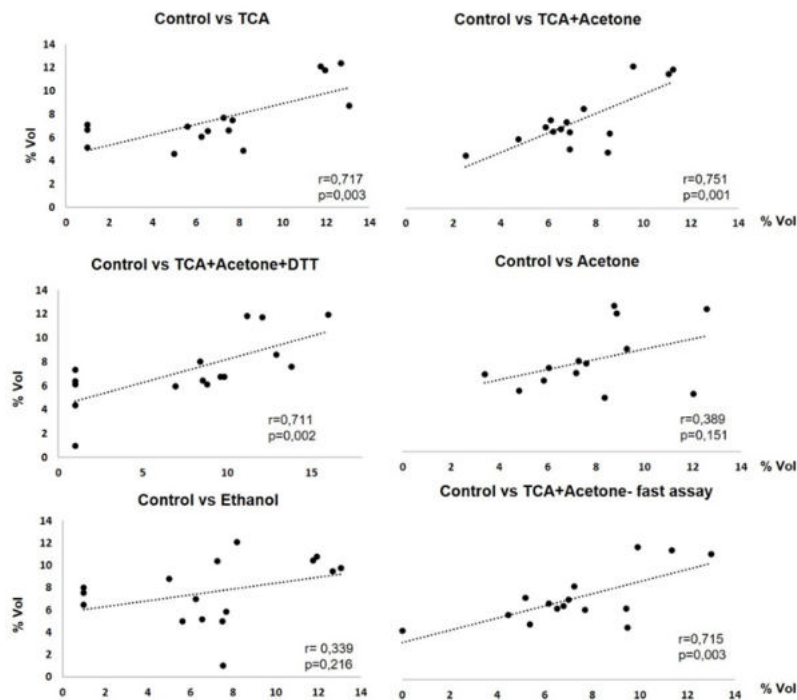
Figure 3. Correlation between the SDS-PAGE profiles of each of the precipitation methods tested and control. Correlations are significant for $p<0.05$.

the one that presented almost no losses, with protein recovery rates near 99%. For human saliva and rat brain samples, protein recovery rates of about 70% and 52.2% with acetone precipitation were reported [17,27], respectively, what suggests that the efficacy of this method depends on the type of biological material. In addition to the reduced losses in total protein, the other great advantage of this protocol is its simple execution, although requiring a high volume of organic solvent.

In our study, TCA-containing precipitation protocols resulted in lower rates of protein recovery (56%), similar to that already observed for human saliva by Jessie et al. (2008) [17], where TCA was observed as one of the precipitating agents leading to higher total protein losses (46.5%). The recovery of the protein depends on the re-solubilisation of the sample, being observed that after protein precipitation with TCA the recovery rate is considerably higher if the samples are sonicated (24% and 77%, without and with sonication, respectively) [27]. In the present work, besides the use of vortex, for resuspension of the precipitate, sonication of the samples was also carried out but even so, no high recovery rates were obtained. However, when acetone and DTT was added to the mixture (method C) higher recovery rates were observed, similarly to what has been reported to human saliva samples [17].

Besides the interest in having a precipitation protocol that allows a high protein recovery rate, it is important that it imposes the minimum of modifications in the protein content of samples, namely in the relative abundance of these proteins. In order to control this aspect, the protein profiles obtained after each precipitation method were

compared with the one from the original sample (without precipitation). This was possible to be done only for SDS-PAGE, since in 2-DE the volume of sample necessary to have the amount of protein needed for spot visualization is too high to be run without concentration. Moreover, the use of Centricon devices for protein concentration did not work in these dog saliva samples, with the filters clogged during centrifugation step (data not shown), with the need of further research to identify the dog saliva components responsible for that. Despite the low protein recovery rate, referred above, a high correlation between the protein profiles from TCA precipitation method and the protein profiles from original sample was observed.

Interestingly, 20% (w/v) TCA solution, representing a concentration of 10% of this acid in sample, induces no major changes in dog saliva. Some previous studies in rats reported that salivary proline rich proteins present the particular characteristic of being soluble in 10% TCA solutions [28], suggesting that this precipitation method would lead to a selective loss of these proteins. In the present work, the high correlation found between the profiles obtained from TCA protocols and the original sample profile suggest that the protein losses resulting from the precipitation occur in the same proportion for the different proteins, with no evidence of some proteins being more affected relatively to others. One hypothesis for explaining these results is that dog saliva may not contain these types of proteins. In fact, some authors report the absence of these proteins in animal species' saliva such as dogs and cats due to their eating patterns [30].

The 90% (v/v) acetone and absolute ethanol protocols
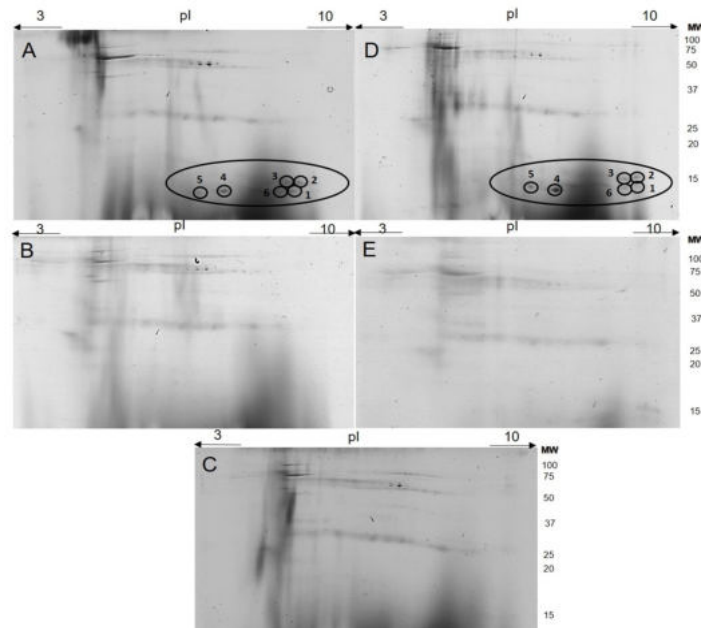
Figure 4. 2-DE protein profiles from dog saliva after various precipitation methods. (A) TCA; (B) TCA+acetone; (C) TCA+acetone+DTT; (D) acetone; (E) ethanol. Gels were stained with CBB-G250 .

were those that gave profiles less correlated with the control profiles. However, in these protocols protein recovery rates were relatively higher. The low correlation between these protocols and the control in SDS-PAGE profile may be due to higher losses of some types of proteins relative to others. Although not identified in the present study (since each band can be constituted by several proteins), proteins with molecular masses around 150 kDa are less intense in acetone and ethanol precipitation methods, whereas proteins with molecular masses around 50 kDa and 25 kDa are highly expressed in acetone and ethanol methods, respectively. The further identification of these proteins are of interest to understand the main changes induced by these protocols.

In 2-DE profiles, precipitations of proteins with TCA or acetone were the methods that allowed the observation of the higher number of spots, with better resolution. It was also only in the gels obtained using these two protocols that spots in the alkaline region were observed. The enrichment in alkaline proteins was already reported for precipitation with TCA [27].

The protein spots observed in the gels obtained following the two methods referred were identified as belonging to two families of dog proteins: keratins and serine-type endopeptidases inhibitors. In the case of keratin 1, which is a constituents of the intermediate filament cytoskeleton in epithelial cells, its presence in saliva from different mammalian species, including dogs, has been reported [31]. It was previously reported, for humans, that the amounts of keratins present in saliva may be a marker of gingival damage [32]. Since, in this study, dog saliva has been collected through cotton rolls, some level of gingival damage may occur, resulting in the identification of this protein in saliva.

Other protein spots, differently expressed among the

precipitation methods tested, were identified as protease inhibitors and, interestingly, one of them was identified as a protein secreted by submandibular glands. The exact role of this protease inhibitor in saliva is not known and further studies would be needed to explore and explain why this protein behaves differentially with different precipitation methods.

5. Concluding Remarks

2-DE is a popular technique for the global analysis and initial profiling of saliva prior to further fractionation and identification with other high throughput techniques such as mass spectrometry. This technique needs a sample preparation that, at the same time, allows cleaning of interfering compounds and protein concentration. Among the several precipitation methods studied in the present work, precipitation using 20% (v/v) TCA (method A) showed best results with dog saliva under studied conditions. Although this method results in considerable amount of total protein loss, the protein profiles are highly correlated with the ones from the original samples, with the advantage of allowing good resolution of spots in 2-DE and the visualization of spots from proteins that may be of interest.

6. Supplementary material

Ethical statement: The study did not involve the manipulation of experimental animals. Saliva was collected from healthy, normal weight, adult dogs of different breeds belonging to staff members of the University of Évora and to two kennels, who gave their informed consent for its collection. The saliva collection and all animal procedures

were carried out by a researcher accredited by the Federation of European Animal Science Associations (FELASA), and conformed to Portuguese law (Decreto-lei nº 113/2013, 7 August), which transposed the directive 2010/63/EU of the European Parliament of the Council of 22 September 2010 on the protection of animals used for scientific purposes. The protocols were also reviewed by the Évora University animal welfare commission.

Acknowledgements

References

1] E. Scarano, A. Fiorita, P.M. Picciotti, G.C. Passali, L. Calò, T. Cabras, R. Inzitari, C. Fanali, I. Messana, M. Castagnola, G., Paludetti Proteomics of saliva: personal experience, Acta Otorhinolaryngol Italica 30 (2010) 125-130.

2] S. Al Kawas, Z.H. Rahim, D.B. Ferguson, Potential uses of human salivary protein and peptide analysis in the diagnosis of disease, Arch Oral Biol 57 (2012)1-9.

3] S.J. Farnaud, O. Kosti, S.J. Getting, D. Renshaw, Saliva: physiology and diagnostic potential in health and disease, Scientific World Journal 10 (2010) 434-456. DOI: 10.1100/tsw.2010.38

4] D. Belstrøm, R.R. Jersie-Christensen, D. Lyon, C. Damgaard, L.J. Jensen, P. Holmstrup, J.V. Olsen, Metaproteomics of saliva identifies human protein markers specific for individuals with periodontitis and dental caries compared to orally healthy controls, PeerJ 4 (2016) e2433. DOI:10.7717/peerj.2433

5] L. Delmonico, M. Bravo, R.T. Silvestre, M.H. Ornellas, C.M. De Azevedo, G.Alves, Proteomic profile of saliva and plasma from women with impalpable breast lesions, Oncol Lett 12 (2016) 2145-2152. DOI: 10.3892/ol.2016.4828

6] N. Nowroozilarki, S. Jamshidi, T.Z. Salehi, S. Kolahian, Identification of Helicobacter and Wolinella spp. in oral cavity of Toy breed dogs with periodontal disease, Topics in Compan An Med (2017). DOI: 10.1053/j.tcam.2017.07.004

7] A. Tvarijonaviciute, C. Castillo, J.J. Ceron, S. Martinez-Subiela, F. Tecles, P.López-Jornet, Leptin and NGF in saliva of patients with diabetes mellitus type 2: A pilot study, J Oral Pathol Med 46 2017 853-855. DOI.org/10.1111/jop.12587

8] I. Miller, A. Preßlmayer-Hartler, R. Wait, K. Hummel, C. Sensi, I. Eberini, E. Razzazi-Fazeli, E. Gianazza, In between - Proteomics of dog biological fluids, J Proteomics 106 (2014) 30-45. DOI: 10.1016/j.jprot.2014.04.016

9] E. Lamy, M. Mau, Saliva proteomics as an emerging, non-invasive tool to study livestock physiology, nutrition and diseases, J Proteomics 75 (2012) 4251-4258. DOI: 10.1016/j.jprot.2012.05.007

10] R. Bergeron, S.L. Scott, J.P. Emond, F. Mercier, N.J. Cook, A.L., Schaefer Physiology and behavior of dogs during air transport, Can J Vet Res 66 (2002) 211-216.

11] B. Beerda, M.B.H. Schilder, J. van Hooff, H.W. de Vries, J.A. Mol, Behavioural, saliva cortisol and heart rate responses to different types of stimuli in dogs, Appl Anim Behav Sci 58 (1998) 365-381.

12] A.J. Kobelt, P.H. Hemsworth, J.L. Barnett, K.L.Butler, Sources of sampling variation in saliva cortisol in dogs, Res Vet Sci 75 (2003) 157-161. DOI: 10.1016/S0034-5288(03)00080-8

13] N.A. Dreschel, D.A. Granger, Methods of collection for salivary cortisol measurement in dogs, Horm Behav 55 (2009) 163-168. DOI: 10.1016/j.yhbeh.2008.09.010

14] E. Lamy, G. da Costa, R. Santos, F. Capela E Silva, J. Potes, A. Pereira, A.V. Coelho, E. Sales Baptista, Sheep and goat saliva proteome analysis: A useful tool for ingestive behavior research?, Physiol Behav, 98 (2009) 393-401. DOI: 10.1016/j.physbeh.2009.07.002

15] R.L. Chen, E.A. Sage, M.J. Dunn, R. Wait, J.F. Preston, Optimising ovine cerebrospinal fluid preparation for two-dimensional gel electrophoresis, Proteomics 6 (2006) 3170-3175. DOI: 10.1002/pmic.200500623

16] U. Jankowska, A. Latosinska, B. Skupien-Rabian, B. Swiderska, M. Dziedzicka-Wasylewska, S. Kedracka-Krok, Optimized procedure of extraction, purification and proteomic analysis of nuclear proteins from mouse brain, J Neurosci Methods 261 (2016) 1-9. DOI: 10.1016/j.jneumeth.2015.12.002

17] K. Jessie, O.H. Hashim, Z.H. Rahim, Protein precipitation method for salivary proteins and rehydration buffer for two-dimensional electrophoresis, Biotechnology 7 (2008) 686-693. DOI: 10.3923/biotech.2008.686.693

18] H. Nakanishi, T. Ohmori, M. Hara, K. Yoneyama, A. Takada, K. Saito, Identification of canine saliva using mRNA-based assay, Int J Legal Med 131 (2017) 39-43. DOI: 10.1007/s00414-016-1391-7

19] C. Boehlke, O. Zierau, C. Hannig, Salivary amylase – The enzyme of unspecialized euryphagous animals, Arch Oral Biol 60 (2015) 1162-1176. DOI: 10.4239%2Fwjd.v7.i6.112

20] E. Lavy, D. Goldberger, M. Friedman, D. Steinberg, pH values and mineral content of saliva in different breeds of dogs, Israel J Vet Med 67 (2012) 244-248.

21] E. Lamy, C. Simões, L. Rodrigues, A.R. Costa, R. Vitorino, F. Amado, C. Antunes, I. do Carmo, Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not, J Physiol Biochem 71 (2015) 691-702. DOI 10.1007/s13105-015-0434-8

22] P. Pomastowski, B. Buszewski, Two-dimensional gel electrophoresis in the light of new developments, Trends Analyt Chem 53 (2014) 167-177. DOI: 10.1016%2Fj.trac.2013.09.010

23] M. Sandri, A. Colussi, M.G. Perrotta, B. Stefanon, Salivary cortisol concentration in healthy dogs is affected by size, sex, and housing context, J Vet Behav 10 (2015) 302-306. DOI: 10.1016/j.jveb.2015.03.011

24] F. Panchout, J. Letendre, F. Bultelle, X. Denier, B. Rocher, P. Chan, D. Vaudry, F. Durand, Comparison of protein-extraction methods for gills of the shore crab, Carcinus maenas (L.), and application to 2DE, J Biomol Tech 24 (2013) 218-223. DOI: 10.7171/jbt.13-2404-002

25] R. Hao, C. Adoligbe, B. Jiang, X. Zhao, L. Gui, K. Qu, S. Wu, L. Zan, An Optimized Trichloroacetic Acid/Acetone

Precipitation Method for Two-Dimensional Gel Electrophoresis Analysis of Qinchuan Cattle Longissimus Dorsi Muscle Containing High Proportion of Marbling, PLoS ONE 10(4) (2015) e0124723. DOI:10.1371/journal.pone.0124723

26] P. Yiou, A. Shaoli, L. Kebin, W. Tao, F. Kui, Z. Hua, S. Yu, Y. Xun, X. Jinghui, Evaluation of extraction procedures for 2-DE analysis of aphid proteins, J Sep Sci 36 (2013) 532-539. DOI: 10.1002/jssc.201200642

27] E. Fic, S. Kedracka-Krok, U. Jankowska, A. Pirog, M. Dziedzicka-Wasylewska, Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis, Electrophoresis 31 (2010) 3573-3579. DOI: 10.1002/elps.201000197

28] H. Mehansho, A. Hagerman, S. Clements, L. Butler, J. Rogler, D.M. Carlson, Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels, Proc Natl Acad Sci 80 (1983) 3948-3952.

29] J.C. Suares, D.K. Shori, G.B., Proctor Expression of proline-rich proteins in the parotid & submandibular glands of the untreated rat, Biochem Soc Trans 25 (1997) 31S.

30] J.B. Harborne, in: Plant Ecology (Ed.), Crawley MJ, Oxford, UK, 1996, pp.132-155.

31] P. de Sousa-Pereira, M. Cova, J. Abrantes, R. Ferreira, F. Trindade, A. Barros, P. Gomes, B. Colaço, F. Amado, P.J. Esteves, R. Vitorino, Cross-species comparison of mammalian saliva using an LC-MALDI based proteomic approach, Proteomics 15 (2015) 1598-1607. DOI: 10.1002/pmic.201400083

32] W.S. McLaughlin, J. Kirkham, M.J. Kowolik, C. Robinson, Human gingival crevicular fluid keratin at healthy, chronic gingivitis and chronic adult periodontitis sites, J Clin Periodontol 23 (1996) 331-135.

THE EFFECT OF BREED, GENDER AND ACID STIMULATION IN DOG SALIVA PROTEOME

Research Article

The Effect of Breed, Gender, and Acid Stimulation in Dog Saliva Proteome

Sónia Lucena,[1,2] **Ana V. Coelho** [iD],[3] **Fernando Capela-Silva** [iD],[1,4]
Asta Tvarijonaviciute [iD],[5] **and Elsa Lamy** [iD][1]

[1]*Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM), Universidade de Évora, 7000-083 Évora, Portugal*
[2]*Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, 7000-083 Évora, Portugal*
[3]*Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, 2780-157 Oeiras, Portugal*
[4]*Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, 7000-671 Évora, Portugal*
[5]*Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), Regional Campus of International Excellence "Campus Mare Nostrum", University of Murcia, 30100 Espinardo, Murcia, Spain*

Correspondence should be addressed to Elsa Lamy; ecsl@uevora.pt

Saliva gained interest as a potential noninvasive source of biomarkers in humans and that interest starts to be extended also to other animal species. For this purpose, the knowledge of the salivary proteome in healthy conditions and the factors that affect it and how they affect it are necessary. The aim of the present study was to assess the effect that gender and breed have in saliva proteome and the changes in it induced by stimulation with acid. Saliva from 4 different purebred dogs (Portuguese Podengo, Greyhound, Rafeiro Alentejano, and Beagle) of both genders was collected without and after stimulation with lemon juice. SDS-PAGE and two-dimensional gel electrophoresis (2-DE) profiles were compared and the proteins of interest in-gel digested and identified by mass spectrometry. Acid stimulation decreased total protein concentration and the relative amounts of some protein bands/spots. Gender appeared to have minimal effect in saliva proteome, whereas the influence of breed varies. Beagles and Portuguese Podengos were the two breeds with higher differences. In conclusion, stimulation procedures and dog breed should be considered in data analysis when using salivary proteins for diagnostic purposes.

1. Introduction

Physiological variables are of added value to assess the welfare and lifespan both in humans and in animals, as they provide important information for interpreting and validating emotional and biological responses, respectively [1]. Saliva has gained interest for biomarker identification, mainly due to the noninvasive nature of its collection; at the same time that it contains glandular and blood-born molecules that can change under different conditions [2]. In dogs, most of the studies have been focused on the evaluation of stress by measuring salivary cortisol levels [3]. Infectious agents, such as *Helicobacter* spp., *Bartonella* spp., or rabies virus, have also been evaluated [4–6]. In addition, canine saliva has been used for quantification of acute phase proteins [7] and allergen measurements [8] and in forensic studies for canine

mRNA determination [9]. Furthermore, recently, healthy dog saliva proteome has been characterized by shotgun proteomics, with the identification of 2,491 proteins and peptides [10]. Despite this characterization, two-dimensional electrophoresis (2-DE) salivary protein profiles of dog saliva have been less explored. Although several researchers consider that gel-based approaches provide limited information, 2-DE continues providing reliable quantitative results on differential protein expressions as they display a high number of protein species, their isoforms, and posttranslational modifications at the same time [11]. It also has the advantage of allowing modifications of the protein mixtures caused by inadequate treatment or endogenous protease activities with physiological relevance to be easily recognized via pattern disturbances by 2D gels [11].

TABLE 1: Dog population for each breed by gender and age.

Breed	Average body weight (Kg)	Age (years)	Gender	
			Female	Male
Portuguese Podengo	4-5	0.5–10	7	6
Greyhound	26–40	1–7	9	6
Rafeiro Alentejano	35–50	0.5–8	8	7
Beagle	9–11	2–11	0	10

In humans, physiological and environmental factors, such as gender, age, interindividual variability, taste stimulation, and circadian rhythms, were identified to cause differences in the human salivary protein profiles [12]. However, to the best of the author's knowledge, in dogs such influences in salivary proteome are not deeply studied. The knowledge of the possible salivary proteome changes due to different factors would later permit correcting data interpretation for disease diagnostics.

Different methods of saliva sampling in dogs have been reported in literature: (1) without stimulation [10, 13]; (2) using different stimulating methods, such as citric acid in swabs [14] or in crystals spreader in the tongue [15], beef-flavoured cotton ropes [16], dogs' snack held in front of the dog's snout [17], or visualization and smell of food [18] what could result in different salivary proteomes. Acid stimulation, which is one of the mostly used methods for stimulating saliva production in humans, has been already reported to influence human salivary proteome [12]. However, its influence in dog saliva composition has not been reported.

The aims of this study were to evaluate the possible influence of biological factors, namely, breed and gender, and different saliva sampling conditions (with and without saliva stimulation with citric acid) on dog's saliva proteome.

2. Materials and Methods

2.1. Ethical Note. Dogs used in this study belong to three kennels and to a university (University of Murcia), whose gave their informed consent and participated in the collection procedures by handling the animals. The saliva collection and all animal procedures were carried out by researcher accredited by the Federation of European Laboratory Animal Science Association (FELASA) and conformed to legislation.

2.2. Dog Population. Dog population for each breed by gender and age is shown in Table 1. All were healthy and normal weight animals. Only male's pure breed Beagle were neutered animals.

2.3. Saliva Collection. Saliva samples were collected in the afternoon between 3:30 and 6:30 pm. Dogs did not eat for 16–18 hours prior to saliva sampling. Water was provided ad libitum. Saliva was collected by rolling a cotton cylinder (Salivette®, Sarstedt) inside each dog's mouth as described previously [19, 20]. The cotton cylinders were inserted under the dog's tongue for chew, until completely soaked with saliva, for a maximum of two minutes [21]. Two to three

sample were collected in all animals, in different days. In one of these sample collections two to three drops of lemon juice were put under the tongue for stimulating saliva flow (acid stimulation). Only for Rafeiro Alentejano breed acid stimulated saliva collection was not possible. After collection, the cotton cylinders were immediately placed on ice, until laboratory arrival, which lasted no more than 30 minutes. In the laboratory saliva was extracted from the cotton roll by centrifugation at 4°C, at 5000 rpm, for 5 min, and immediately stored at −20°C for further analysis.

2.4. Total Protein Concentration. Bradford method protein assay [22] with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA) was performed to determine the total protein concentration of each sample. Standards and samples were run in triplicate, in 96-well microplates. Absorbance was read at 600 nm in a microplate reader (Glomax, Promega).

2.5. SDS-PAGE. Proteins from individual saliva samples of all animals (both without and with acid stimulation) were separated by SDS-PAGE electrophoresis in 14% acrylamide gels in a mini-protean apparatus (BioRad) as described before [23]. Briefly, a total of 15 μg protein from each saliva sample was run in each lane. The samples were resuspended in sample buffer [Tris–HCl 0.125 M pH 6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoetanol, 20% (v/v) glycerol traces of bromophenol blue], heated at 95°C for 5 minutes, and run at a constant voltage of 140 V until the dye front reaches the end of the gel. Gels were fixed in 40% methanol, 20% acetic acid, for one hour, stained with Coomassie Brilliant Blue (CBB) G-250 (0.125% CBB G-250, 20% ethanol) for two hours and destained in several washes with distilled water. A scanning Molecular Dynamics densitometer with internal calibration and LabScan software (GE, Healthcare) were used to acquire gel images and to determine the percentage of volume of each protein band; GelAnalyzer software (http://www.gelanalyzer.com/) was used to analyze the gel images. Molecular masses were determined in accordance with molecular mass standards (Bio-Rad Precision Plus Protein Dual Color 161–0394) run with protein samples.

2.6. Two-Dimensional Gel Electrophoresis (2-DE)

2.6.1. Protein Precipitation. Due to the limited amount of individual saliva samples, the unstimulated and acid stimulated saliva samples from each breed and gender were mixed in pools, constituting a total of 12 pools: (1) unstimulated

female Portuguese Podengo; (2) unstimulated male Portuguese Podengo; (3) stimulated female Portuguese Podengo; (4) stimulated male Portuguese Podengo; (5) unstimulated female Greyhound; (6) unstimulated male Greyhound; (7) stimulated female Greyhound; (8) stimulated male Greyhound; (9) unstimulated female Rafeiro Alentejano; (10) unstimulated male Rafeiro Alentejano; (11) unstimulated male Beagle; (12) stimulated male Beagle. Volumes of saliva from each pool containing 250 μg of total protein were used. The volume of each pool was mixed with equal volume of TCA 20% (m/v), incubated overnight, at −20°C, followed by centrifugation at 15,000 g, 30 min, and two cold-acetone washes. This protocol as previously observed by us allows satisfactory results for preparation of dog saliva samples for 2-DE [24].

2.6.2. 2-DE Protein Separation. For 2-DE, the precipitates were mixed with 250 μL rehydration buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v), 60 mM DTT and traces of bromophenol blue] +5 μL IPG buffer +5 μL NaOH. Then the precipitates were sonicated until total resuspension and incubated during 1 h at room temperature, being subsequently centrifuged for 5 min at 10000 rpm. IPG strips (13 cm, pH 3–10 NL; GE, Healthcare) were passively rehydrated overnight with this solution. Focusing was performed in a Multiphor II (GE, Healthcare) at 20°C, with the programme (gradient): (1) 0–300 V for 2 h; (2) 300 V for 2 h; 300 V to 3500 V for 6 h; 3500 V for 6 h. Focused strips were equilibrated in two steps of 15 min each with equilibration buffer [50 mM Tris–HCl, pH 8.8; 6 M urea; 30% (v/v) glycerol and 2% (w/v) SDS], with the addition of 1% (w/v) DTT and 65 mM iodoacetamide in the first and second steps, respectively. After equilibration the strips were applied in the top of a SDS-PAGE gel 14% acrylamide and run at 150 V constant voltage in a mini-protean system (BioRad). Staining with CBB-G250 and destaining were done through the same protocol described for SDS-PAGE gels. Gel images were acquired using the same scan method and apparatus described for SDS-PAGE gels. ImageMaster 2D Platinum v7 software was used to analyze these gel images. Spot editing and the match were performed automatically and corrected manually. Spot volume was normalized to the total spot volume. Three laboratorial replicates of each pool were run.

2.7. Protein Identification. Bands and spots that differed among the factors tested were manually excised from gels and digested with trypsin following the protocol already described [25]. MALDI-TOF/-TOF mass spectrometry was used for protein identifications. Tryptic peptide mixtures were acidified with 5% (V/V) formic acid, desalted, and concentrated using home-made reversal phase (R2 pores-Applied Biosystems) microcolumns (R2 pores-Applied Biosystems). Peptides were eluted with the matrix solution (α-cyano-4-hydroxycinnamic acid Fluka) 5 mg/mL in 50% (v/v) acetonitrile and 5% (v/v) formic acid. MS and MS/MS data were acquired in positive reflector mode in a 4800 Plus AB SCIEX using the software 4000 Series Explorer, version 3.5.3.3 (Applied Biosystems).

Peptide mass spectra were acquired using a MALDI-TOF/TOF 4800 plus MS/MS (Applied Biosystems® Life Technologies, Carlsbad, United States of America). Data were acquired in positive MS reflector using a PepMix1 (LaserBio Labs, Sophia-Antipolis, France) to calibrate the instrument. Each reflector MS spectrum was collected in a result independent acquisition mode, using 750 shots per spectra in 800–4000 m/z range and fixed laser intensity to 3100 V. Fifteen of the strongest precursors were selected for MS/MS. MS/MS analyses were performed using CID (Collision Induced Dissociation) assisted with a collision energy of 1 kV and a gas pressure of $1 \times 10-6$ Torr. For each MS/MS spectrum, 1400 laser shots were collected, using fixed laser intensity of 4400 V. Processing and interpretation of MS and MS/MS spectra were performed with the 4000 Series Explored™ Software (Applied Biosystems® Life Technologies, Carlsbad, United States of America).

Protein identification was performed using MS and MS/MS spectral data and ProteinPilot (Applied Biosystems, version 3.0, rev. 114732) on Canis canis database (85118 sequences; 46,697,962 residues) retrieved from NCBI (downloaded in October 2017). Searches included trypsin as digesting enzyme; peptide mass tolerance of 50 ppm; fragment mass tolerance of 0.5 Da and possible oxidation, carbomidomethylation, or deamidation as variable amino acid modifications with one missed cleavage. Peptides were only considered if the ion score indicated extensive homology ($p < 0.05$). Proteins were considered if the protein score indicated significant statistical confidence ($p < 0.05$). Protein identifications with only one matched peptide were considered if they were identified with >95% confidence.

2.8. Statistical Analysis. Multivariate analyse of protein bands, on one hand, and protein spots, on the other, were performed with MetaboAnalyst 3.6 to evaluate clustering of individuals or groups [26]. Data normalization was used when normal distribution was not observed, using transformation (log10) or scaling methods, alone or combined. The method chosen was the one that allowed data to be normally distributed. For univariate analysis, t-test, one-way ANOVA, and two-way ANOVA were used for comparison of protein profiles (band percentage volume or spots percentage volume) between unstimulated and acid stimulated saliva and among breeds and genders. For Multivariate Analysis, partial least squares discriminant analysis (PLS-DA) was used. Discriminant variables selection was done using variable importance in the projection (VIP) with a threshold of 1.0. Finally, paired-samples t-test was used for comparison of total protein concentration between saliva samples with and without stimulation. Statistical significance was considered for $p < 0.05$.

3. Results

3.1. Effect of Acid Stimulation on Salivary Proteome

3.1.1. Total Protein Concentration. Total protein concentration decreased significantly in stimulated saliva in males of both pure breeds Portuguese Podengo and Beagle. In females,

TABLE 2: Comparison of total protein concentration (mean ± standard error) between saliva with acid stimulation and saliva without stimulation, for each dogs breed and gender.

| | Total protein concentration (μg/mL) | | |
	with acid stimulation	without stimulation	p
Breed			
Portuguese Pondego ($n = 6$)	843.0 ± 163.6	2385.7 ± 482.9	0.036[*]
Greyhound ($n = 7$)	961.7 ± 72.3	1146.7 ± 504.7	0.354
Beagle ($n = 7$)	1273.3 ± 161.8	1811.8 ± 246.3	0.033[*]
Gender			
Female (Podengo, $n = 4$, Greyhound, $n = 4$)	950.1 ± 115.3	1743.3 ± 404.3	0.112
Male (Beagle, $n = 7$, Podengo, $n = 2$, Greyhound, $n = 3$)	1049.2 ± 110.5	1737.3 ± 170.7	0.001[*]

[*]Statistically significant differences for $p < 0.05$.

(a)

(b)
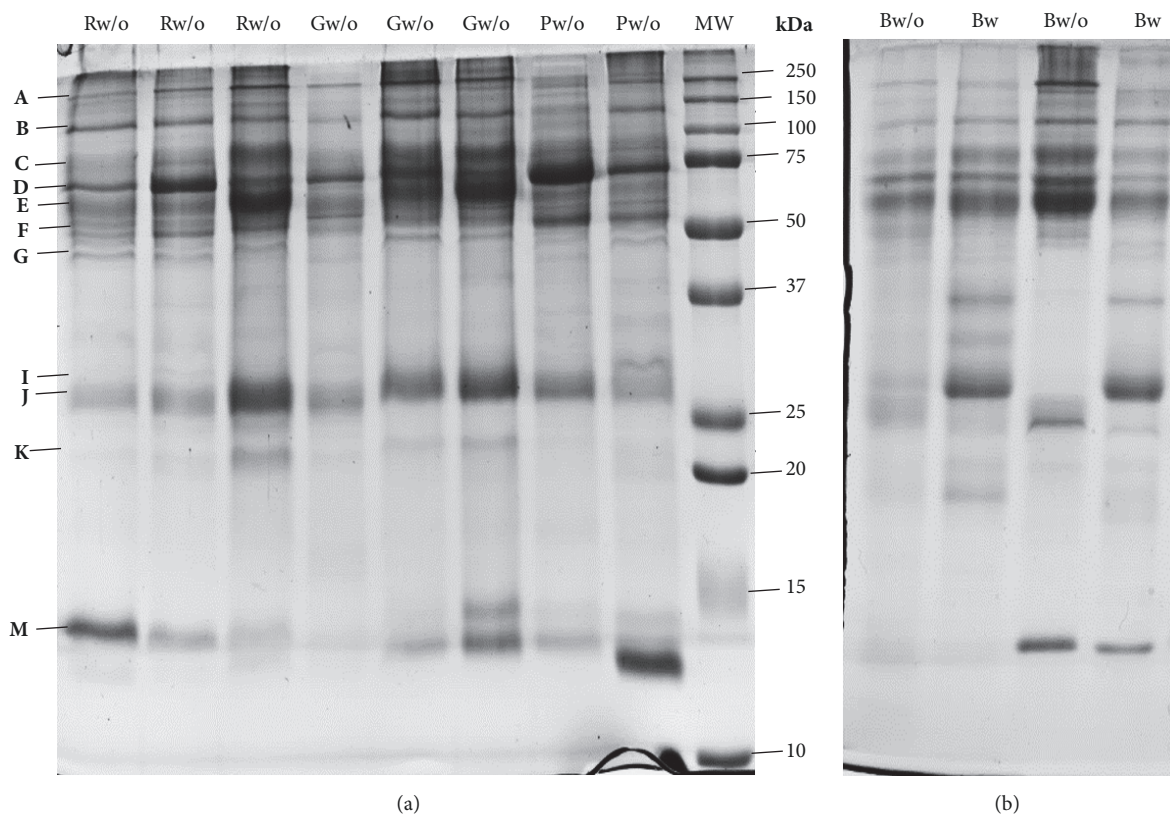
FIGURE 1: Representative SDS-PAGE profile of dog saliva: (a) from different breeds without stimulation (R: Rafeiro; G: Greyhound; P: Portuguese Podengo); (b) from Beagles without (w/o) and with (w) acid stimulation; MW: molecular mass marker; upper letters indicate the different protein bands.

no statistically significant differences were observed for saliva collected under the two conditions (Table 2). Concerning salivary flow rate, although this was not measured, it was possible to observe a tendency for higher salivary flow rates in big, comparatively to small breeds and higher salivary flow rate after lemon juice induction, in all breeds.

3.1.2. SDS-PAGE Profile. Among the 16 protein bands, with molecular masses between 20 and 245 kDa, observed in SDS-PAGE protein profiles (Figure 1), some presented changes

in their intensities/volumes, which were induced by acid stimulation. Some of these changes were observed to be dependent on the dogs' breed and/or gender. Considering the total of the animals, 2 of the protein bands decreased (F and J) and one increased (I1) with acid stimulation (Table 3). Concerning bands F and J, the decreased levels were observed only in males and not in females.

By considering the dog breeds separately, changes induced by stimulation were observed only for Beagles: decreased expression levels of 4 protein bands (B, D, F, and J)

TABLE 3: Protein bands differently expressed (mean ± standard error) between saliva collected with and without acid stimulation.

| Bands | % vol | | p |
	Without acid stimulation	With acid stimulation	
	Total of animals ($n = 20$)		
F	8.26 ± 0.46	5.61 ± 0.58	0.002*
I1	3.69 ± 0.51	7.62 ± 1.01	0.002*
J	12.81 ± 0.54	9.24 ± 0.63	0.0008*
	Beagles (only males) ($n = 7$)		
B	9.10 ± 0.79	6.13 ± 0.39	0.004*
D	10.28 ± 1.04	6.9 ± 0.39	0.004*
F	8.34 ± 0.91	4.15 ± 0.64	0.002*
I1	3.79 ± 1.00	10.10 ± 1.6	0.010*
J	11.88 ± 0.98	8.43 ± 0.70	0.002*
	Males (three breeds) ($n = 12$)		
F	8.34 ± 0.54	5.29 ± 0.69	0.003*
J	12.88 ± 0.68	8.56 ± 0.79	0.0003*

*Statistically significant differences for $p < 0.05$.

TABLE 4: Mass spectrometry identification of proteins present in bands from saliva SDS-PAGE profiles.

Band	Protein	NCBI Accession Code Accession n	Estim/theoret MW (kDa)#	ID Score*	Seq. Cov. (%)	Matched Peptides MS (MS/MS)
A	Mucin-19	XP_022267206.1	240.6/340.8	201	11	21 (5)
C	IgGFc-binding protein	XP_022261796.1	75/318.0	187	14	14 (9)
D	Chain A, Crystal Structure Analysis Of Canine Serum Albumin	pdb|5GHK|A	67.8/65.7	815	52	15 (11)
E	Serum albumin isoform X1	XP_005628024.1	61.3/68.6	661	44	12 (10)
F	IgGFc-binding protein	XP_022261796.1	52.6/318.0	313	8	13 (6)
M	Full-double-headed protease inhibitor, submandibular gland	sp|P01002.1|IPSG_CANLF	12.2/12.8	166	46	6 (3)

#MW values observed in gel versus theoretical ones. *Protein score is $-10*\log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 62 are significant ($p < 0.05$).

and increased expression level of 1 protein band (I1) (Table 3). Information about mass spectrometry details of identified proteins is present in Table 4.

Although, in the pure breeds Portuguese Podengo and Greyhound, none of the individual bands from SDS-PAGE protein profiles showed statistical significant intensity differences, between the saliva collected with and without acid stimulation, the multivariate PLS-DA model clustered separately unstimulated saliva from acid stimulated saliva, in these two breeds (Figure 2). The protein bands J, K, and M were the major contributors for the differences in Greyhounds. Band M was identified as containing full-double-headed protease inhibitor, whereas the other two bands resulted in no confident identification. The protein bands C, E, and G, identified as containing IgGFc-binding protein and serum albumin, were the major contributors for differences in Portuguese Podengos (Supplementary Figure 1).

3.1.3. Two-Dimensional Protein Profile (2-DE). By analyzing 2-DE salivary protein profiles (Figure 3), 3 protein spots were observed to be present in lower volume in the saliva collected after stimulation: spot 0 (34.4 ± 5.14 and 13.9 ± 2.97% vol., saliva without and with stimulation, respectively), spot 5 (0.73 ± 0.02 and 0.47 ± 0.05% vol., saliva without and with stimulation, respectively) and spot 81 (0.5 ± 0.24 and 0.21 ± 0.20% vol., saliva without and with stimulation). These spots were identified as serum albumin subunit A, cytoskeletal keratin, and one unknown protein (Table 5).

3.2. Effect of Dog's Breeds and Genders on Salivary Proteome

3.2.1. Total Protein Concentration. The four different breeds did not differ among them for the total protein concentration of saliva, as shown by univariate statistical analysis. Also, no differences were observed between genders, neither for saliva collected without nor saliva collected with acid stimulation.

3.2.2. SDS-PAGE Profile. Salivary protein profiles of the 4 dog breeds studied were compared for the saliva collected without acid stimulation. Six protein bands showed a different volume among dog breeds (Table 6): bands containing serum

(a)

(b)

(c)

(d)
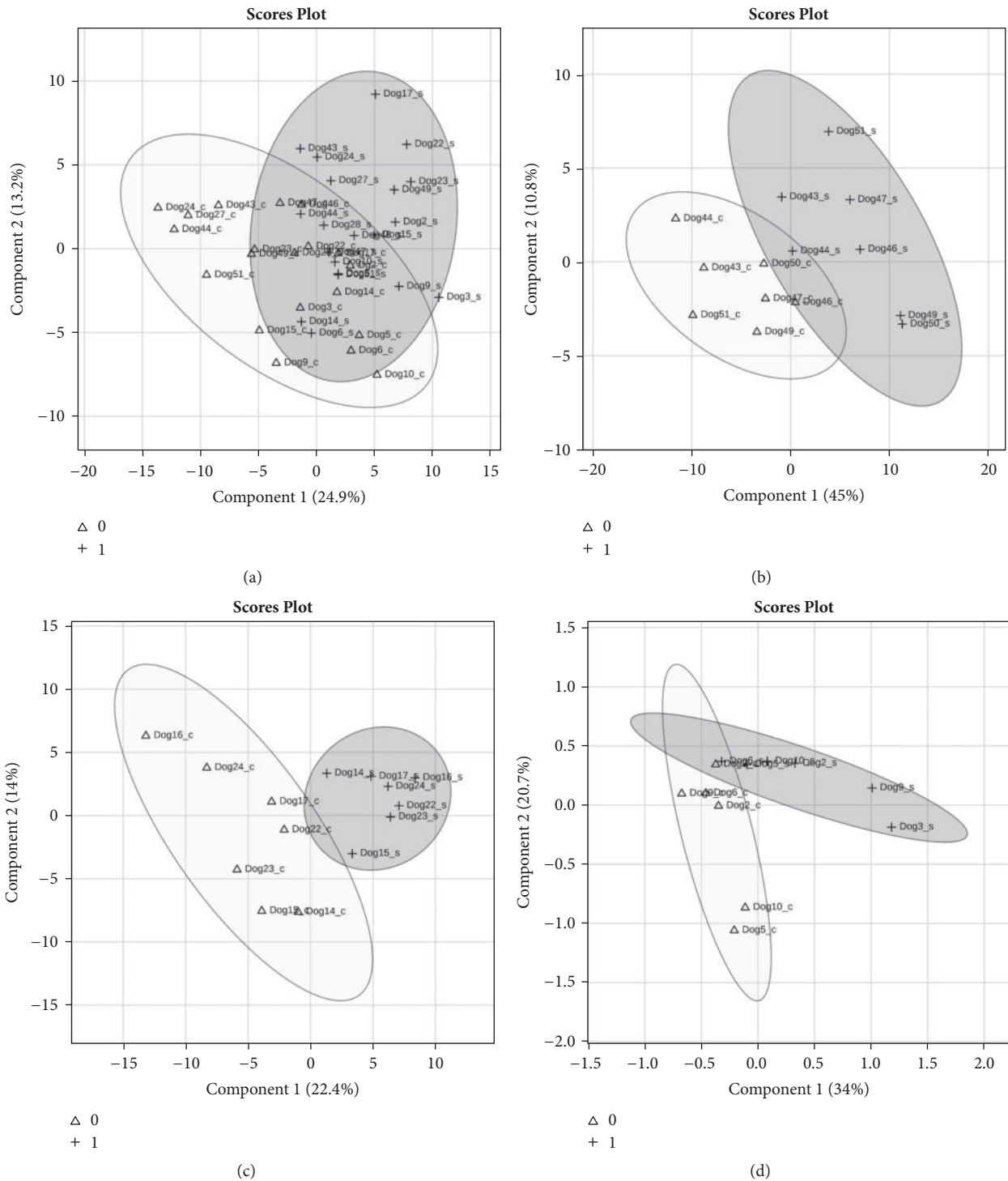
Figure 2: PLS-DA of saliva samples SDS-PAGE bands for all dogs (a), Beagles ($n = 7$) (b), Greyhounds ($n = 7$) (c), and Portuguese Podengos ($n = 6$) (d). Scaling was applied to rows when needed; X and Y axes show principal component 1 (PC1) and principal component 2 (PC2), respectively, and the total variance explained by each of them. Δ: with acid stimulation; +: without stimulation.

albumin were observed to be increased in Beagles, whereas a band containing a full-double-headed protease inhibitor was decreased, comparatively to the other breeds; bands containing albumin and IgGFc-binding protein were increased and one not identified was decreased in Portuguese Podengo. No

trends for gender were found and no relationship between breed and gender was found, as well.

Through the multivariate PLS-DA model, that has into account the interrelationship among variables, it was possible to cluster Portuguese Podengos and Beagles more distant,

TABLE 5: Mass spectrometry identification of proteins present in spots from saliva 2-DE profiles differing between stimulation conditions and/or among breeds.

Spots	Protein	Entry reference	Estim/ theoret MW (kDa)	Estim/ theor pI	Score ID*	% Seq. Cov.	Matched Peptides MS (MS/MS)
0	Chain A, Crystal Structure Analysis Of Canine Serum Albumin	pdb|5GHK|A (NCBI)	78.1/65.7	4.9/5.3	263	42	18 (2)
5	Keratin, type I cytoskeletal 10	Q6EIZ0 (Uniprot)	18.5/57.7	7.8/5.1	207	30	10 (5)
8	double-headed protease inhibitor, submandibular gland	XP_022264993.1 (NCBI)	17.9/15.7	6.0/8.6	428	58	4 (8)
12	Immunoglobulin J chain	XP_532398.2 (NCBI)	30.1/18.3	4.4/4.7	125	40	3 (2)
16	Immunoglobulin lambda-1 light chain isoform X34	XP_005636600.1 (NCBI)	30.0/24.8	6.0/6.4	326	33	6 (4)
18	Immunoglobulin lambda-1 light chain isoform X25	XP_022266294.1 (NCBI)	31.0/24.9	5.5/5.1	198	35	8 (4)
37	IgGFc-binding protein	XP_022261796.1 (NCBI)	59.9/318.0	4.9/5.2	267	4	7 (4)
45	Uncharacterized protein	J9P732 (Uniprot)	25.0/21.4	5.8/6.0	192	28	4 (5)
81	Uncharacterized protein	F1PW98 (Uniprot)	19.1/55.0	8.0/5.7	111	29	15 (2)

*Protein score is $-10*\log(P)$, where P is the probability that the observed match is a random event. Protein scores greater than 62 are significant ($p < .05$).
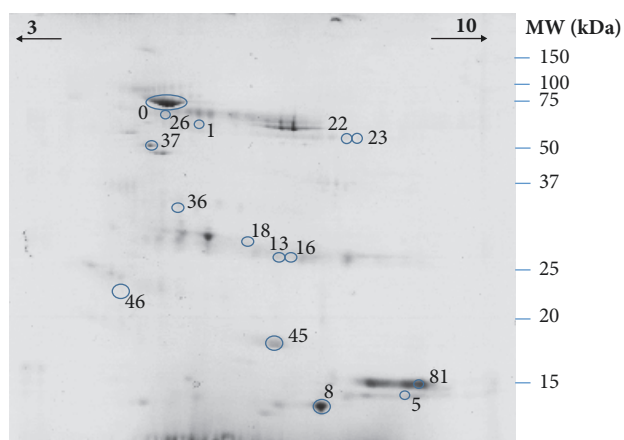
FIGURE 3: Representative dog's saliva 2-DE gel. Spots excised for digestion and identification by MS are numbered.

comparatively to the other breeds (Figure 4(a) and Supplementary Figure 2). The differences between Portuguese Podengo and Beagles for nonstimulated saliva were confirmed in the saliva collected after acid stimulation, by univariate analysis. In this case, it was also possible to observe that these breeds differ in saliva protein profile, with five proteins bands (E, F, I1, J, and M) observed to be differently expressed (Table 7 and Figure 4(b)).

3.2.3. 2-DE Saliva Profile. 2-DE salivary protein profiles of the several dog breeds evaluated presented differences in the percentage volumes of 7 protein spots. Through ANOVA (univariate analysis) it was observed that Portuguese Podengo presented higher levels of 5 salivary protein spots [1 ($p = 0.034$), 18 ($p = 0.041$), 22 ($p = 0.046$), 36 ($p = 0.036$), and 46 ($p = 0.014$)], comparatively to the other breeds. Among them, only spot 18 was identified (as a light-chain

of immunoglobulin lambda-1). Spots 8 ($p = 0.043$) and 26 ($p = 0.015$) were present at different levels in Beagles, the spot 8 (identified as double-headed protease inhibitor) being in lower levels than in Greyhounds and the spot 26 (not identified) in higher levels than in the other breeds.

Besides these spots, multivariate PLS-DA model clustered Portuguese Podengo distinctly from Beagles and Rafeiro Alentejano breeds in 2-DE protein profiles (Figure 5). Spots 1, 18, 23, 45, and 82 were the ones that most contributed to these differences (Supplementary Figure 3). Detailed information about MS/MS identification of the referred spots is presented in Table 5.

In the case of spots 45 and 81, the identification resulted in unknown proteins. However, through BLAST analysis, it was possible to observe 83% homology between the protein present in spot 45 and a S100 calcium binding protein A9 and 83% homology between the protein present in spot 81 and keratin 8.

4. Discussion

In this study, the influence of gender and acid stimulation on the normal dog salivary proteome of different breeds was studied through in-gel based proteomics approach. For all the breeds, animals with a wide range of ages were included in the study. The number of proteins observed and identified in dog saliva through this methodology is much lower than the one reported in other studies, using LC-MS/MS [10, 27]. Nevertheless, in this study, dog gel protein profiles presented what can be of utility for studies where protein isoforms and/or posttranslational modifications (PTMs) are of interest [11]. SDS-PAGE and 2-DE protein separations were simultaneously performed in this study due to the limited amount of individual saliva. As such, SDS-PAGE was used for assessing variability and to make comparisons using individual information. Since this approach only allows

TABLE 6: Protein bands differently expressed (mean ± standard error) between dog breeds, in saliva collected without acid stimulation.

Bands	Breed		% vol		p
B	Beagle	Port. Pod.	9.02 ± 0.63[a]	6.07 ± .31[b]	0.005
		Greyhound		6.71 ± .44[b]	
		Raf. Alent.		6.86 ± .38[b]	
D	Port. Pod.	Greyhound	12.59 ± 1.32[a]	8.16 ± 0.80[b]	0.005
		Raf. Alent.		8.2 ± 0.46[b]	
		Beagle		10.71 ± 0.48	
E	Beagle	Port. Pod.	13.7 ± 1.29[a]	8.14 ± 0.53[b]	0.005
		Greyhound		11.5 ± 0.98[a,b]	
		Raf. Alent.		9.97 ± 0.45[b]	
F	Port. Pod.	Greyhound	9.66 ± 0.75[a]	7.05 ± 0.62[a,b]	0.01
		Raf. Alent.		6.39 ± 0.56[b]	
		Beagle		8.53 ± 0.90[a,b]	
G	Port. Pod.	Greyhound	3.73 ± 0.72[a]	6.32 ± 0.51[a,b]	0.005
		Raf. Alent.		6.35 ± 0.35[b]	
		Beagle		8.43 ± .99[b]	
M	Beagle	Port. Pod.	1.71 ± 0.005[a]	8.64 ± 1.04[b]	0.005
		Greyhound		7.44 ± 1.25[b]	
		Raf. Alent.		5.98 ± .83[b]	

Different letters mean statistically significant differences between pairs, for $p < 0.05$. Beagle ($n = 10$); Portuguese Podengo ($N = 7$); Greyhound ($n = 11$); Rafeiro Alentejano ($n = 13$).
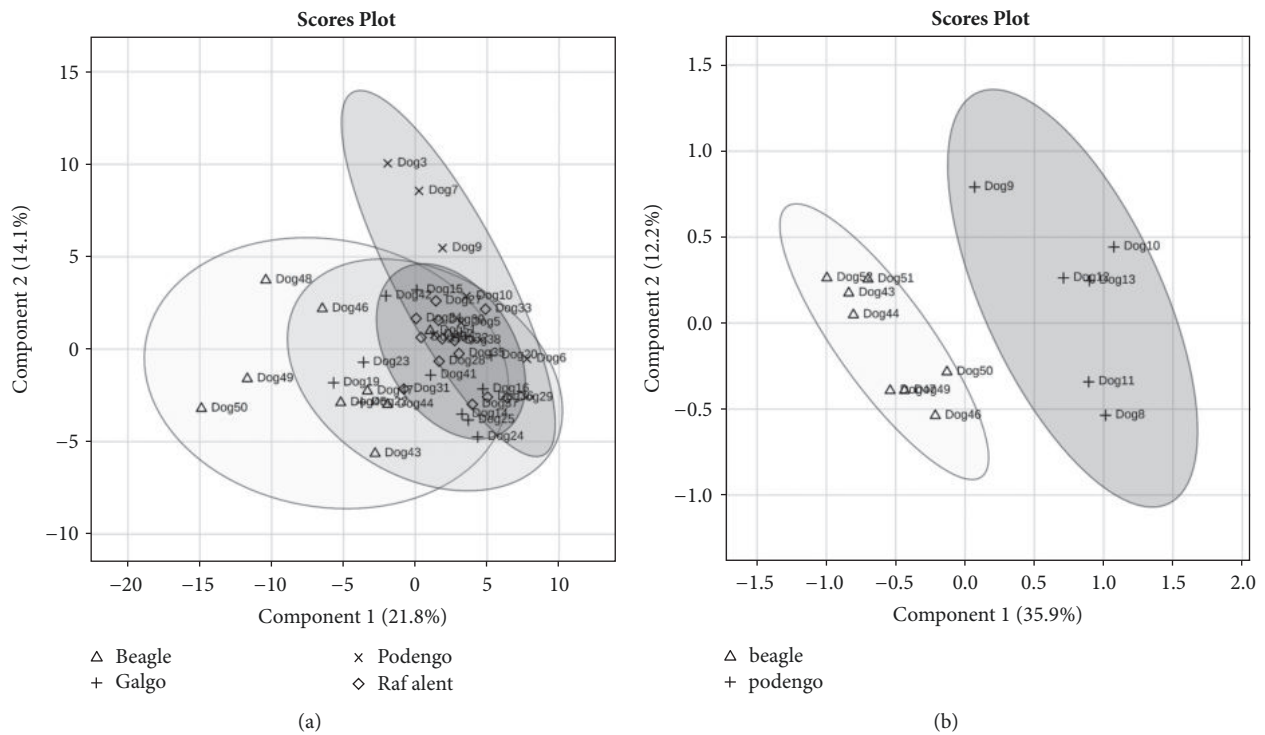
FIGURE 4: Partial Least Square Determinant Analysis (PLS-DA) model for all dog unstimulated saliva samples SDS-PAGE bands [Δ: Portuguese Podengo ($n = 7$); +: Greyhound ($n = 11$); ◊: Rafeiro Alentejano ($n = 13$); and x: Beagles ($n = 10$)] (a) and for stimulated saliva samples SDS-PAGE bands [+: Portuguese Podengo ($n = 6$) and Δ: Beagles ($n = 8$)] (b). Scaling was applied to rows when needed; X and Y axes show principal component 1 (PC1) and principal component 2 (PC2), respectively, and the contribution of each of them for explaining the total variance.
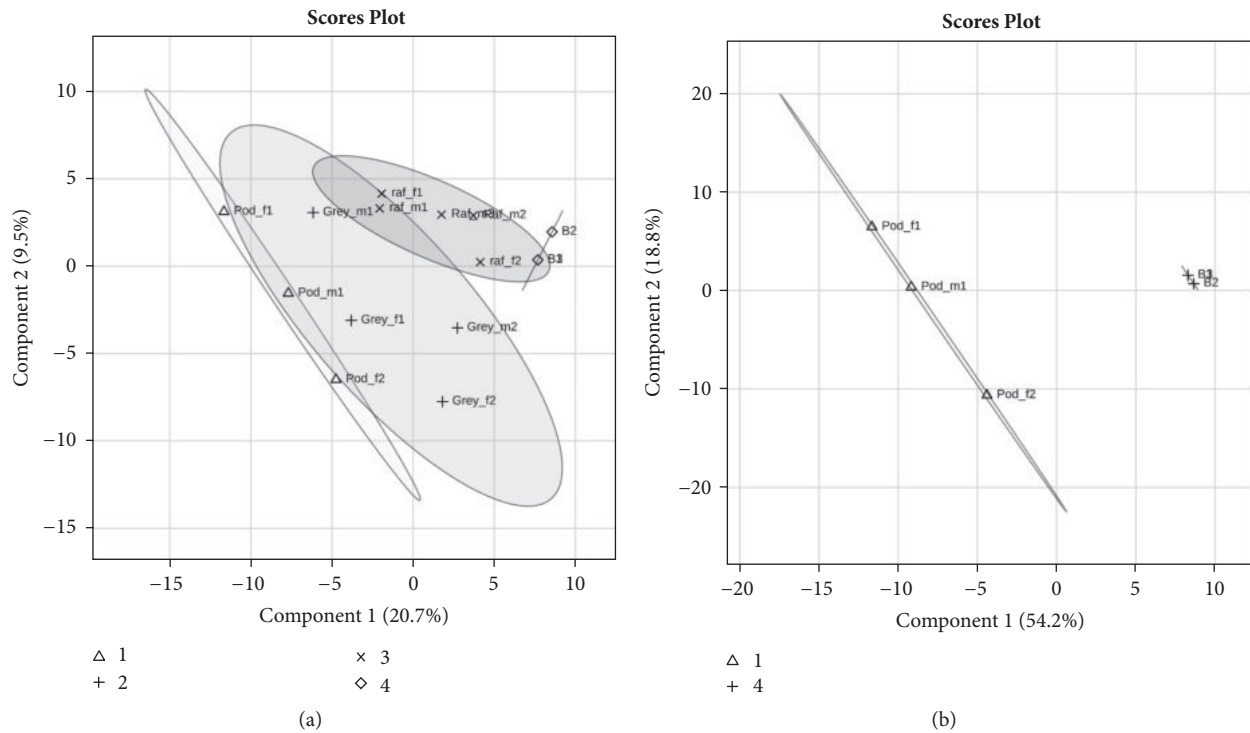
FIGURE 5: PLS-DA of dog saliva pool samples 2-DE spots of each breed (a) or considering only Portuguese Podengo and Beagles (b). Log transformation was applied to rows; X and Y axis show principal component 1 (PC1) and principal component 2 (PC2), and the respective % of explanation for the total variance. 1 – Portuguese Podengo; 2- Greyhound; 3- Rafeiro Alentejano; 4- Beagle.

TABLE 7: Protein bands differently expressed (mean ± standard error) in saliva samples with acid stimulation between Portuguese Podengo ($n = 6$) and Beagles ($n = 8$)[#].

Bands	% vol		P^*
	Portuguese Podengo	Beagle	
E	7.39 ± 1.64	13.21 ± 0.54	0.003
F	8.89 ± 0.57	3.98 ± 0.60	9.075e − 05
I1	2.72 ± 1.07	10.64 ± 1.50	0.002
J	12.87 ± 1.21	8.40 ± 0.60	0.004
M	9.12 ± 0.68	2.85 ± 0.60	1.6369e − 05

[*]Statistically significant differences for $p < 0.05$. [#] N of Beagles used for comparison was different that the one reported in Table 3, since for 1 animal only saliva from the collection after stimulation contained enough amount for analysis, impeding that animal for being included in paired analysis reported in Table 3.

separation according to molecular masses, several proteins must be present in each band, making it difficult to know the one (or several) responsible for changes. 2-DE profiles of saliva pools were used to add such detail.

No significant differences among breeds or between genders were observed on total protein concentration of normal dog's saliva. However, a decrease in the total protein concentration after acid stimulation was observed, especially in males of both pure breeds Portuguese Podengo and Beagle. In terms of profiles, proteins such as cytoskeletal keratin, serum albumin, and IgGFc-binding proteins were

identified in bands and/or spots whose levels decreased with acid stimulation. IgG Fc-binding protein has been recently identified as one of the more abundant proteins in dog saliva [10] being a protein involved in binding IgG on mucosal surfaces [28]. To our knowledge, there are no other reports, in the literature, concerning the effect of acid stimulation on salivary proteome of dogs or other animals. But, our results are in accordance with studies performed in humans [12], where it was observed that acid stimulation produced considerable major changes, namely, in proteins related to immune function, inflammation, and cell movement [12]. Also Lorenz et al. (2011) [29] observed significant decreases on the relative abundance of several protein spots, in human saliva, after citric acid stimulation. It is curious that keratin is a protein from the cytoskeleton and IgG Fc-binding protein is a gel-like component of the mucosa. Stimulation with lemon juice raised the total volume of saliva produced and, as such, the cotton roll needed less time in the mouth for getting enough saliva amounts. Such decreased time of saliva collection, associated with fewer movements, may have resulted in a lower incorporation of components from the epithelium in the samples. In fact, the possibility of variations in the levels of these proteins being done to this effect was recently suggested [13].

In dogs, saliva collection without stimulation has the constraint of allowing obtaining only limited volumes of saliva for performing some laboratorial techniques [30]. However, if stimulation is needed it is important to have in

mind the referred differences in protein composition that such stimulation is producing.

In the present study, we could observe that salivary protein composition varies among different dog breeds, but no major differences were observed between genders. The reduced impact of gender in dog salivary proteome observed is in agreement with others recently published [13]. Our results go in accordance with these observations.

Two of the breeds that most differed between them were Portuguese Podengo and Beagle. According to Federation Cynologique Internationale (FCI) (http://www.fci.be, accessed on January 31, 2018) purebred Portuguese Podengo is a primitive type of breed, hunting dog probably originating from the ancient dogs, traditionally used for helping in rabbit or birds hunting, but without working trial [31]. This breed is also used as a watch and companion dog. Despite being a pure breed, it is expected that individuals present higher genetic variability than Beagles, since this last has been bred in a controlled way, also for use in laboratory studies. Also according to FCI, purebred Beagles belong to a small-sized hound group with working trial. By using clustering analysis, to define phylogenetic tree, this breed belongs to a cluster comprised mostly by modern breeds used in hunting [32].

Bands containing chains of canine serum albumin and IgGFc-binding protein were proteins differently expressed among dog's breeds. One of the proteins observed to be present in lower amounts in Beagles, both in SDS-PAGE and in 2-DE protein profiles, was the full-double-headed protease inhibitor from the submandibular glands. This protein is a serine type endopeptidase, which has been assumed to protect mucosal cells in mouth and oesophagus against the action of proteinases from microbial origin and/or ingested with food [33].

In the present study only a limited number of proteins were observed to differ with stimulation and/or among breeds. Even some protein spots failed a positive identification, which can be related to a lower number of proteins present in curated protein databases, comparatively to other species, such as humans. On the other hand, in this study, dogs were available from pure breed kennels and some of the differences observed for the different breeds can be done to different types of dog food consumed. Further studies, with a higher number of animals per breed, higher number of breeds and controls for type of food, and other treatments are necessary to have a better characterization of each breed saliva proteome.

5. Conclusions

This work, in line with what was hypothesized, allowed us to conclude that dog salivary protein composition is influenced by different factors. Despite the need of procedures that allow the collection of higher amounts of saliva, it is necessary to be aware that techniques such as acid stimulation not only induce higher salivary flow rates, but also change the levels/proportion of various salivary proteins. It is also of interest to retain that dog salivary proteome should be considered according to dog breed, since this was observed to be a factor responsible for variations in the proportion of different salivary proteins. In fact, breed appears to have even more influence than gender. Nevertheless, that does not mean that gender should be ignored, in dog saliva analysis. Despite males and females presenting minimal differences in salivary profiles, in this study differences in the way each gender responded to stimulation were observed.

From our knowledge this is one of the first studies evaluating factors affecting dog saliva electrophoretic protein profiles. More studies are needed to increase the knowledge about dog saliva proteome, in order to use it in research and diagnosis.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Supplementary Materials

Supplementary 1. Supplementary Figure 1: PLS-DA loading plots (left) of the first two components for analysis of SDS-PAGE bands of profiles from saliva collected with and without acid stimulation in Beagles (a), Greyhound (b), and Portuguese Podengo (c); for each case, variable importance in the projection (VIP) is presented, with 1.5 score considered as thresholder (right).

Supplementary 2. Supplementary Figure 2: PLS-DA loading plots (left) of the first two components for analysis of protein bands of salivary profiles from the different dog breeds. Variable importance in the projection (VIP) is presented (right).

Supplementary 3. Supplementary Figure 3: PLS-DA loading plots (left) of the first two components for analysis of protein

spots of salivary profiles from the different dog breeds. Variable importance in the projection (VIP) is presented (right).

References

[1] C. S. Chee, K. M. Chang, M. F. Loke, V. P. Angela Loo, and V. Subrayan, "Association of potential salivary biomarkers with diabetic retinopathy and its severity in type-2 diabetes mellitus: a proteomic analysis by mass spectrometry," *PeerJ*, vol. 4, article e2022, 2016.

[2] M. Castagnola, E. Scarano, G. C. Passali et al., "Salivary biomarkers and proteomics: Future diagnostic and clinical utilities," *Acta Otorinolaryngologica Italica*, vol. 37, no. 2, pp. 94–101, 2017.

[3] M. L. Cobb, K. Iskandarani, V. M. Chinchilli, and N. A. Dreschel, "A systematic review and meta-analysis of salivary cortisol measurement in domestic canines," *Domestic Animal Endocrinology*, vol. 57, pp. 31–42, 2016.

[4] U. Jankowska, A. Latosinska, B. Skupien-Rabian, B. Swiderska, M. Dziedzicka-Wasylewska, and S. Kedracka-Krok, "Optimized procedure of extraction, purification and proteomic analysis of nuclear proteins from mouse brain," *Journal of Neuroscience Methods*, vol. 261, pp. 1–9, 2016.

[5] B. Lösch and R. Wank, "Life-threatening angioedema of the tongue: the detection of the RNA of B henselae in the saliva of a male patient and his dog as well as of the DNA of three Bartonella species in the blood of the patient," *BMJ Case Reports*, vol. 2014, Article ID 203107, 2014.

[6] P. P. Mshelbwala, A. B. Ogunkoya, and B. V. Maikai, "Detection of rabies antigen in the saliva and brain of apparently healthy dogs slaughtered for human consumption and its public health implications in Abia State, Nigeria," *ISRN Veterinary Science*, vol. 2013, Article ID 468043, 5 pages, 2013.

[7] M. D. Parra, F. Tecles, S. Martínez-Subiela, and J. J. Cerón, "C-reactive protein measurement in canine saliva," *Journal of Veterinary Diagnostic Investigation*, vol. 17, no. 2, pp. 139–144, 2005.

[8] C. Breitenbuecher, J. M. Belanger, K. Levy et al., "Protein expression and genetic variability of canine Can f 1 in golden and Labrador retriever service dogs," *Canine Genetics and Epidemiology*, vol. 3, no. 1, article 3, 2016.

[9] H. Nakanishi, T. Ohmori, M. Hara, K. Yoneyama, A. Takada, and K. Saito, "Identification of canine saliva using mRNA-based assay," *International Journal of Legal Medicine*, vol. 131, no. 1, pp. 39–43, 2017.

[10] S. M. F. Torres, E. Furrow, C. P. Souza et al., "Salivary proteomics of healthy dogs: an in depth catalog," *PLoS ONE*, vol. 13, no. 1, Article ID e0191307, 2018.

[11] D. Kang and M. H. Moon, "Development of non-gel-based two-dimensional separation of intact proteins by an on-line hyphenation of capillary isoelectric focusing and hollow fiber flow field-flow fractionation," *Analytical Chemistry*, vol. 78, no. 16, pp. 5789–5798, 2006.

[12] X. Xiao, Y. Liu, Z. Guo et al., "Comparative proteomic analysis of the influence of gender and acid stimulation on normal human saliva using LC/MS/MS," *PROTEOMICS—Clinical Applications*, vol. 11, no. 7-8, Article ID 1600142, 2017.

[13] S. Pasha, T. Inui, I. Chapple, S. Harris, L. Holcombe, and M. M. Grant, "The saliva proteome of dogs: variations within and between breeds and between species," *Proteomics*, vol. 18, no. 3-4, Article ID 1700293, 2018.

[14] A. J. Kobelt, P. H. Hemsworth, J. L. Barnett, and K. L. Butler, "Sources of sampling variation in saliva cortisol in dogs," *Research in Veterinary Science*, vol. 75, no. 2, pp. 157–161, 2003.

[15] R. Bergeron, S. L. Scott, J.-P. Émond, F. Mercier, N. J. Cook, and A. L. Schaefer, "Physiology and behavior of dogs during air transport," *Canadian Journal of Veterinary Research*, vol. 66, no. 3, pp. 211–216, 2002.

[16] N. A. Dreschel and D. A. Granger, "Methods of collection for salivary cortisol measurement in dogs," *Hormones and Behavior*, vol. 55, no. 1, pp. 163–168, 2009.

[17] J. A. M. van der Borg, B. Beerda, M. Ooms, A. S. de Souza, M. van Hagen, and B. Kemp, "Evaluation of behaviour testing for human directed aggression in dogs," *Applied Animal Behaviour Science*, vol. 128, no. 1–4, pp. 78–90, 2010.

[18] L. M. Glenk, O. D. Kothgassner, B. U. Stetina, R. Palme, B. Kepplinger, and H. Baran, "Salivary cortisol and behavior in therapy dogs during animal-assisted interventions: A pilot study," *Journal of Veterinary Behavior: Clinical Applications and Research*, vol. 9, no. 3, pp. 98–106, 2014.

[19] P. Mongillo, E. Pitteri, P. Carnier, G. Gabai, S. Adamelli, and L. Marinelli, "Does the attachment system towards owners change in aged dogs?" *Physiology & Behavior*, vol. 120, pp. 64–69, 2013.

[20] C. Pastore, F. Pirrone, F. Balzarotti, M. Faustini, L. Pierantoni, and M. Albertini, "Evaluation of physiological and behavioral stress-dependent parameters in agility dogs," *Journal of Veterinary Behavior: Clinical Applications and Research*, vol. 6, no. 3, pp. 188–194, 2011.

[21] E. Lamy, G. da Costa, R. Santos et al., "Sheep and goat saliva proteome analysis: A useful tool for ingestive behavior research?" *Physiology & Behavior*, vol. 98, no. 4, pp. 393–401, 2009.

[22] K. Jessie, O. H. Hashim, and Z. H. A. Rahim, "Protein precipitation method for salivary proteins and rehydration buffer for two-dimensional electrophoresis," *Biotechnology*, vol. 7, no. 4, pp. 686–693, 2008.

[23] E. Lamy, C. Simões, L. Rodrigues et al., "Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not," *Journal of Physiology and Biochemistry*, vol. 71, no. 4, pp. 691–702, 2015.

[24] S. Lucena, M. C. Carreira, L. Rodrigues, F. Capela-Silva, A. Tvarijonaviciute, and E. Lamy, "Comparison of protein precipitation methods for two-dimensional electrophoresis of dog salivary proteins," *Journal of Integrated Omics*, In press.

[25] L. Rodrigues, G. da Costa, C. Cordeiro, C. C. Pinheiro, F. Amado, and E. Lamy, "Relationship between saliva protein composition and 6-*n*-Propylthiouracil bitter taste responsiveness in young adults," *Journal of Sensory Studies*, vol. 32, no. 4, Article ID e12275, 2017.

[26] J. Xia and D. S. Wishart, "Using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis: using MetaboAnalyst 3.0 for comprehensive metabolomics data analysis," in *Current Protocols in Bioinformatics*, A. Bateman, W. R. Pearson, L. D. Stein, G. D. Stormo, and J. R. Yates, Eds., pp. 14.10.1–14.10.91, John Wiley & Sons, Hoboken, NJ, USA, 2016.

[27] P. de Sousa-Pereira, M. Cova, J. Abrantes et al., "Cross-species comparison of mammalian saliva using an LC-MALDI based proteomic approach," *PROTEOMICS*, vol. 15, no. 9, pp. 1598–1607, 2015.

[28] K. Kobayashi, H. Ogata, M. Morikawa et al., "Distribution and partial characterisation of IgG Fc binding protein in various mucin producing cells and body fluids," *Gut*, vol. 51, no. 2, pp. 169–176, 2002.

[29] K. Lorenz, M. Bader, A. Klaus, W. Weiss, A. Görg, and T. Hofmann, "Orosensory stimulation effects on human saliva proteome," *Journal of Agricultural and Food Chemistry*, vol. 59, no. 18, pp. 10219–10231, 2011.

[30] C. M. M. Lensen, C. P. H. Moons, and C. Diederich, "Saliva sampling in dogs: How to select the most appropriate procedure for your study," *Journal of Veterinary Behavior: Clinical Applications and Research*, vol. 10, no. 6, pp. 504–512, 2015.

[31] C. San José, M. J. Cárcel, M. T. Tejedor, and L. V. Monteagudo, "Microsatellite DNA markers applied to the classification of the Podenco Valenciano canine breed," *Italian Journal of Animal Science*, vol. 17, no. 1, pp. 49–52, 2017.

[32] E. A. Ostrander and R. K. Wayne, "The canine genome," *Genome Research*, vol. 15, no. 12, pp. 1706–1716, 2005.

[33] H. Fritz, E. Jaumann, R. Meister, P. Pasquay, K. Hochstrasser, and E. Fink, "Proteinase inhibitors from dog submandibular glands—isolation, amino acid composition, inhibition spectrum," in *Proceedings of the International Research Conference on Proteinas Inhibitors*, pp. 257–270, Walter de Gruyter, Berlin, Germany, 1970.

STUDY 4

COMPARATIVE PROTEOMIC ANALYSIS OF SALIVA FROM DOGS WITH AND WITHOUT OBESITY-RELATED METABOLIC DYSFUNCTION

PREPARED TO SUBMISSION

COMPARATIVE PROTEOMIC ANALYSIS OF SALIVA FROM DOGS WITH AND WITHOUT OBESITY-RELATED METABOLIC DYSFUNTION

Sónia Lucena[1,2], Ana Varela Coelho[3], Sandra I. Anjo[4,5], Bruno Manadas[4], Vladimir Mrljak[6], Fernando Capela e Silva[1,7], Elsa Lamy[1], Asta Tvarijonaviciute[8]

[1]*Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM);* [2]*Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, Portugal;* [3]*Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Portugal;* [4]*CNC - Center for Neuroscience and Cell Biology, University of Coimbra, Portugal;* [5]*Faculty of Medicine, University of Coimbra, Portugal;* [6]*Clinic for Internal Dieaases, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10 000 Zagreb, Croatia;* [7]*Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Portugal;* [8]*Interlab-UMU, Regional Campus of International Excellence Campus Mare Nostrum, University of Murcia, Murcia 30100, Espinardo, Spain.*

ABSTRACT

Dogs develop only some of the components of the human metabolic syndrome (MetS). Thus, in order to study possible MetS-related alterations in dogs, human MetS criteria were adapted to define canine MetS or so called obesity-related metabolic dysfunction (ORMD). The main objective of this study was to identify changes in the salivary proteome of obese dogs with ORMD in comparison with obese dogs without ORMD which may constitute potential salivary biomarkers for assessing ORMD diagnosis in obese dogs. Twelve adult obese dogs without ORMD (N=6) and with ORMD (N=6) were included in the study. Body condition score was determined using a 5-point scale in both groups. Saliva samples were subjected to a quantitative proteomics analysis and the levels of eight salivary proteins were found to be significantly different between groups, among them those which had greatest fold-change were Kallikrein-1 precursor, GC, vitamin D binding protein and BPIFA2. In conclusion, the proteins identified in the present study, were related to obesity and/or type 2 diabetes, and suggested the implication of inflammation and prothrombotic state in dogs with ORMD.

Key-words: dog, saliva, proteome, obesity, metabolic dysfunction

1. Introduction

In humans, the term 'metabolic syndrome' (MetS) describes a clustering of obesity (especially visceral obesity), hypertension, insulin resistance or hyperglycaemia, and hyperlipidaemia, especially increased fasting triglycerides and decreased High-Density Lipoprotein Cholesterol (HDL-C) [1]. The MetS itself causes no clinical signs, but comprises a set of important risk factors for diseases that cause morbidity and mortality in humans, including atherosclerosis, coronary heart disease, stroke, and type 2 diabetes [2]. Dogs develop some of the components of the human MetS: obesity [3], insulin resistance [4], increased blood pressure [5], and hyperlipidaemia [6]. Nevertheless, the most important consequences of the MetS in humans such as type 2 diabetes (T2D), stroke and coronary heart disease, either do not exist or are very rare in dogs suggesting that dogs could have protective mechanisms or that dogs lack pathophysiological elements present in humans [7]. In order to study possible MetS-related alterations in dogs, human MetS criteria were adapted to define canine MetS or so called obesity-related metabolic dysfunction (ORMD) [8]. Approximately 20% of dogs with naturally occurring obesity were described to suffer ORMD [8] associated with insulin resistance and hypoadiponectinemia [8–10].

Saliva has gained interest for biomarker identification, mainly due to the non-invasive nature of its collection, at the same time that it contains glandular and blood-born molecules whose levels can change under different conditions [11]. In humans, various MetS biomarkers were found in saliva samples, such as salivary HDL-C and fasting glucose levels, adipokines (such as adiponectin, leptin, resistin) and proinflammatory markers such as C-reactive protein (CRP), insulin, ghrelin, tumour necrosis factor alpha (TNF-α), interleukins, either in adults or adolescents and children [12–14]. The results of those studies provide useful information about the development of this metabolic disease and establish that saliva may be a fluid of interest in the study of this syndrome [13].

In this study, we hypothesized that dogs with metabolic dysfunction could have a different salivary protein composition comparatively to dogs without ORMD. Thus, the main objective of this study was to identify changes in the salivary proteome in obese dogs with ORMD that can help to understand the metabolic/pathophysiological changes related to this condition together with the identification of potential biomarkers for its diagnosis.

2. Materials and methods

2.1. Ethical note

The study protocol adhered to the University of Murcia Animal Ethics Guidelines, and was approved by the University of Murcia Research Ethics Committee (323/2017) and Water, Agriculture, Livestock and Fisheries Counselling of Murcia Region Ethics Committee (A13170806).

2.2. Dog population

Six castrated adult obese dogs without ORMD (non-ORMD group) and six castrated adult obese dogs with ORMD (ORMD group) were included in the study. All dogs were evaluated for their general health status and only those that did not present signs of other than obesity diseases, were included in the study. CRP was measured in all dogs in order to discard active inflammation; only dogs with CRP < 12 mg/L were considered for the inclusion in the study. Body condition score (BCS) was determined using a validated 5-point body condition score system in both groups [15]. Blood pressure in all dogs was measured non-invasively using an oscillometric method. All dogs were fully conscious. A cuff of the appropriate size (e.g. the cuff chosen had a width of ~40% circumference of the leg) was placed on the right forelimb. Once the dog was calm and still, at least five systolic arterial pressure (SAP) readings were taken and averaged. The descriptive clinical data of dogs from both groups are detailed in Table 1.

2.3. Definition of obesity-related metabolic dysfunction

Dogs were considered as having ORMD if met previously described criteria [8]: (a) BCS 4-5/5; and (b) any two of the following: 1) plasma triglycerides >200 mg/dl; 2) plasma cholesterol >300 mg/dl; 3) SAP > 160 mmHg; 4) fasting plasma glucose >100 mg/dl, or previously diagnosed diabetes mellitus [8].

2.4. Serum analysis

Serum total cholesterol, triglycerides and glucose were measured in an automated biochemistry analyser (Olympus AU600, Beckman Coulter, Brea, USA) using commercially available reagents and following the instructions of the manufacturer.

2.5. Saliva analysis

2.5.1 Saliva collection

Saliva samples were collected as previously described [16]. A sponge was placed in each dog's mouth, left in contact with the cheek mucosa for 1–2 min, and was then placed into the Salivette device for centrifugation (3000g, 10 min, 4ºC). After

centrifugation, saliva was transferred to 1.5mL polyethylene tubes and stored at -80°C until analysis.

2.5.2. Total protein concentration

Bradford method protein assay with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA) was performed to determine the total protein concentration of each sample. Standards and samples were run in triplicate, in 96 wells microplates. Absorbance was read at 600 nm in a microplate reader (Glomax, Promega).

2.5.3. Protein digestion in solution

The volume of each saliva sample correspondent to a total of 50 µg of protein was added to 10 µL of 6M Urea, 50 mM ammonium bicarbonate (AB). Then 1 µL of NaOH 0.5M was also added to adjust pH to 8-8.5. To perform the reduction 1.43 µL of dithiothreitol (DTT) 700 mM was added and the samples incubated for 1h at room temperature. Then alkylation was made adding 4.29 µL of iodoacetamida (IAA) 700 mM, with a posterior incubation of 30 min at room temperature in the dark. To quench the excess of IAA 7.5 µL of 500 mM N-acetyl cysteine (NAC) was added to the samples and incubated for 15 min at room temperature. A volume of 486.8 µL of AB 50mM was added to samples to dilute the urea concentration to 1 M. The digestion with 10 µL of trypsin (stock 1µg / µL) added to each sample was done for 18 hours at 37 ºC. To stop digestion a volume of 3 µL of formic acid (FA) was added to each sample. Subsequently, a cleaning/concentration step was performed using OMIX C18 tips (Agilent Technologies), according to manufacturer recommendations, by passing the mixture through the tips and eluting the peptides by adding 70% acetonitrile (ACN) in 0.1% FA solution. The flow was transferred to a new 1.5mL polyethylene tube and the mixture dried using a speed vac (LabConco, CentriVap micro IR).

2.5.4. SWATH-MS analysis – data acquisition

Saliva samples were analysed on a TripleTOF™ 6600 System (Sciex®) using information-dependent acquisition (IDA) of pooled samples for protein identification and Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) acquisition of each individual sample for protein quantification [17]. Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column Halo Fuse Core™ (300 µm ID × 15 cm length, 2.7 µm particles, 90 Å pore size, Eksigent®) at 5µL/min with 45 min linear gradient from 5% to 30% of ACN in 0.1% FA and 5% DMSO. Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, ABSciex®) with a 50 µm internal

diameter (ID) stainless steel emitter (NewObjective). For IDA experiments, the mass spectrometer was set to scanning full spectra (*m/z* 350-1250) for 250 ms, followed by up to 100 MS/MS scans (*m/z* 100–1500 from a dynamic accumulation time – minimum 30 ms for precursor above the intensity threshold of 2000 – in order to maintain a cycle time of 3.3 s). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 15 seconds (mass spectrometer operated by Analyst® TF 1.7, Sciex®). Rolling collision was used with a collision energy spread of 5. For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode [18] and the same chromatographic conditions used as in the IDA run described above. A set of 168 windows of variable width (containing an *m/z* of 1 for the window overlap) was constructed covering the precursor mass range of *m/z* 350-1250. A 50 ms survey scan (*m/z* 350-1250) was acquired at the beginning of each cycle for instrument calibration and SWATH MS/MS spectra were collected from *m/z* 100–1500 for 20 ms resulting in a cycle time of 3.29 s from the precursors ranging from *m/z* 350 to 1250. The collision energy (CE) applied to each *m/z* window was determined considering the appropriate CE for a +2 ion centred upon this window and the collision energy spread (CES) was also adapted to each m/z window.

2.5.5. SWATH-MS data analysis – protein identification and quantification

A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments (one pool per group of samples), and used for subsequent SWATH processing.

Peptide identification and library generation were performed with ProteinPilot software (v5.0, Sciex®), using the following parameters: i) search against a database composed by the Uniprot's reference proteome UP000002254 from *Canis lupus familiaris* (85188 entries, release at December 2017) and MBP-GFP; ii) iodoacetamide alkylated cysteines as fixed modification; iii) trypsin as digestion type. An independent False Discovery Rate (FDR) analysis, using the target-decoy approach provided by ProteinPilot™, was used to assess the quality of the identifications and confident identifications were considered when identified peptides and proteins reached a 5% local FDR [19,20]. Data processing was performed using SWATH™ processing plug-in for PeakView™ (v2.0.01, ABSciex®). After retention time adjustment using the MBP-GFP peptides, up to 15 peptides, with up to 5 fragments each, were chosen per protein,

and quantitation was attempted for all proteins in library file that were identified from ProteinPilot™ search. Peptides' confidence threshold was determined based on a FDR analysis using the target-decoy approach and those that met the 1 % FDR threshold in at least three biological replicates were retained, and the peak areas of the target fragment ions of those peptides were extracted across the samples using an extracted-ion chromatogram (XIC) window of 4 minutes and 100 ppm error. The levels of the proteins were estimated by summing all the filtered transitions from all the filtered peptides for a given protein and normalized to the total intensity obtained in each sample.

2.6. BLAST Search and Gene Ontology (GO) Classification

Proteins for which differential accumulation was found by LC MS/MS analysis were submitted to functional classification by PANTHER (protein annotation through evolutionary relationship) (http://www.pantherdb.org/). *Canis lupus familiaris* was the selected organism. Molecular function and biological processes pie-charts were constructed.

The BLAST program downloaded from The National Centre for Biotechnology Information (NCBI) was used for annotation of "uncharacterized proteins" and to assess homologies with proteins with known functions.

2.7. Statistical analysis

Each variable (i.e., quantified protein) was tested for normality using the Shapiro-Wilk test. Outlier's values were removed, when existent. Univariate analysis, for comparison of protein levels between ORMD and non-ORMD groups, was performed using the Student's t-test or the non-parametric Mann-Whitney test, depending if the data follow a normal distribution. Pearson test correlation was performed between baseline characteristics (triglycerides, cholesterol and glucose) and the two studied groups (ORMD and non-ORMD). Statistical significance was considered for $p<0.05$ and analysis procedures were achieved using the SPSS 21.0 software package (SPSS Inc., Chicago, USA).

For Multivariate Analysis, partial least squares discriminant analysis (PLS-DA) was used taking into account the interdependence among proteins. Data normalization was performed by a pooled sample from group non-ORMD and cube root transformation was used. Discriminant variables selection was done using variable importance in the projection (VIP) with a threshold of 2.0. This was done using Metaboanalyst 4.0 [21].

3.   Results

3.1. Dog characteristics

The baseline characteristics of dogs with and without ORMD included in the present study are shown in Table 1. Five dogs with ORMD presented total cholesterol above upper reference limit, five increased glucose concentrations; two dogs had increased serum triglycerides concentration, and one increased urine protein creatinine ratio concentration. None of them had increased SAP. Some of the dogs without ORMD presented also one of these parameters increased, but never two at the same time. No statistically significant changes were detected between the two groups in terms of age, body weight (BW), biochemical data and SAP.

Table 1 The baseline characteristics of dogs included in salivary proteomic study with and without obesity-related metabolic dysfunction (ORMD).

Variable	Non-ORMD	ORMD	P
Breed	German Shepherd, Mongrel	Beagle, Mongrel, Shih Tzu, Scotish terrier, Cocker spaniel	
Age, years	6.75 (2.5-11)	8 (3-11.6)	0.228
Sex	3 females and 3 males	3 females and 3 males	
Body condition score, 5- point scale	4	5	
Body Weight, kg	15.75 (8.85-24.7)	11.95 (8.7-18.7)	0.254
Total cholesterol, mg/dL	244.5(209-649)	381 (269-433)	0.119
Triglycerides, mg/dL	83.5 (66-96)	88 (69.7-355)	0.333
Glucose, mg/dL	94 (82-144)	107.45 (90-111)	0.083
Systolic arterial pressure (SAP)	140.9 (137.3-157.5)	139.75 (94.5-149)	0.329

Data are presented as median (interquartile range). *P* is from Mann-Whitney U with statistically significant differences being considered for *P*<0.05.

3.2. Salivary proteomics

In the present study, 300 proteins were identified in dog saliva (Supplementary Table 1). These proteins have different molecular functions, with 47.3% having catalytic activity and 37.2% being involved in binding. A lesser percentage of dog salivary proteins have diverse molecular functions, such as structural molecule activity (6.9%), antioxidant (4.8%), signal transducer (1.6%), receptor (1.1%) and translation regulator (1.1%) activities. From the proteins identified, 28.3% are involved in cellular processes and 24.1% in metabolic processes (Supplementary Figure 1). To the best of author's knowledge, 51 of the identified proteins were not previously reported in canine saliva (Supplementary Table 2).

Amongst the identified proteins, 257 were quantified, and their salivary levels were correlated with serum concentrations of triglycerides, cholesterol and glucose, from which 14%, 5% and 1.2% were, correlated with triglycerides, cholesterol and glucose, respectively (Supplementary Table 3 and Supplementary Figure 2 and 3). The majority of these proteins have a catalytic activity (45.9%) and/or are involved in binding (43.2%), and with lesser percentage having structural molecule (8%) and antioxidant (3%) activities.

The levels of salivary proteins correlated positively with total cholesterol levels in serum, with the exception of the superoxide dismutase and uncharacterized protein (E2R886 – with almost 80% sequence homology with Kininogen 1 from different mammalian species) that showed negatively correlation. Salivary proteins significantly associated with serum total cholesterol were involved in binding (45.5%), and catalytic activity (36.4%). A high percentage of salivary proteins that correlated with serum triglyceride concentrations (31%) were involved in metabolic processes, although the proteins that showed strongest correlations were proteins with catalytic activity, namely elastase neutrophil expressed (R=0.852), thioredoxin (R=0.862), peptidyl-prolyl cis-trans isomerase (R=0.867) and protein S100 (R=0.919).

Univariate analysis of the 257 quantified proteins revealed eight proteins with significant differential levels between the two study groups (Table 2). These proteins were related to hydrolase/peptidase and reductase enzymatic pathways and with transport and storage functions. Most of these proteins levels were decreased in the ORMD group and only Kallikrein-1 precursor (F1Q0B9) level was found to be increased in this group.

Table 2 Salivary proteins with significantly different abundance between dogs with (ORMD) and without metabolic dysfunction (non-ORMD) and proteins that contributed to the clustering of group's by multivariate PLS-DA model.

Protein Name	Accession Number (Uniprot)	Max no. peptides	Protein function	Fold-change (ratio ORMD/non-ORMD)	p
Leukotriene A(4) hydrolase	F6Y290	1	Hydrolysis of Leukotriene A4 [22]	0.21	0.021*
Kallikrein-1 precursor	F1Q0B9	11	kallikrein – kinin system [23]	1.86	0.021*
Biliverdin reductase B	E2QVU9	3	Reduce flavins and biliverdin [24]	0.27	0.027*
Carbonic Anhydrase I	F1PBK6	1	Ion transport and host defence [25,26]	0.26	0.011*
GC, vitamin D binding	F1P841	7	Transport and storage	0.48	0.045*

protein			[27]		
¡ Secretoglobin family 1D member 4	A0A2K6QVB0	4	IgE-binding protein [28]	0.08	0.020*
¡ Hemoglobin subunit beta-like	P60524	3	Transport of oxygen, carbon dioxide, nitric oxide [29]	0.09	0.024*
Allergen Feld 4-like precursor	F1Q3K7	15	IgE-binding protein [30]	0.49	0.037*
# Uncharacterized protein	J9NXL3	15		0.1	#
# BPI fold containing family A member 2	E2QXJ0	15	Innate immune response and olfaction [31]	0.53	#

*Statistical significant differences for univariate analysis (p<0.05). *P* is from Student's t-test or the non-parametric Mann-Whitney test.

Proteins without statistically significant differences between the studied groups, but which contributed to the clustering of group's by multivariate PLS-DA model.

¡ Proteins identified through BLAST analysis.

The multivariate PLS-DA model shows a clear separation of the experimental groups (ORMD *vs*. non-ORMD) (Figure 1). The proteins that mostly contributed to this separation (VIP≥2) were BPI fold containing family A member 2 (E2QXJ0), allergen Fel d 4-like precursor (F1Q3K7) and an uncharacterized protein (J9NXL3) (Figure 2). All these proteins were decreased in ORMD group. BLAST analysis revealed that the identified "uncharacterized protein" (J9NXL3) presented 100% homology with the haemoglobin alpha subunit from *Canis lupus familiaris*.

Furthermore, the BLAST analysis showed that the uncharacterized protein (J9P7B6) had 73% homology of the sequence with a secretoglobin family 1D member 4, from *Rhinopithecus roxellana* (golden snub-nosed monkey). Concerning the uncharacterized protein (J9JHZ3), the same type of analysis allowed an observation of 95.4% homology with haemoglobin subunit beta-like, from *Canis lupus familiaris*.
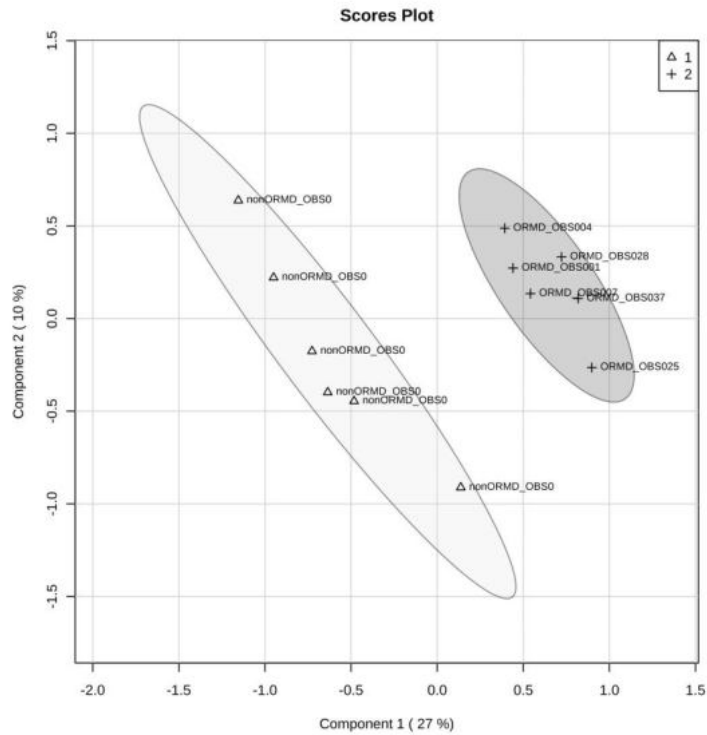
Figure 1 - Partial Least Square Determinant Analysis (PLS-DA) model for all dog saliva samples [ORMD (n=6,△) vs. non-ORMD (n=6, +)]. X and Y axis show principal component 1 (PC1) and principal component 2 (PC2), respectively, and the contribution of each of them for explaining the total variance.
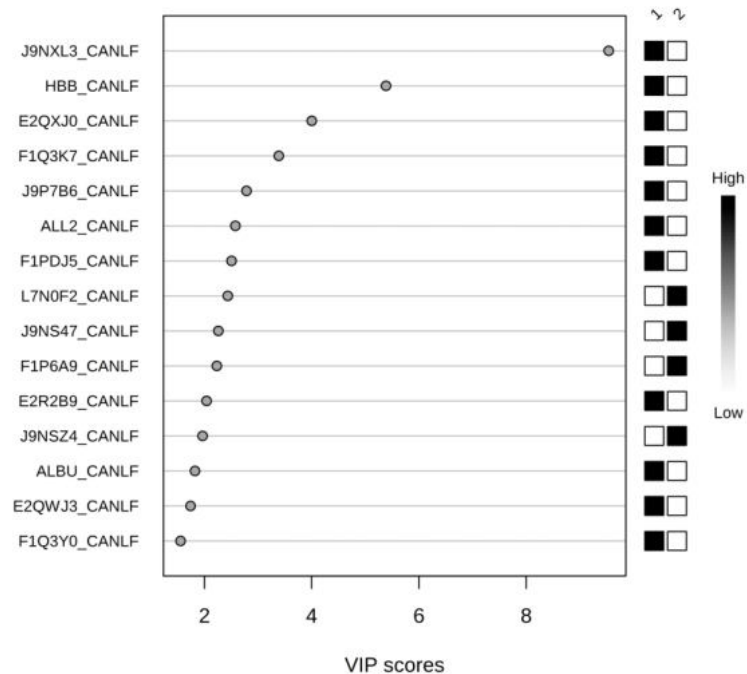
Figure 2 - Variable Importance in Projection (VIP) represents the contribution score of the proteins that mostly contribute to the separation between Non-ORMD and ORMD groups. The black and white boxes on the right indicate the relative concentrations of the corresponding protein in each group under study.1 – Non-ORMD; 2 –ORMD

4. Discussion

This is the first study evaluating the salivary proteome in obese dogs with and without obesity-related metabolic dysfunction (ORMD and non-ORMD, respectively).

In the present study, 83% of the detected proteins were reported previously in canine saliva using proteomic approaches [32]. While 51 identified proteins have never been reported before for dog saliva. Some differences in the results could be attributed to different methodologies and different dog breeds used among the diverse studies, since different salivary proteomes were described in dogs of different breeds [32-34].

In the present work, correlations between some of the salivary proteins and total serum cholesterol and/or triglycerides were detected. The proteins presenting a strongest correlation included elastase neutrophil expressed, thioredoxin, peptidyl-prolylcis-trans isomerase (Pin1) and protein S100. All these proteins were previously related to dyslipidaemia and/or inflammatory processes. Elastase neutrophil protein (NE) was identified within the atherosclerotic plaque suggesting a role in positive arterial remodelling or in promotion of atherosclerotic plaque rupture in humans subjects [35]. It is predominantly present in neutrophils and has an important role in defence against infection. In addition to its classic substrate, elastin, NE can also digest a number of other biologically important proteins, such as collagens type I-IV and VI, fibronectin, laminin and other extra cellular matrix components [35]. Thioredoxin protein has been reported as an antioxidant which combined with others antioxidant proteins leads to cancer cell death *in vitro* and *in vivo*, demonstrating the importance of this antioxidant to tumour progression and as potential target for therapeutic intervention in humans [36]. Peptidyl-prolylcis-trans isomerase (Pin1) protein enhanced the uptake of triglycerides and the differentiation of fibroblasts cells into adipose cells in response to insulin stimulation [37]. Pin1 down-regulation was reported that could be a potential approach in human's obesity-related dysfunctions, such as in conditions like high blood pressure and diabetes. S100 proteins are found both intra cells and extracellularlyand are often named S100/calgranulins [38]. These proteins have a variety of target proteins and receptors and are involved in several functions, such as cellular proliferation and differentiation, energy metabolism, calcium homeostasis, regulation of the cell cytoskeleton, apoptosis, and inflammation [38]. S100A12 is a member of the S100 family being associated with coronary atherosclerosis, diabetes mellitus, and chronic kidney disease [38]. Moreover, this protein has also been linked to several autoimmune

diseases such as rheumatoid and psoriatic arthritis and to cardiovascular complications in lupus [38].

Univariate analysis allowed the observation of eight salivary proteins differently in their abundance between groups. Leukotriene A(4) hydrolase (LTA$_4$H) was increased in ORMD dogs. This protein is responsible for the hydrolysis of leukotriene A$_4$ to leukotriene B$_4$, which are biologically active metabolites of arachidonic acid that act as potent lipid mediators implicated in a range of acute and chronic inflammatory diseases such as asthma and rhinitis [39]. LTA$_4$H is a monomeric, soluble intracellular 69-kDa zinc metallohydrolase and it has a bi-functional nature using either a peptidase or a hydrolase pathway [39]. In studies with humans, this protein presented abundant levels in tissues of individuals with atherosclerotic lesions and their levels correlated with symptoms of atherosclerotic plaque instability [40]. The increase LTA$_4$H verified in ORMD group, in the present study, could be related to the presence of a low grade inflammatory state in obese dogs with metabolic dysfunction. Further studies are needed to confirm or deny this observation.

In the present study, Carbonic anhydrase I (CA I) was another salivary protein observed to be lower levels in ORMD dogs. Carbonic anhydrases (CA) are metals-enzymes containing zinc that catalyses the rapid hydration of bicarbonate and the dehydration of carbonic acid. At least, 16 distinct isoenzymes of CA have been identified in mammals [41]. The decreased levels of CAI in the saliva samples of the ORMD dogs are in accordance with previous results in humans, where decreases in the serum activity of CAI have been associated with the altered metabolism, especially in type 2 diabetes mellitus [42]. More studies are needed to clarify the association of CA I in saliva with ORMD and other obesity-related pathologies in dogs.

Biliverdin reductase B (BVRB), another protein identified in the present study was decreased in dogs suffering from ORMD, is an isozyme that belongs to biliverdin reductase (BVR) protein family along with biliverdin reductase A (BVRA) [43]. These two isozymes are major regulators of metabolic processes. Just for instance, in mammals, BVR is responsible for reducing rapidly biliverdin to bilirubin, which is produced by hemodegradation [44]. In humans, decreased plasma levels of bilirubin and BVR were associated with increased adipocyte size and decreased production of anti-inflammatory adipokines (e.g. adiponectin) resulting in increased risk of T2D and coronary artery disease (CVD) [45].

Gc vitamin D-binding (VDBP) protein was decreased in saliva of ORMD dogs in present study. This protein is a 52–59 kDa serum glycoprotein, from albumin superfamily, which is secreted by monocytes and the liver [46]. VDBP was previously identified in human saliva samples [47]. However, to our knowledge, the X3 was the only isoform of this protein previously reported in dog saliva [32]. The physiological functions of the VDBP include vitamin D (VD) and its metabolites transport and storage, fatty acid transport, scavenging of extracellular G-actin and enhancement of the chemotactic activity for neutrophils in inflammation and macrophage activation [27]. The lower levels of VDBP in the ORMD group, goes in accordance with recent studies, both in humans and mice, which observed lower blood levels of VDBP and VD in diabetes [48].

Kallikreins (KLK) are a group of serine proteases that are present in diverse tissues and biological fluids [23]. KLK1 (plasma kallikrein) and prekallikrein (kallikrein precursor, PK) has already been reported both in human and dogs saliva samples [32,49]. The plasma 'kallikrein/kinin system' (KKS) refers to KAL cleavage high-molecular-weight kininogen (HK) to liberate a biologically active peptide, bradykinin (BK). The KKS is an inflammatory response mechanism since it's activation occurs in inflammatory states with little clinical thrombin formation [23]. Studies in mice, showed that activation of the KKS result in the activation of a defence system that responds to foreign materials and several disease states [50]. In this study PK was increased in the ORMD group which might be related to low grade chronic inflammatory state. Moreover, according to Feener *et al*. [51], KKS has been associated with coagulation, vascular, and metabolic abnormalities in diabetes mellitus. On the other hand, the presence of prolylcarboxy peptidase and circulating microparticles which activate PK in KAL have been related to patients with metabolic syndrome, obesity and diabetes mellitus [52].

Through PLS-DA it was also observed the contribution of Bactericidal/permeability increasing protein fold containing family A member 2 (BPIFA2) and a protein homolog of haemoglobin subunit alpha to separate the two groups (ORMD *vs*. Non ORMD). BPI fold-containing family A member 2 (BPIFA2) has already been reported in both dog and human saliva samples [32,33]. This antimicrobial peptides from the lipid transfer/lipopolysaccharide binding protein (LT/LBP) gene family of the innate immune response is involved in the recognition of bacterial products (Gram-negative bacteria), and activation of phagocytic cells and olfaction [31]. In humans, BPIFA2 has already been associated with proliferative diabetic retinopathy (PDR) and its severity in T2D,

being suggested that the up-regulation of BPIFA2 has the potential to be considered a salivary biomarker of these diseases [53]. In dogs saliva this protein has been reported as parotid secretory protein which is involved in mucosal host defence and considered a major allergen IgE-binding protein [30]. In the present work, BPIFA2 was decreased in the ORMD group, suggesting that innate immune response may be compromised in dogs with ORMD. Other hypothesis could be related to olfaction disorders in dogs with ORMD since smell dysfunction has been associated both with age and degenerative complications of diabetes in humans subjects [54]. In dogs, further research is needed to better understand the possible negative association between BPIFA2 and dogs with ORMD.

The limitations of this study include a relatively low number of animals used, although it is in line with previously reported studies based on proteomic approach [33]. Furthermore, the included animals were from different households with different diets, environmental conditions and care. However, this permitted to evaluate a naturally occurring ORMD and get a true clinical picture. Furthermore, ideally a validation of the analytes of interest detected in this study would be desirable in a population of 200-300 dogs, with ORMD, to get an estimated power of 75%.

5.   Conclusion

In the present study, direct correlation between abundance of some salivary proteins and serum total cholesterol and triglycerides were detected, suggesting the direct relationship between saliva and serum in dogs. When salivary proteomes of dogs with and without ORMD were compared, eight proteins with different abundance levels were identified, namely LTA4H, CAI, BVRB, VDPB, Allergen Feld 4-like precursor and PK. Furthermore, multivariate PLS-DA model permitted inclusion of one additional protein (BPIFA2) responsible for clustering non-ORMD and ORMD groups. The identified proteins were related to obesity and/or type 2 diabetes, and suggested the implication of inflammation and prothrombotic state in dogs with ORMD. However, further long-scale studies are needed to confirm these findings and to increase the knowledge about possible associations between these proteins and ORMD in dogs, and to identify the possible differences between human and canine metabolic syndrome.

6.   Acknowledgments

8. References

1. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009 Oct 20;120(16):1640–5.

2. Simmons RK, Alberti KGMM, Gale EAM, Colagiuri S, Tuomilehto J, Qiao Q, et al. The metabolic syndrome: useful concept or clinical tool? Report of a WHO Expert Consultation. Diabetologia. 2010 Apr;53(4):600–5.

3. Lund EM, Armstrong PJ, Kirk CA, Klausner JS. Prevalence and risk factors for obesity in adult dogs from private US veterinary practices. Int J Appl Res Vet Med. 2006;4(2):177.

4. Verkest KR, Fleeman LM, Morton JM, Ishioka K, Rand JS. Compensation for obesity-induced insulin resistance in dogs: assessment of the effects of leptin, adiponectin, and glucagon-like peptide-1 using path analysis. Domest Anim Endocrinol. 2011 Jul 1;41(1):24–34.

5. Bodey AR, Michell AR. Epidemiological study of blood Sressure in omestic dogs. J Small Anim Pract. 1996 Mar 1;37(3):116–25.

6. Chikamune T, Katamoto H, Ohashi F, Shimada Y. Serum Lipid and Lipoprotein Concentrations in Obese Dogs. J Vet Med Sci. 1995 Aug 15;57(4):595–8.

7. Verkest KR. Is the metabolic syndrome a useful clinical concept in dogs? A review of the evidence. Vet J. 2014 Jan;199(1):24–30.

8. Tvarijonaviciute A, Ceron JJ, Holden SL, Cuthbertson DJ, Biourge V, Morris PJ, et al. Obesity-related metabolic dysfunction in dogs: a comparison with human metabolic syndrome. BMC Vet Res. 2012 Aug 28;8:147.

9. Tvarijonaviciute A, Ceron JJ, de Torre C, Ljubić BB, Holden SL, Queau Y, et al. Obese dogs with and without obesity-related metabolic dysfunction – a proteomic approach. BMC Vet Res [Internet]. 2016 Sep 20;12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5028949/

10. Piantedosi D, Di Loria A, Guccione J, De Rosa A, Fabbri S, Cortese L, et al. Serum biochemistry profile, inflammatory cytokines, adipokines and cardiovascular findings in obese dogs. Vet J. 2016 Oct;216:72–8.

11. Castagnola M, Scarano E, Passali GC, Messana I, Cabras T, Iavarone F, et al. Salivary biomarkers and proteomics: future diagnostic and clinical utilities. Acta Otorhinolaryngol Ital. 2017 Apr;37(2):94–101.

12. Shi P, Goodson JM, Hartman M-L, Hasturk H, Yaskell T, Vargas J, et al. Continuous Metabolic Syndrome Scores for Children Using Salivary Biomarkers. PLoS ONE [Internet]. 2015 Sep 29;10(9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4587796/

13. Goodson JM, Kantarci A, Hartman M-L, Denis GV, Stephens D, Hasturk H, et al. Metabolic Disease Risk in Children by Salivary Biomarker Analysis. PLoS ONE [Internet]. 2014 Jun 10;9(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4051609/

14. Chauhan A, Yadav SS, Dwivedi P, Lal N, Usman K, Khattri S. Correlation of Serum and Salivary Cytokines Level With Clinical Parameters in Metabolic Syndrome With Periodontitis. J Clin Lab Anal. 2016 Sep 1;30(5):649–55.

15. Laflamme D. Nutritional management. Vet Clin North Am Small Anim Pract. 1997 Nov;27(6):1561–77.

16. Tvarijonaviciute A, Carrillo-Sanchez JD, García-Martinez JD, Tecles F, Martinez-Subiela S, German AJ, et al. Measurement of salivary adiponectin concentrations in dogs. Vet Clin Pathol. 2014;43(3).

17. Short GeLC-SWATH: A fast and reliable quantitative approach for proteomic screenings - Anjo - 2015 - PROTEOMICS - Wiley Online Library [Internet]. Available from: http://onlinelibrary.wiley.com/doi/10.1002/pmic.201400221/abstract;jsessionid=858AF2C17EC8FA92F5E8969AD91BAEE0.f04t01

18. Gillet LC, Navarro P, Tate S, Röst H, Selevsek N, Reiter L, et al. Targeted Data Extraction of the MS/MS Spectra Generated by Data-independent Acquisition: A New Concept for Consistent and Accurate Proteome Analysis. Mol Cell Proteomics MCP [Internet]. 2012 Jun;11(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3433915/

19. Sennels L, Bukowski-Wills J-C, Rappsilber J. Improved results in proteomics by use of local and peptide-class specific false discovery rates. BMC Bioinformatics. 2009 Jun 12;10:179.

20. Tang WH, Shilov IV, Seymour SL. Nonlinear fitting method for determining local false discovery rates from decoy database searches. J Proteome Res. 2008 Sep;7(9):3661–7.

21. Xia J, Wishart DS. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis: Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. In: Bateman A, Pearson WR, Stein LD, Stormo GD, Yates JR, editors. Current Protocols in Bioinformatics [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2016. p. 14.10.1-14.10.91. Available from: http://doi.wiley.com/10.1002/cpbi.11

22. Haeggström JZ. Structure, Function, and Regulation of Leukotriene A4 Hydrolase. Am J Respir Crit Care Med. 2000 Feb 1;161(supplement_1):S25–31.

23. Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. J Thromb Haemost. 2016 Jan 1;14(1):28–39.

24. Watchko JF, Daood MJ, Biniwale M. Understanding neonatal hyperbilirubinaemia in the era of genomics. Semin Neonatol. 2002 Apr;7(2):143–52.

25. Sly WS, Hewett-Emmett D, Whyte MP, Yu YS, Tashian RE. Carbonic anhydrase II deficiency identified as the primary defect in the autosomal recessive syndrome of osteopetrosis with renal tubular acidosis and cerebral calcification. Proc Natl Acad Sci U S A. 1983 May;80(9):2752–6.

26. Heo S-M, Choi K-S, Kazim LA, Reddy MS, Haase EM, Scannapieco FA, et al. Host Defense Proteins Derived from Human Saliva Bind to Staphylococcus aureus. Infect Immun. 2013 Apr;81(4):1364–73.

27. Nagasawa H, Uto Y, Sasaki H, Okamura N, Murakami A, Kubo S, et al. Gc Protein (Vitamin D-binding Protein): Gc Genotyping and GcMAF Precursor Activity. Anticancer Res. 2005 Nov 1;25(6A):3689–95.

28. Scheurer S, Toda M, Vieths S. What makes an allergen? Clin Exp Allergy. 2015 Jul 1;45(7):1150–61.

29. Hsia CCW. Respiratory function of hemoglobin. N Engl J Med. 1998 Jan 22;338(4):239–47.

30. Polovic N, Wadén K, Binnmyr J, Hamsten C, Grönneberg R, Palmberg C, et al. Dog saliva – an important source of dog allergens. Allergy. 2013 May 1;68(5):585–92.

31. Bingle L, Barnes FA, Lunn H, Musa M, Webster S, Douglas CWI, et al. Characterisation and expression of SPLUNC2, the human orthologue of rodent parotid secretory protein. Histochem Cell Biol. 2009 Sep 1;132(3):339–49.

32. Torres SMF, Furrow E, Souza CP, Granick JL, de Jong EP, Griffin TJ, et al. Salivary proteomics of healthy dogs: An in depth catalog. Guo X, editor. PLOS ONE. 2018 Jan 12;13(1):e0191307.

33. Pasha S, Inui T, Chapple I, Harris S, Holcombe L, Grant MM. The Saliva Proteome of Dogs: Variations Within and between Breeds and between Species. PROTEOMICS. 2018 Jan 12;1700293.

34. Lucena S, Coelho AV, Capela-Silva F, Tvarijonaviciute A, Lamy E. The Effect of Breed, Gender, and Acid Stimulation in Dog Saliva Proteome. BioMed Res Int. 2018 Jun 3;2018:1–12.

35. Dollery CM. Neutrophil Elastase in Human Atherosclerotic Plaques: Production by Macrophages. Circulation. 2003 Jun 10;107(22):2829–36.

36. Harris IS, Treloar AE, Inoue S, Sasaki M, Gorrini C, Lee KC, et al. Glutathione and Thioredoxin Antioxidant Pathways Synergize to Drive Cancer Initiation and Progression. Cancer Cell. 2015 Feb 9;27(2):211–22.

37. Uchida T, Furumai K, Fukuda T, Akiyama H, Takezawa M, Asano T, et al. Prolyl Isomerase Pin1 Regulates Mouse Embryonic Fibroblast Differentiation into Adipose Cells. Rota R, editor. PLoS ONE. 2012 Mar 7;7(3):e31823.

38. Oesterle A, Bowman MAH. S100A12 and the S100/CalgranulinsSignificance: Emerging Biomarkers for Atherosclerosis and Possibly Therapeutic Targets. Arterioscler Thromb Vasc Biol. 2015 Dec 1;35(12):2496–507.

39. Khim S-K, Bauman J, Evans J, Freeman B, King B, Kirkland T, et al. Discovery of novel and potent aryl diamines as leukotriene A4 hydrolase inhibitors. Bioorg Med Chem Lett. 2008 Jul 15;18(14):3895–8.

40. Qiu H, Gabrielsen A, Agardh HE, Wan M, Wetterholm A, Wong C-H, et al. Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. Proc Natl Acad Sci U S A. 2006 May 23;103(21):8161–6.

41. Imtaiyaz Hassan M, Shajee B, Waheed A, Ahmad F, Sly WS. Structure, function and applications of carbonic anhydrase isozymes. Bioorg Med Chem. 2013 Mar;21(6):1570–82.

42. Ghosh C, Banik GD, Maity A, Som S, Chakraborty A, Selvan C, et al. Oxygen-18 isotope of breath CO2 linking to erythrocytes carbonic anhydrase activity: a biomarker for pre-diabetes and type 2 diabetes. Sci Rep [Internet]. 2015 Jan 30;5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4311236/

43. O'Brien L, Hosick PA, John K, Stec DE, Hinds TD. Biliverdin Reductase Isozymes in Metabolism. Trends Endocrinol Metab TEM. 2015 Apr;26(4):212–20.

44. Abraham NG, Junge J, Drummond GS. Translational Significance of Heme Oxygenase in Obesity and Metabolic Syndrome. Trends Pharmacol Sci. 2016 Jan;37(1):17–36.

45. Rao PV, Reddy AP, Lu X, Dasari S, Krishnaprasad A, Biggs E, et al. Proteomic Identification of Salivary Biomarkers of Type-2 Diabetes. J Proteome Res. 2009 Jan 2;8(1):239–45.

46. Yousefzadeh P, Shapses SA, Wang X. Vitamin D Binding Protein Impact on 25-Hydroxyvitamin D Levels under Different Physiologic and Pathologic Conditions [Internet]. International Journal of Endocrinology. 2014. Available from: https://www.hindawi.com/journals/ije/2014/981581/abs/

47. Krayer JW, Emerson DL, Goldschmidt-Clermont PJ, Nel AE, Werner PA, Galbraith RM. Qualitative and quantitative studies of Gc (vitamin D-binding protein) in normal subjects and patients with periodontal disease. J Periodontal Res. 1987 Jul 1;22(4):259–63.

48. Jain SK, Kanikarla-Marie P, Warden C, Micinski D. L-cysteine supplementation upregulates glutathione (GSH) and vitamin D binding protein (VDBP) in hepatocytes cultured in high glucose and in vivo in liver, and increases blood levels of GSH, VDBP, and 25-hydroxy-vitamin D in Zucker diabetic fatty rats. Mol Nutr Food Res. 2016 May;60(5):1090–8.

49. Tagashira S, Yamaguchi K, Matsunaga T, Toda K, Hayashi Y. Salivary prekallikrein output during the ranger training-induced stress. Stress Health. 2004 Dec 1;20(5):249–53.

50. Stavrou EX, Fang C, Merkulova A, Alhalabi O, Grobe N, Antoniak S, et al. Reduced thrombosis in Klkb1−/− mice is mediated by increased Mas receptor, prostacyclin, Sirt1, and KLF4 and decreased tissue factor. Blood. 2015 Jan 22;125(4):710–9.

51. Feener EP, Zhou Q, Fickweiler W. Role of plasma kallikrein in diabetes and metabolism. Thromb Haemost. 2013;110(09):434–41.

52. Xu S, Lind L, Zhao L, Lindahl B, Venge P. Plasma Prolylcarboxypeptidase (Angiotensinase C) Is Increased in Obesity and Diabetes Mellitus and Related to Cardiovascular Dysfunction. Clin Chem. 2012 Jul 1;58(7):1110–5.

53. Chee CS, Chang KM, Loke MF, Angela Loo VP, Subrayan V. Association of potential salivary biomarkers with diabetic retinopathy and its severity in type-2 diabetes mellitus: a proteomic analysis by mass spectrometry. PeerJ [Internet]. 2016 May 12;4. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4893325/

54. Floch J-PL, Lièvre GL, Labroue M, Paul M, Peynegre R, Perlemuter L. Smell Dysfunction and Related Factors in Diabetic Patients. Diabetes Care. 1993 Jun 1;16(6):934–7.

EFFECT OF BODY WEIGHT LOSS IN THE SALIVARY PROTEOME OF OBESE BEAGLE DOGS

PREPARED TO SUBMISSION

EFFECT OF BODY WEIGHT LOSS IN THE SALIVARY PROTEOME OF OBESE BEAGLE DOGS

Sónia Lucena[1,2], Ana Varela Coelho[3], Alberto Muñoz-Prieto[4], Sandra I. Anjo[5,6], Bruno Manadas[5], Fernando Capela e Silva[1,7,] Elsa Lamy[1], Asta Tvarijonaviciute[4]

[1]Instituto de Ciências Agrárias e Ambientais Mediterrânicas (ICAAM); [2]Departamento de Medicina Veterinária, Escola de Ciências e Tecnologia, Universidade de Évora, Portugal; [3]Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Portugal; [4]Interlab-UMU, Regional Campus of International Excellence "Mare Nostrum", University of Murcia, Espinardo, 30100 Murcia, Spain; [5]CNC - Center for Neuroscience and Cell Biology, University of Coimbra, Portugal; [6]Faculty of Medicine, University of Coimbra, Portugal; [7]Departamento de Biologia, Escola de Ciências e Tecnologia, Universidade de Évora, Portugal.

ABSTRACT

Obesity has become probably the most important health issue of humans and pet dogs, with a dramatic increase of prevalence in both all over the world. The aim of this study was to investigate changes in the salivary proteome of obese dogs after induced weight loss and to identify the salivary proteins related to this weight loss. Five obese neutered males of pure breed Beagles were used. The weight loss protocol over a 3 month period was based on one previously described. Serum and saliva analysis were performed to all dogs before and after weight loss. Quantitative proteomics analysis using SWATH-MS was used to evaluate the salivary proteome changes induced by weight loss. Among the 23 salivary proteins changed after weight loss, the levels of four of them, which are related with immune system, inflammation status, oxidative stress and glucose metabolism, were strongly correlated with weight loss percentage. These were peptidyl-prolyl cis-trans isomerase, fructose-bisphosphate aldolase C, 78kDa glucose regulated protein and Angiopoietin-like 5. The first three were negatively correlated, whereas angiopoietin-like 5 was increased in the animals loosing higher body weight percentage. These variations suggest that weight loss results in improved physiological status in dogs.

Key words: dog, saliva, proteome, obesity, weight loss

1. Introduction

During the last 20 years obesity became one of the most important health issue of humans and pet dogs, with a dramatic increase of prevalence world-wide (1). In European countries, recent estimates suggest that 22–56% of dogs are overweight/obese (2).

The list of health problems associated with obesity in dogs is increasing with new studies identifying relationships between alterations in adipokines and disorders such as osteoarthritis, respiratory distress, abnormalities in circulating lipid profiles, diabetes mellitus, hypertension, dystocia, heat intolerance, urinary and reproductive disorders, some forms of cancer and dermatological diseases, as well as anaesthetic complications (3). These pathologies not only shorten the expected lifespan of the affected animals, but also reduce their health-related quality of life (3). Thus, the prevention and management of obesity is essential (4). Controlled weight loss in obese dogs was considered challenging (5), but it was associated with improved metabolism, especially in inflammation and in insulin resistance-related parameters (6–8) resulting in alleviated obesity-related pathologies (9).

Proteomic analyses of serum have been used to compare healthy and obese conditions in dogs and humans, as well as the effect of weight loss. Data observed in serum of dogs submitted to an experimental weight loss suggested improved insulin resistance and inflammatory status (6,7). Moreover, the identified proteins (retinol binding protein 4, clusterin, among others) were suggested to be potential biomarkers of obesity and its metabolic comorbidities, as well as measurement of therapeutic effectiveness of weight loss.

In the last years, saliva proved to be a bio-fluid of interest in physiology and pathophysiology studies. The main benefits of saliva are the non-invasive nature of its collection, and at the same time, its content in glandular and blood-born molecules that can change under different conditions (10). The potential of saliva as a source of biomarkers of inflammation, such as C-reactive protein, tumour necrosis factor-α, interleukin-6 and interferon-γ, and insulin resistance, such as insulin and glucose, has been also reported in humans saliva (11,12). In human medicine, obesity-related changes in salivary proteome as well as changes after weight loss have been studied reporting alterations in proteins involved in taste perception among others (13–15). However, to the best of authors' knowledge, the effect of weight loss in salivary proteome of obese dogs has not been previously studied.

The aim of this study was to investigate changes in the salivary proteome of obese Beagle dogs undergoing weight loss in order to identify the salivary proteins that could reflect the physiological changes occurring during weight loss and serve as potential non-invasive biomarkers associated with overweight/obesity.

2. Materials and methods

2.1. Ethical note

The study protocol adhered to the University of Murcia Animal Ethics Guidelines, and was approved by the University of Murcia Research Ethics Committee (323/2017) and Water, Agriculture, Livestock and Fisheries Counselling of Murcia Region Ethics Committee (A13170806).

2.2. Dog population

Five neutered male Beagle dogs with ages ranging from 1 to 4 years were used in the study. At the beginning of this experiment, all animals were obese with body weight (BW) ranging from 14 to 22 kg and Body Condition Score (BCSs) from 4 to 5, on a validated 5-point scale of BCSs system (16). Physical examination with complete blood count (CBC) and complete serum biochemistry were performed to all dogs weekly in order to discard other than obesity pathologies and acute inflammation.

2.3. Weight loss protocol

The weight loss program over a 3 month period followed a previously defined protocol (8). In brief, the dogs received a strictly controlled amount of a hypo-energetic commercial diet (Obesity Management, Royal Canin) once daily with the aim to induce a rapid weight loss (2–3% of BW/week), yet providing minimal protein requirements (17). Drinking water was available *ad libitum*. BCSs and BWs were assessed weekly. Saliva and serum samples were collected from all dogs before (PRE) and after (POST) weight loss period the morning after overnight fast.

2.4. Serum analysis

For serum total cholesterol, triglycerides and glucose analysis, blood samples were collected the morning after an overnight fast of at least 12 h by puncture of the jugular or saphenous vein and placed in tubes containing a clotting accelerator (TapVal; Aquisel, Barcelona, Spain). Samples were centrifuged at 2000 3 *g* for 10 min at room temperature to obtain serum, which was passed to plastic vials and analyzed in fresh. Serum total cholesterol, triglycerides and glucose were measured in the automated

biochemistry analyser (Olympus AU600, Beckman Coulter, Brea, USA) using commercially available reagents and following the instructions of the manufacturer.

2.5. Saliva analysis

2.5.1. Saliva collection

Saliva samples were collected by placing a sponge in each dog's mouth during 1–2 min, which was then passed into the Salivette device for centrifugation (3000g, 10 min, 4ºC) (18). Afterwards, saliva present in the lower part of device was transferred to polyethylene tubes and stored at -80°C until analysis.

2.5.2. Total protein concentration

Bradford protein assay, with BSA as the standard protein (Pierce Biotechnology, Rockford, IL, USA), was performed to determine the total protein concentration of each sample. Different dilutions of the standards and samples were run in triplicate, in 96 wells microplates. Absorbance was read at 600 nm in a microplate reader (Glomax, Promega).

2.5.3. Protein digestion in solution

The volume of each saliva sample correspondent to a total of 50 µg of protein was added to 10 µL of 6M Urea 50mM ammonium bicarbonate. Then 1 µL of NaOH 0.5M was also added to adjust pH to 8-8.5. To perform the reduction 1.43 µL of ditiotreitol (DTT) 700 mM was added and the samples incubated for 1h at room temperature. Then alkylation was made adding 4.29 µL of iodoacetamide (IAA) 700 mM, with a posterior incubation of 30 min at room temperature in the dark. To quench the excess of IAA, 7.5 µL of N-acetyl cysteine (NAC) 500 mM was added to the samples and incubated for 15 min at room temperature. A volume of 486.76 µL of ammonium bicarbonate 50mM was added to samples to dilute the urea concentration to 1 M. The digestion with 10 µL of trypsin in HCl 1mM (stock 1µg/µL) added to each sample was done overnight at 37ºC. To stop digestion a volume of 3.05 µL of formic acid (FA) was added to each sample. Subsequently, a cleaning/concentration step was performed using OMIX C18 tips (Agilent Technologies), according to manufacturer recommendations, by passing the mixture through the tips and eluting the peptides by adding 70% acetonitrile (ACN) in 0.1% FA solution. The flow was transferred to a new 1.5mL polyethylene tube and the mixture allowed to dry using a speed vac (LabConco, CentriVap micro IR).

2.5.4. SWATH-MS analysis – data acquisition

Saliva samples were analysed on a TripleTOF™ 6600 System (Sciex®) using information-dependent acquisition (IDA) of pooled samples for protein identification

and Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) acquisition of each individual sample for protein quantification (19). Peptides were resolved by liquid chromatography (nanoLC Ultra 2D, Eksigent®) on a MicroLC column Halo Fuse Core™ (300 μm ID × 15 cm length, 2.7 μm particles, 90 Å pore size, Eksigent®) at 5μL/min with 45 min linear gradient from 5% of ACN in 0.1% FA and 5% dimethyl sulfoxide (DMSO). Peptides were eluted into the mass spectrometer using an electrospray ionization source (DuoSpray™ Source, ABSciex®) with a 50 μm internal diameter (ID) stainless steel emitter (NewObjective). For IDA experiments, the mass spectrometer was set to scanning full spectra (*m/z* 350-1250) for 250 ms, followed by up to 100 MS/MS scans (*m/z* 100–1500 from a dynamic accumulation time – minimum 30 ms for precursor above the intensity threshold of 2000 – in order to maintain a cycle time of 3.3 s). Candidate ions with a charge state between +2 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation and one MS/MS spectra was collected before adding those ions to the exclusion list for 15 seconds (mass spectrometer operated by Analyst® TF 1.7, Sciex®). Rolling collision was used with a collision energy spread of 5. For SWATH-MS based experiments, the mass spectrometer was operated in a looped product ion mode (20) and the same chromatographic conditions used as in the IDA run described above. A set of 168 windows of variable width (containing an *m/z* of 1 for the window overlap) was constructed covering the precursor mass range of *m/z* 350-1250. A 50 ms survey scan (*m/z* 350-1250) was acquired at the beginning of each cycle for instrument calibration and SWATH MS/MS spectra were collected from *m/z* 100–1500 for 20 ms resulting in a cycle time of 3.29 s from the precursors ranging from *m/z* 350 to 1250. The collision energy (CE) applied to each *m/z* window was determined considering the appropriate CE for a +2 ion centred upon this window and the collision energy spread (CES) was also adapted to each *m/z* window.

2.5.5. SWATH-MS data analysis – protein identification and quantification.

A specific library of precursor masses and fragment ions was created by combining all files from the IDA experiments (one pool per group of samples), and used for subsequent SWATH processing.

Peptide identification and library generation were performed with ProteinPilot software (v5.0, Sciex®), using the following parameters: i) search against a database composed by the Uniprot's reference proteome UP000002254 from *Canis lupus familiaris* (85188 entries, release at December 2017) and MBP-GFP ii) iodoacetamide alkylated cysteines

as fixed modification; iii) trypsin as digestion type. An independent False Discovery Rate (FDR) analysis, using the target-decoy approach provided by ProteinPilot™, was used to assess the quality of the identifications and confident identifications were considered when identified proteins reached a 5% local FDR (21,22). Data processing was performed using sequential window acquisition of all theoretical fragment-ion spectra (SWATHTM) processing plug-in for PeakViewTM (v2.0.01, ABSciex®). After retention time adjustment using the malE-GFP peptides, up to 15 peptides, with up to 5 fragments each, were chosen per protein, and quantitation was attempted for all proteins in library file that were identified from ProteinPilotTM searches. Peptides' confidence threshold was determined based on a FDR analysis using the target-decoy approach and those that met the 1% FDR threshold in at least three biological replicates were retained, and the peak areas of the target fragment ions of those peptides were extracted across the experiments using an extracted-ion chromatogram (XIC) window of 4 minutes with 100 ppm XIC width. The levels of the proteins were estimated by summing all the filtered transitions from all the filtered peptides for a given protein and normalized to the total intensity obtained for samples.

2.6. BLAST Search and Gene Ontology (GO) Classification

The list of the identified proteins was submitted to gene function analysis by using the PANTHER (protein annotation through evolutionary relationship) classification system (http://www.pantherdb.org/) and *Canis lupus familiaris* selected as the organism. Molecular function and biological processes pie-charts were constructed.

The BLAST program downloaded from NCBI was used for annotation of "uncharacterized proteins" and to assess homologies with proteins with known functions.

2.7. Statistical analysis

Due to the reduced number of animals, Wilcoxon test (related samples) was used for comparison between before (PRE) and after (POST) BW loss. Spearman correlation test was performed between the levels of the salivary proteins changed after weight loss and the percentage of BW loose (n=5), as well as, between the levels of salivary proteins and serum biochemical data (total cholesterol, triglycerides, and glucose) collected in both periods. Statistical significance was considered for $p < 0.05$. All statistical analysis procedures were achieved using the SPSS 21.0 software package (SPSS Inc., Chicago, USA).

For Multivariate Analysis, partial least squares discriminant analysis (PLS-DA) was used, to test the possibility of interdependence among some proteins using Metaboanalyst 4.0 (23). Data normalization was performed by a pooled sample from group PRE BW loss and auto scaling was used. Discriminant variables selection was done using variable importance in the projection (VIP) with a threshold of 1.5.

3. Results

3.1. Dog characteristics

Weight loss protocol resulted in BW loss of 10 to 21.3% (mean, 16.8 %). The baseline characteristics of dogs, before and after weight loss are shown in Table 1. Statistically significant changes were detected in BCSs, BW and serum total cholesterol concentration.

Table 1. Characteristics of dogs before (PRE) and after (POST) a 3 month period of experimentally-induced weight loss.

Parameter	PRE Median; Mean (Range)	POST Median; Mean (Range)	p-value
Body condition score, BCS	4.5	3.5	0.024*
Body weight, Kg	17.2; 17.7 (14-21.6)	13.9; 14.66 (12.60-17.00)	0.0048*
Triglycerides, mg/dL	70.4; 88.28 (61.12 – 135.90)	92.2; 88.27 (48.00 – 127.50)	0.68
Total Cholesterol, mg/dL	183.4; 214.5 (178.4 – 271.6)	131.7; 154.0 (109.4 – 206.1)	0.043*
Glucose, mg/dL	86.2; 85.8 (79.5 – 93.7)	85.4; 82.94 (74.30 – 90.70)	0.080

P is from Wilcoxon (2 related samples). *Statistically significant differences for $p<0.05$.

3.2. Salivary proteomics

In the present study, 248 proteins were quantified in dog saliva (Supplementary Table 1). These proteins have catalytic activity (48.3%) and binding functions (35.6%), being involved in cellular (27.1%) and metabolic (23.8%) processes, in response to stimulus (11.4%), cellular component organization or biogenesis (9.2%), biological regulation (7.7%) and localization (6.6%), among others (Supplementary Figures 1,2).

Out of all identified proteins in saliva, 21.8%, 23.4% and 29% were correlated with triglycerides, cholesterol and glucose, respectively (Supplementary Table 2 and Supplementary Figures 3 and 4). The proteins correlated with triglycerides have a catalytic activity (43.8%) and are involved in binding (40.6%), and with lesser percentage having structural molecule (6.3%), antioxidant (6.3%) and receptor (3.1%)

activities. The proteins correlated with total cholesterol have also a great proportion involved in catalytic activity (45.2%) and in binding (35.7%), and with lesser percentage involved in structural molecule (11.9%), antioxidant (2.4%), receptor (2.4%) and signal transducer (2.4%) activities. The proteins correlated with glucose, as well as the others ones, have a catalytic activity (46.9%) and are involved in binding (37.4%), structural molecule (10.2%), antioxidant (4.1%), receptor (2%) and translation regulator (2.0%) activities.

The levels of 21 salivary proteins were simultaneously positively correlated with serum triglycerides (all of them with R=1.0) and negatively correlated with total cholesterol and glucose (all of them with R=-1.0), among which joining chain of multimeric IgA and IgM and matrix metalloproteinase were identified (Supplementary Table 2). The levels of another 18 salivary proteins were correlated with both cholesterol and glucose concentrations in serum, among which actinin alpha 4 (both negatively), NAD(P) H quinone dehydrogenase 1 (both negatively), peptidyl-prolyl cis trans-cis isomerase (both negatively) and GC, vitamin D binding protein and stratifin (positively correlated with cholesterol and negatively correlated with glucose) showed strongest correlations (Supplementary Table 2). The levels of the protein clusterin were only correlated with serum total cholesterol concentration, being this correlation strongly positive, whereas ankyrin repeat domain 13B presented a strong negative correlation. On the other hand, the levels of antioxidant 1 copper transport protein (copper transport protein ATOX1) and 27 referred in Table 2 were strongly correlated only with triglycerides.

Univariate analysis of proteomic data revealed 23 proteins that were significantly different in their abundance between PRE and POST BW loss (Table 2). Through Panther Classification System (PCS), these proteins were related to several biological processes: biological adhesion (n=1) and regulation (n=2), cellular component organization or biogenesis (n=2), cellular (n=6; communication, cycle and recognition), developmental (n=1; mesoderm development) , immune system (n=1), localization (n=2; transport), metabolic (n=4; catabolic process, nitrogen compound metabolic process, phosphate-containing compound metabolic process, primary metabolic process) and multicellular organismal (n=1; single-multicellular organism process) , and response to stimulus (n=2; defence response to bacterium, immune response, response to biotic and abiotic stimulus and to stress) (Supplementary Figure 5). These proteins have different molecular functions, with 27.3% having catalytic activity, 63.6% being

involved in binding and 9.1% in structural molecule activity (Supplementary Figure 6). Almost all of these proteins were increased after weight loss, except Tubulin beta 1 class VI (J9P716), which decreased.

Table 2. Mass spectrometry (LC-MS/MS) identification of the salivary proteins of obese Beagles changed after a 3 month period of experimentally-induced body weight loss.

Protein Name	Accession Number (Uniprot)	Max.no. of quantified peptides	Protein function	Fold-change (ratio POST/PRE)	p-value
Peptidyl-prolyl cis-trans isomerase	J9NV93	3	IgE antibodies binding and chemotactic activity	5	0.043[a]
Ankyrin repeat domain 13B	J9P1D5	1	Binding activity (24)	5	0.043[a]
Carboxypeptidase D	E2R830	1	Metallopeptidase and serine-type activity	2.5	0.043[a]
Immunoglobulin heavy constant Alpha 1-related	J9P9J6	15	Antigen and receptor binding	2.94	0.043[a]
Polymeric Immunoglobulin receptor (pIgR)	F6Y6T8	14	Transport of polymeric Igs	2.54	0.043[a]
Minor allergen Can f 2	O18874	14	Binding activity, isomerase	2.9	0.043[a]
†Angiopoietin-like 5	G9KZX8	15	Regulation of glucose and lipid homeostasis	2.8	0.043[a]
Actinin alpha 4	L7N071	4	Actinin binding proteins, cytoskeleton organization and cell adhesion and migration	2.8	0.043[a]
NAD(P)H quinone dehydrogenase1	F1PBZ4	10	Chemoprotection, cancer susceptibility, and antitumor agents	3	0.043[a]
Stratifin	F1PQ93	6	Binding phosphorylated serine and threonine motifs in other proteins	2.75	0.043[a]
Joining chain of multimeric IgA and IgM	J9JHH5	9	Receptor binding	2.6	0.043[a]
BPI fold containing family A member 1	E2QXE7	11	Innate immunity	6	0.043[a]
78 kDa Glucose regulated protein	F1PIC7	4	Catalytic activity, Signalling/regulation Nucleotide and protein binding/folding	2	0.043[a]
Fructose-bisphosphate aldolase C	F1PBT3	2	Catalytic activity over fructose 1,6- (bis)phosphate and fructose 1-phosphate	2	0.043[a]
Cysteine rich secretory protein 2	F1PSF3	4	Immunoglobulin receptor, defence	2.25	0.043[a]
Protein S100 (subfamily S100-A6)	E2R5P5	7	Calcium, calmodulin and receptor binding	2	0.043[a]
Alkaline phosphatase	F1PF95	1	Catalytic activity,	3.5	0.043[a]

			Hydrolyses of phosphomonoesters		
Tubulin beta 1 class VI	J9P716	1	Nucleotide binding, constituent of cytoskeleton	0.45	0.043[a]
Mucin-4	F1PUY9	4	Lubrication, signal transduction in forming chemical and pathogen barriers(25)	4	0.043[a]
Alpha-lactalbumin	F1PTQ7	2	Calcium binding activity (26)	3.5	0.043[a]
Protein disulphide-isomerase A6	E2RB37	1	Catalytic activity, protein folding and turn-over of ECM	2	0.043[a]
Copper transport protein ATOX1	F1PBC8	1	Copper transport	5	0.043[a]
LY6/PLAUR domain containing 3	J9NRF9	2	Plasma membrane component	2	0.028[a]
#Immunoglobulin heavy constant mu	J9NVC6	2	Antigen and receptor binding	0.75	#
#Alpha-1-glycoprotein	F1PCK2	2	Immunoglobulin receptor activity, protease inhibitor	0.75	#
#Hemopexin	F1PZR4	6	Metalloproteinase, ECM component	1	#
#Matrix metalloproteinase-9	F1PYF5	5	Catalytic activity	2	#

PANTHER Classification System.

P is from Wilcoxon test (related samples).

[a]Statistically significant differences for $p < 0.05$.

†Protein obtained through BLAST analysis.

Responsible for the clustering of PRE and POST BW loss periods (PLS-DA).

Among the salivary proteins changed after weight loss, the levels of 4 of them were strongly correlated with weight loss percentage. The proteins peptidyl-prolyl cis-trans isomerase (R=-0.9, *p*=0.037), fructose-bisphosphate aldolase C (R=-1.0, *p*=0.0005) and 78kDa glucose regulated protein (R=-0.9, *p*=0.037) were negatively correlated with the percentage of weight loss. This means that, although increased after weight loss, the increases were lower in the animals that loose a higher percentage of body weight. On the other hand, 1 uncharacterized protein (F1P931), presenting 83% homology with Angiopoietin-like 5 from *Mustela putorius furo* (BLAST analysis), was strongly positive correlated (R=1.0, *p*<0.0005) with the percentage of weight loss.

Through the multivariate PLS-DA model, it was possible to separate PRE *vs.* POST BW loss periods (Figure 2). Besides the proteins already observed to differ between periods, through univariate analysis, four additional proteins were responsible for clustering the two periods (PRE and POST) among which immunoglobulin heavy constant mu

(J9NVC6), alpha-1-glycoprotein (F1PCK2) and hemopexin (F1PZR4) were decreased and matrix metalloproteinase-9 was increased after BW loss.

The proteins that most contributed to this clustering were 78 kDa glucose regulated protein (F1PIC7), immunoglobulin heavy constant mu (J9NVC6), copper transport protein ATOX1 (F1PBC8), alpha-1-glycoprotein (F1PCK2), tubulin beta 1 class VI (J9P716), stratifin (F1PQ93), alkaline phosphatase (F1PF95), protein disulphide-isomerase A6 (E2RB37), hemopexin (F1PZR4) and matrix metalloproteinase-9 (F1PYF5) (Figure 3).
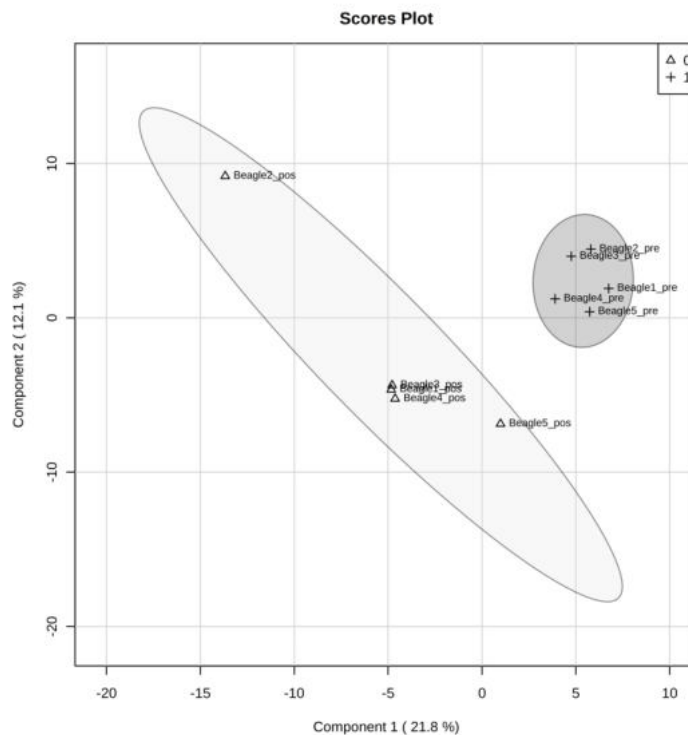
Figure 2. Partial Least Square Determinant Analysis (PLS-DA) model for all dog saliva samples [PRE (n=5) *vs*. POST BW loss (n=5)]. X and Y axis show principal component 1 (PC1) and principal component 2 (PC2), respectively, and the contribution of each of them for explaining the total variance. Δ– POST and + - PRE BW loss

Protein	Accession		VIP scores
78 kDa Glucose regulated protein	F1PIC7	■□	
Immunoglobulin heavy constant mu	J9NVC6	□□■	
Copper transport protein ATOX1	F1PBC8	■□	
Alpha-1-glycoprotein	F1PCK2	□■	
Tubulin beta 1 class VI	J9P716	□■	
Stratifin	F1PQ93	■□	
Alkaline phosphatase	F1PF95	■□	
Protein disulphide-isomerase A6	E2RB37	■□	
Hemopexin	F1PZR4	□■	
Matrix metalloproteinase-9	F1PYF5	■□	
Uncharacterized protein	F1Q0B9	□■	
NAD(P)H quinone dehydrogenase 1	F1PBZ4	■□	
Uncharacterized protein	J9NXL3	□■	
Prostaglandin reductase 1	E2R002	■□	
6-phosphogluconate dehydrogenase, decarboxylating	F1PE09	■□	

4. Discussion

Canine saliva proteome has already been studied in dogs for different purposes including the effect of breed, gender and acid stimulation (27–29). However, this is the first study evaluating salivary proteome changes related to weight loss in dogs.

One of the salivary proteins whose levels correlated with serum total cholesterol was clusterin. The levels of this protein were lower after BW loss, what was in accordance with previous studies, where circulating concentrations of this protein were also decreased after weight loss in dogs (6,30). Clusterin, partly associates with high-density lipoprotein (HDL) cholesterol, functioning in lipoprotein transport, existing a positive correlation between its levels and body fat mass in humans (31). Furthermore, as clusterin has been described to be biomarker of renal function, since it increases in plasma and urine in renal damage, in both humans and dogs (30), it was hypothesised that decreased concentrations of clusterin after weight loss would indicate improved renal function.

Salivary proteins peptidyl-prolyl cis–trans isomerase, ankyrin repeat domain 13B, NAD(P)H quinone dehydrogenase1, BPI fold containing family A member 1, alkaline phosphatase, mucin-4, alpha-lactalbumin and copper transport protein ATOX1 showed the highest fold changes after an experimental weight loss. Among these, peptidyl-prolyl cis–trans isomerase, NAD(P)H quinone dehydrogenase1,

108

bactericidal/permeability increasing protein (BPI), alkaline phosphatase and copper transport protein ATOX1 have already been related to obesity in different studies.

Peptidyl-prolyl cis–trans isomerase (cyclophilin) and BPI are related with innate immunity and/or inflammation and their variation in this study goes in line with improvements in inflammatory status when obese dogs lose weight.

Peptidyl-prolyl cis–trans isomerase (cyclophilin), although being increased after weight loss, presented a strong negative correlation with the percentage of weight lost, i.e., higher levels in dogs loosing less body weight. This enzyme accelerates protein folding and has been referred as probably related to some pathological situations like chronic inflammation (32). Furthermore, cyclophilin has been referred as an adipogenic factor, potentially implicated in development of obesity (33). BPI increased after BW loss, in the present work. It has been observed not only associated with immune system and inflammatory pathways but also potentially linked with insulin action, being positively correlated with this last (34). In line with this, the BPI family A member 1 (BPIFA1) was suggested as a sensitive biomarker of Type 2 Diabetes Mellitus, being decreased in this condition (35). Recently, a study reported a negative association between plasma BPI and body mass index (BMI), waist circumference, body fat, systolic blood pressure (SBP) and insulin resistance, in prepubertal children (36). Despite of the reported studies, which go in line with our observation of salivary BPI after weight loss, some studies reported opposite results. Some authors failed to observe an association between increased plasma BPI levels and improvements in insulin sensitivity due to the massive weight loss, in morbidly obese women (37). Further studies are needed to better understand the relation between the changed salivary levels of these proteins and dog obesity.

The proteins NAD(P)H dehydrogenase quinone 1, copper chaperone ATOX1, alkaline phosphatase and 78kDa glucose regulated protein are proteins related to oxidative stress and were associated with the weight loss. In the case of NAD(P)H dehydrogenase quinone 1 also identified as NAD(P)H quinone oxidoreductase 1, a significant increased after weight loss and a negative correlation with total cholesterol was observed, in the present study. Besides the function of this protein in protection against oxidative *stress* (38), studies showed that this protein may play a role in lipid metabolism and insulin resistance in mice (39). In a study reported by Capel *et al*. (40), NAD(P)H dehydrogenase quinone 1 was described as a cytosolic enzyme highly present in human adipose tissue and adipocytes, with its levels being positively correlated with adiposity,

and markers of liver dysfunction. Diet-induced weight loss resulted in decreased levels of this protein, in some studies (38).

The increases in the salivary levels of copper chaperone ATOX1 and alkaline phosphatase, in the present study, go in line with a study with humans, where weight loss resulted in inhibition of oxidative *stress* occurrence and in elevation in antioxidant defence markers in serum, such as the referred proteins (41). Copper chaperone ATOX1 is a small cytosolic protein involved in the delivery of copper, which functions as an antioxidant defence marker against superoxide and hydrogen peroxide (42). Alkaline phosphatase is an enzyme that has a catalytic function which hydrolyses phosphomonoesters and is involved in nervous system, skeletal tissues, liver, kidney and intestinal (fat absorption) development (43).

Considering 78kDa glucose regulated protein, the changes in its amounts, induced by weight loss, agree with the potential beneficial effects that weight loss has in terms of endoplasmic reticulum *stress*. This protein increased in the saliva collected after weight loss. However, it was negatively correlated with this last one, indicating lower levels in the dogs that lose higher body weight percentages. Weight loss is known to induce reduction in endoplasmic reticulum *stress* (44), what, consequently, will reduce the levels of 78kDa glucose regulated protein (45). As such, our results support the thought of the benefits of the weight loss treatment performed in this study.

Two proteins whose levels were correlated with the percentage of weight loss were angiopoietin like 5 (ANGPTL5) and fructose-biphosphate aldolase C. These proteins are involved in glucose metabolism, suggesting positive effects of weight loss in glucose regulation. The salivary levels of ANGPTL5 were strongly positively correlated with the percentage of weight loss, in accordance with a reported opposite association between the levels of serum angiopoietin like 3 and 4 (ANGPTL3 and ANGPTL4) proteins and body weight, diabetes status, and parameters of glucose control across a wide range of BMI (46). Concerning fructose-bisphosphate aldolase C, which was negatively correlated with the percentage of body weight loss, is expressed primarily in brain, smooth muscle and neuronal tissue, but it was also reported to be present in human saliva (47). This protein has a catalytic activity over fructose 1,6- (bis)phosphate and fructose 1-phosphate (48) and was suggested to be a potential biomarker associated with obesity, decreasing after weight loss induced by a very low calorie diet, in overweight/obese subjects (49), similarly to what was observed in our study.

In this study we used an experimental model of a weight-loss in dogs that produced an improvement of the BW and BCSs of the animals. A limitation of this study could be that the experimental model used does not reflect a real clinical situation of canine obesity, nevertheless, the experimental protocol aims to avoid different variations that can occur in clinical situations such as the use of different diets and environmental conditions, different lengths in the onset of obesity, medications used, and eventually secondary diseases. Moreover, no controls without weight loss were included in this study. Nevertheless, since saliva was collected at the same day time and in the same conditions in both days, and since we related the levels of the proteins with the percentage of body weight loose, the possibility of effects of major non-controllable factors are reduced.

5. Conclusions

In the present study, a considerable amount of salivary proteins have changed with body weight loss, some of which were also found to be correlated with serum total cholesterol, triglycerides and glucose. The salivary proteins, the abundance of which was changed after weight loss, were related with immune system/inflammation, oxidative stress and glucose metabolism. The variations suggest that weight loss results in physiological improvements in dogs. Overall, the results obtained in the present study highlight the potential of saliva for obesity and weight loss studies in dogs.

6. Funding

7. References

1. Day MJ. One Health: the small animal dimension. Vet Rec. 2010 Nov 27;167(22):847–9.

2. Muñoz-Prieto A, Nielsen LR, Dąbrowski R, Bjørnvad CR, Söder J, Lamy E, et al. European dog owner perceptions of obesity and factors associated with human and canine obesity. Sci Rep. 2018 Sep 6;8(1):13353.

3. Sandøe P, Palmer C, Corr S, Astrup A, Bjørnvad CR. Canine and feline obesity: a One Health perspective. Vet Rec. 2014 Dec 20;175(24):610–6.

4. Zoran DL. Obesity in Dogs and Cats: A Metabolic and Endocrine Disorder. Vet Clin North Am Small Anim Pract. 2010 Mar;40(2):221–39.

5. German A j., Titcomb J m., Holden S l., Queau Y, Morris P j., Biourge V. Cohort Study of the Success of Controlled Weight Loss Programs for Obese Dogs. J Vet Intern Med. 2015 Nov 1;29(6):1547–55.

6. Tvarijonaviciute A, Gutiérrez AM, Miller I, Razzazi-Fazeli E, Tecles F, Ceron JJ. A proteomic analysis of serum from dogs before and after a controlled weight-loss program. Domest Anim Endocrinol. 2012 Nov 1;43(4):271–7.

7. Tvarijonaviciute A, Tecles F, Martínez-Subiela S, Cerón JJ. Effect of weight loss on inflammatory biomarkers in obese dogs. Vet J. 2012 Aug 1;193(2):570–2.

8. Park H-J, Lee S-E, Kim H-B, Isaacson RE, Seo K-W, Song K-H. Association of Obesity with Serum Leptin, Adiponectin, and Serotonin and Gut Microflora in Beagle Dogs. J Vet Intern Med. 2015 Jan;29(1):43–50.

9. Yamka RM, Friesen KG, Frantz NZ. Identification of canine markers related to obesity and the effects of weight loss on the markers of interest. Int J Appl Res Vet Med. 2006;4(4):282.

10. Castagnola M, Scarano E, Passali GC, Messana I, Cabras T, Iavarone F, et al. Salivary biomarkers and proteomics: future diagnostic and clinical utilities. Acta Otorhinolaryngol Ital. 2017 Apr;37(2):94–101.

11. Qvarnstrom M, Janket S-J, Jones JA, Jethwani K, Nuutinen P, Garcia RI, et al. Association of salivary lysozyme and C-reactive protein with metabolic syndrome. J Clin Periodontol. 2010 Sep;37(9):805–11.

12. Desai GS, Mathews ST. Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance. World J Diabetes. 2014 Dec 15;5(6):730–8.

13. Lamy E, Simões C, Rodrigues L, Costa AR, Vitorino R, Amado F, et al. Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not. J Physiol Biochem. 2015 Dec;71(4):691–702.

14. Simões CS da S. Avaliação de alterações na composição e função proteica da saliva induzidas por perda de peso [Master's Thesis]. Universidade de Évora; 2014.

15. Rodrigues L, da Costa G, Cordeiro C, Pinheiro CC, Amado F, Lamy E. Relationship between saliva protein composition and 6- n -Propylthiouracil bitter taste responsiveness in young adults: RODRIGUES et al. J Sens Stud. 2017 Jul 21;e12275.

16. Laflamme D. Nutritional management. Vet Clin North Am Small Anim Pract. 1997 Nov;27(6):1561–77.

17. Blanchard G, Nguyen P, Gayet C, Leriche I, Siliart B, Paragon B-M. Rapid Weight Loss with a High-Protein Low-Energy Diet Allows the Recovery of Ideal Body Composition and Insulin Sensitivity in Obese Dogs. J Nutr. 2004 Aug 1;134(8):2148S-2150S.

18. Tvarijonaviciute A, Carrillo-Sanchez JD, García-Martinez JD, Tecles F, Martinez-Subiela S, German AJ, et al. Measurement of salivary adiponectin concentrations in dogs. Vet Clin Pathol. 2014 Sep 1;43(3):416–21.

19. Short GeLC-SWATH: A fast and reliable quantitative approach for proteomic screenings - Anjo - 2015 - PROTEOMICS - Wiley Online Library [Internet]. Available from: http://onlinelibrary.wiley.com/doi/10.1002/pmic.201400221/abstract;jsessionid=858AF2 C17EC8FA92F5E8969AD91BAEE0.f04t01

20. Gillet LC, Navarro P, Tate S, Röst H, Selevsek N, Reiter L, et al. Targeted Data Extraction of the MS/MS Spectra Generated by Data-independent Acquisition: A New Concept for Consistent and Accurate Proteome Analysis. Mol Cell Proteomics MCP [Internet]. 2012 Jun;11(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3433915/

21. Sennels L, Bukowski-Wills J-C, Rappsilber J. Improved results in proteomics by use of local and peptide-class specific false discovery rates. BMC Bioinformatics. 2009 Jun 12;10:179.

22. Tang WH, Shilov IV, Seymour SL. Nonlinear fitting method for determining local false discovery rates from decoy database searches. J Proteome Res. 2008 Sep;7(9):3661–7.

23. Xia J, Wishart DS. Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis: Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis. In: Bateman A, Pearson WR, Stein LD, Stormo GD, Yates JR, editors. Current Protocols in Bioinformatics [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2016. p. 14.10.1-14.10.91. Available from: http://doi.wiley.com/10.1002/cpbi.11

24. Tanno H, Yamaguchi T, Goto E, Ishido S, Komada M. The Ankrd 13 family of UIM-bearing proteins regulates EGF receptor endocytosis from the plasma membrane. Mol Biol Cell. 2012 Apr 1;23(7):1343–53.

25. Behera SK, Praharaj AB, Dehury B, Negi S. Exploring the role and diversity of mucins in health and disease with special insight into non-communicable diseases. Glycoconj J. 2015 Nov;32(8):575–613.

26. Permyakov EA, Berliner LJ. α-Lactalbumin: structure and function. FEBS Lett. 473(3):269–74.

27. Lucena S, Coelho AV, e Silva FC, Tvarijonaviciute A, Lamy E. The effect of breed, gender and acid stimulation in dog saliva proteome.

28. Torres SMF, Furrow E, Souza CP, Granick JL, de Jong EP, Griffin TJ, et al. Salivary proteomics of healthy dogs: An in depth catalog. Guo X, editor. PLOS ONE. 2018 Jan 12;13(1):e0191307.

29. Pasha S, Inui T, Chapple I, Harris S, Holcombe L, Grant MM. The Saliva Proteome of Dogs: Variations Within and between Breeds and between Species. PROTEOMICS. 2018 Jan 12;1700293.

30. Tvarijonaviciute A, Ceron JJ, Holden SL, Biourge V, Morris PJ, German AJ. Effect of Weight Loss in Obese Dogs on Indicators of Renal Function or Disease. J Vet Intern Med. 27(1):31–8.

31. Oberbach A, Blüher M, Wirth H, Till H, Kovacs P, Kullnick Y, et al. Combined Proteomic and Metabolomic Profiling of Serum Reveals Association of the Complement System with Obesity and Identifies Novel Markers of Body Fat Mass Changes. J Proteome Res. 2011 Oct 7;10(10):4769–88.

32. Roumans NJ, Vink RG, Fazelzadeh P, Baak V, A M, Mariman EC. A role for leukocyte integrins and extracellular matrix remodeling of adipose tissue in the risk of weight regain after weight loss. Am J Clin Nutr. 2017 May 1;105(5):1054–62.

33. Zhang L, Li Z, Zhang B, He H, Bai Y. PPIA is a novel adipogenic factor implicated in obesity. Obesity. 23(10):2093–100.

34. Gubern C, López-Bermejo A, Biarnés J, Vendrell J, Ricart W, Fernández-Real JM. Natural Antibiotics and Insulin Sensitivity: The Role of Bactericidal/Permeability-Increasing Protein. Diabetes. 2006 Jan 1;55(1):216–24.

35. Guo Y, Guo L-N, Zhu J-F, Tang C-Y, Feng Y-Z, Zhou H-D. Associations of Salivary BPIFA1 Protein in Chronic Periodontitis Patients with Type 2 Diabetes Mellitus. Int J Endocrinol [Internet]. 2017;2017. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5646319/

36. Prats-Puig A, Gispert-Saüch M, Carreras-Badosa G, Osiniri I, Soriano-Rodríguez P, Planella-Colomer M, et al. α-Defensins and bacterial/permeability-increasing protein as new markers of childhood obesity. Pediatr Obes. 2016 Mar 1;12(2):e10–3.

37. Manco M, Fernandez-Real JM, Equitani F, Vendrell J, Mora V, Elena M, et al. Effect of Massive Weight Loss on Inflammatory Adipocytokines and the Innate Immune System in Morbidly Obese Women. J Clin Endocrinol Metab. 2007 Feb 1;92(2):483–90.

38. Palming J, Sjöholm K, Jernås M, Lystig TC, Gummesson A, Romeo S, et al. The Expression of NAD(P)H:Quinone Oxidoreductase 1 Is High in Human Adipose Tissue, Reduced by

Weight Loss, and Correlates with Adiposity, Insulin Sensitivity, and Markers of Liver Dysfunction. J Clin Endocrinol Metab. 2007 Jun 1;92(6):2346–52.

39. Zhu H, Li Y. NAD(P)H:Quinone Oxidoreductase 1 and its Potential Protective Role in Cardiovascular Diseases and Related Conditions. Cardiovasc Toxicol. 2012 Mar;12(1):39–45.

40. Capel F, Viguerie N, Vega N, Dejean S, Arner P, Klimcakova E, et al. Contribution of Energy Restriction and Macronutrient Composition to Changes in Adipose Tissue Gene Expression during Dietary Weight-Loss Programs in Obese Women. J Clin Endocrinol Metab. 2008 Nov 1;93(11):4315–22.

41. Bawahab MA, Assiri AS, Maksoud WA, Patel A, Kadoumi O, Zaman GS, et al. Effects of Weight Reduction After Sleeve Gastrectomy on Metabolic Variables in Saudi Obese Subjects in Aseer Province of Kingdom of Saudi Arabia. Obes Surg. 2017 Aug 1;27(8):2005–14.

42. Montes S, Rivera-Mancia S, Diaz-Ruiz A, Tristan-Lopez L, Rios C. Copper and Copper Proteins in Parkinson's Disease [Internet]. Oxidative Medicine and Cellular Longevity. 2014. Available from: https://www.hindawi.com/journals/omcl/2014/147251/abs/

43. Millán JL. Alkaline Phosphatases: Structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. Purinergic Signal. 2006 Jun;2(2):335–41.

44. Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, et al. Endoplasmic Reticulum Stress Is Reduced in Tissues of Obese Subjects After Weight Loss. Diabetes. 2009 Mar 1;58(3):693–700.

45. Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ. 2008 Sep;15(9):1460–71.

46. Cinkajzlová A, Mráz M, Lacinová Z, Kloučková J, Kaválková P, Kratochvílová H, et al. Angiopoietin-like protein 3 and 4 in obesity, type 2 diabetes mellitus, and malnutrition: the effect of weight reduction and realimentation. Nutr Diabetes. 2018 Apr 25;8(1):21.

47. Baliban RC, Sakellari D, Li Z, DiMaggio PA, Garcia BA, Floudas CA. Novel protein identification methods for biomarker discovery via a proteomic analysis of periodontally healthy and diseased gingival crevicular fluid samples. J Clin Periodontol. 2012 Mar 1;39(3):203–12.

48. Arakaki TL, Pezza JA, Cronin MA, Hopkins CE, Zimmer DB, Tolan DR, et al. Structure of human brain fructose 1,6-(bis)phosphate aldolase: Linking isozyme structure with function. Protein Sci. 2004 Dec 1;13(12):3077–84.

49. Bouwman FG, Claessens M, van Baak MA, Noben J-P, Wang P, Saris WHM, et al. The Physiologic Effects of Caloric Restriction Are Reflected in the in Vivo Adipocyte-Enriched Proteome of Overweight/Obese Subjects. J Proteome Res. 2009 Dec 4;8(12):5532–40.

CHAPTER III

GENERAL DISCUSSION

As in humans, obesity is probably one of the most important health issues of pet dogs (73) which has been associated with conditions that not only shorten expected lifespan, but also reduce health-related quality of life (76). To the best of our knowledge, the first study of dog's obesity prevalence, in Portugal, is reported in study 1 of the present thesis. This work was included in a multi-country study (10 European countries) cross-sectional questionnaire-based, targeted to owners of at least one dog. Although, in Portugal, dogs' overweight/obesity prevalence (30.9% according to owner estimated body condition score) was in line with values reported worldwide (19.7-59.3%) (9,10,17,30,257), these values were among the highest reported for these countries (249). Pet obesity has been suggested to be linked with human obesity due to their shared lifestyle and environment (1,17,88,258). Moreover, some of the "risk factors" may be similar between humans and pets (1,39,107). Nevertheless, in Portugal, obese dogs were not observed to be consistently linked to obese owners. Although further studies are needed to elucidate why our country differ from others where human and dog obesity is related, it is possible to hypothesize that awareness about human and dog obesity is not the same, with people concerned in avoiding their own obesity being no such concerned for dog's condition.

Obesity and obesity-related diseases have been studied through plasma and serum samples analysis; however there are others biological fluids that had gained greater interest, as well. In the last few years, the growing interest in the characterization of salivary proteome has increased, mainly due to proteomic techniques advances (155,259). In humans, saliva has been used for the diagnosis not only of salivary gland disorders but also of oral diseases and several systemic conditions (186,193,260–262). However, saliva of different animal species, namely the dog, is still little studied. Only recently, characterization of dog salivary proteome, by shotgun proteomics, under healthy conditions were reported in two different studies (169,187).

Two-dimensional gel electrophoresis (2-DE) is one of the most popular techniques for the global analysis and initial profiling of saliva prior to further fractionation and identification using techniques such as mass spectrometry. One of the greatest advantages of 2-DE in salivary protein study relates to its capacity of separating proteins with different post-translational modifications (PTMs), allowing their separate quantification (263). Nonetheless, it has been reported that one of the limitations of using 2-DE in animal saliva samples is the difficulty to obtain saliva volumes sufficient to achieve the relatively high amount of proteins needed for performance of this technique (143). In the case of dogs, and particularly in small sized breed dogs, the amount of saliva produced is limited and stimulation might be necessary. However, stimulation may dilute the protein content of the samples. To accomplish the goal of having enough amount of protein in a small volume protein precipitation/concentration methods are necessary. However, one of the limitations of precipitation protocols is the protein loss (158). In the present work, it was observed that precipitation using 20% (w/v) TCA solution showed best results with dog saliva under studied conditions (Study 2). Although this method did also result in considerable amount of total protein loss

(46.5%), the proportion of the different protein species (protein profiles) are highly correlated with the ones from the original/control samples. Furthermore, it showed the advantage of allowing good resolution of spots in 2-DE and the visualization of spots from proteins that may be of interest, namely spots in the alkaline region.

Another question that emerged, before analysing dog saliva in obesity context, concerns the factors that can influence dog saliva protein composition. In fact, the use of animal saliva in disease diagnostics first requires the characterisation of the salivary proteome under healthy conditions as well as the knowledge of possible influence factors. In humans, factors such as gender, age, inter-individual variability and acid stimulation have been reported (153,188,189). In the study 3, of this thesis, the influence of gender and acid stimulation on normal dog salivary proteome from different pure breed dogs (Portuguese Podengo, Greyhound, Rafeiro Alentejano and Beagle) was studied, through in-gel based proteomics approach. No major trends for gender effect were found, which is in agreement with others studies recently published (187). Two of the breeds that most differed between them were Portuguese Podengo and Beagle. According to Federation Cynologique Internationale (FCI) (http://www.fci.be, accessed on January 31, 2018) purebred Portuguese Podengo is a primitive type of breed, probably originating from the ancient dogs (264) and purebred Beagles belongs to a cluster comprised mostly by modern breeds (265). Thus, it is not surprising that this two breeds present differences, neither that Portuguese Podengo dogs present higher genetic variability than Beagles, since this last has been bred in a controlled way, for use in laboratory studies. Also according to FCI, both breeds are traditionally used for hunting, but Portuguese Podengo is a breed without working trial whereas pure breed Beagles has working trial. The proteins that contributed for the major differences among dog's breeds were chains of canine serum albumin and IgG Fc-binding protein. IgG Fc-binding protein has been recently identified as one of the more abundant proteins in dog saliva (169) being a protein involved in binding IgG on mucosal surfaces (266). Study 3 allowed also testing the effect of acid stimulation on salivary proteome of dogs. From our knowledge, no studies have been performed to access the effect of this type of stimulation in salivary protein composition of animals. Stimulation with lemon juice, which is one of the mostly used methods for stimulating saliva production in humans (146), raised the total volume of saliva produced in dogs, as expected, and, as such, the cotton roll needed to remain less time in the mouth for getting enough saliva amounts (267). However, changes in salivary proteome from dogs were in accordance with in the ones reported for human saliva (146), where it was observed that acid stimulation produced reductions in the total protein amount and considerable changes in proteins related to immune function, inflammation, and cell movement (146). In terms of profiles, proteins such as cytoskeletal keratin, serum albumin, and IgG Fc-binding proteins were identified in bands and/or spots whose levels decreased with acid stimulation.

Although, breed was found to be a factor influencing salivary proteome, several breeds were used for evaluating the salivary proteome in obese dogs with and without obesity-

related metabolic dysfunction (ORMD *vs*. non-ORMD, respectively) (study 4). This occurred for different reasons: on one hand, this was a study performed with animals obtained in veterinarian clinics, and so being difficult to get animals from an unique breed; on the other hand, because the objective of the study was to identify salivary proteins that could contribute to the understanding of this disease, independent of the dog breed. Interestingly, the proteins whose levels were found to be significantly different in this condition were not proteins found to differ among dogs' breed studied. The objective of studying ORMD, in this thesis, was because this is a condition that greatly accompanies dog obesity. In humans, the term 'metabolic syndrome' (MetS) describes a clustering of obesity (especially visceral obesity), insulin resistance or hyperglycaemia, hyperlipidaemia, especially increased fasting triglycerides and decreased High-Density Lipoprotein Cholesterol (HDL-C), and hypertension (52). Obese dogs develop some of these components such as insulin resistance (49), increased blood pressure (61), and hyperlipidaemia (53). In line with these findings, human MetS criteria were adapted to define canine MetS or so called obesity-related metabolic dysfunction (ORMD) (10). Thus, dogs were considered as having ORMD if obesity was present, BCS 4-5 (in a 5-point scale); and any two of the following: increased plasma triglycerides (>200 mg/dl), and plasma total cholesterol (>300 mg/dl), fasting plasma glucose (>100 mg/dl), or previously diagnosed diabetes mellitus and increased systolic arterial pressure (> 160 mmHg). In this study, saliva samples were subjected to a quantitative proteomics analysis using protein digestion in solution and followed by liquid chromatography (LC-MS/MS) analysis, which provides the possibility of to compare a high number of proteins from a complex protein mixture, such as saliva (268). Moreover, this approach has the advantage, over in-gel based approaches, to allow the comparison, between groups, of proteins that might fail to enter in gels due to molecular masses, or other reasons.

In this ORMD study, amongst the 300 identified proteins, 83% of them were reported previously in canine saliva using proteomic approaches (169), although 51 of the identified proteins were reported in dog's saliva for the first time. Amongst the identified proteins, 257 were quantified and several of them showed strong correlation between their salivary levels and total serum cholesterol and/or triglycerides, among which elastase neutrophil expressed, thioredoxin, peptidyl-prolylcis-trans isomerase and protein S100. These proteins were previously related to dyslipidaemia and/or inflammatory processes (269–272).

When salivary proteomes of dogs with and without ORMD were compared, eight of the identified proteins presented different abundance levels, namely Leukotriene A(4) hydrolase (LTA4H), Carbonic anhydrase I, Biliverdin reductase B, Gc vitamin D-binding (VDBP), Allergen Feld 4-like precursor and kallikrein precursor. Through multivariate analysis, the contribution of Bactericidal/permeability increasing protein fold containing family A member 2 (BPIFA2), for separating ORMD from non-ORMD groups was also verified. The identified proteins were related to obesity and/or type 2 diabetes, and suggested the implication of inflammation and prothrombotic state in dogs

with ORMD (215,273–277), what goes in accordance with studies of plasma samples in humans and mice (215,274,278,279). The exception was for LTA4H and BPIFA2 proteins levels (277,280) which were decreased. These differences from the studies in humans and animal models could be related to the absence of atherosclerotic lesions and a compromised innate immune system in obese dogs with ORMD, respectively. Although study 4 had the limitation of to be based in a relatively low number of animals (N=6 from each group), it included animals from different households, with different diets, environmental conditions and care, allowing to evaluate a naturally occurring ORMD and to get a true clinical picture.

Contrary to study mentioned above, in study 5 changes in the salivary proteome of obese dogs undergoing weight loss, pure breed Beagles was used. In this case, although with the possibility of not reflecting what happens in all breeds, the experimental protocol aimed to control the conditions of weight loss, avoiding variations in the onset of obesity, medications used, and eventually secondary diseases. Another limitation could be the absence of no controls without weight loss. However, since saliva collection was at the same day time and in the same conditions in both days and since the proteins levels were analysed in relation to the percentage of body weight loose, effects of major non-controllable factors was reduced.

The results obtained after weight loss was compared to the results of ORMD study (study 4). As in the study of ORMD (study 4), also in animals underwent weight loss, correlations between salivary levels of several proteins and serum triglycerides, total cholesterol and glucose concentrations were found. Besides a positive correlation between Peptidyl-prolyl cis trans-cis isomerase levels and serum triglycerides concentrations, in study 4, a negatively correlation of this protein with serum total cholesterol and glucose concentrations, was found in study 5. This protein enhanced the uptake of triglycerides and the differentiation of fibroblasts cells into adipose cells in response to insulin stimulation, accelerates protein folding and has been referred as an adipogenic factor potentially implicated in development of obesity and in some pathological situations like chronic inflammation (272,281,282). However, this difference when comparing groups before and after weight loss may be due to an external effect. For example, the effect of collection day, since one of the major limitations of the experimental design of study 5 was the lack of control dogs, not subjected to weight loss programme. In fact, the levels of this protein presented a negative correlation with the percentage of weight loss, indicating that its levels are lower in the animals that loose higher percentages of body weight. As such, it is possible to hypothesize that the levels of Peptidyl-prolyl cis trans-cis isomerase are decreased with loss of adiposity.

Vitamin D binding protein (VDBP) levels were decreased in saliva of ORMD dogs and were strongly positive and negative correlated with serum total cholesterol and glucose concentrations, respectively, in dogs participating in the study of weight loss. The physiological functions of the VDBP include vitamin D and its metabolites transport and storage, fatty acid transport, scavenging of extracellular G-actin and enhancement

of the chemotactic activity for neutrophils in inflammation and macrophage activation (275). The lower salivary levels of VDBP in the ORMD group, goes in accordance with recent studies of blood samples in diabetic humans and mice, where these levels were decreased (279). The decreased levels of VDBP in ORMD dogs is also in accordance with a recent study, where this protein was pointed as a marker of risk for the development of metabolic syndrome, in humans (283). Moreover, in this species, this protein was also reported to be positively correlated with total cholesterol in circulation (284). Taking together, these convergent results reinforce that saliva can reflect what happens in circulation, having potential in the study of obesity.

Another salivary protein whose levels were also positively correlated with serum total cholesterol was clusterin. The levels of this protein were lower after BW loss, what was in accordance with previous studies, using blood, where circulating concentrations of this protein were also decreased after weight loss in dogs (66,243). Clusterin has been described to be biomarker of renal function in both humans and dogs (66) and its levels correlate well with body fat mass in humans (203). Besides peptidyl-prolyl cis-trans isomerase protein, discussed above, fructose-bisphosphate aldolase C and 78 kDa glucose regulated protein were negatively correlated with the percentage of weight loss. This means that dogs losing more weight have lower levels of these proteins in their saliva. In both cases, this agrees with bibliography. Concerning 78kDa glucose regulated protein, its function is related to oxidative stress, especially endoplasmic reticulum stress (285). Thus, the changes in its amounts, induced by weight loss, agree with the potential beneficial effects that weight loss has in decreasing the stress of the endoplasmic reticulum (286), what, consequently, will reduce the levels of 78kDa glucose regulated protein. Concerning fructose-bisphosphate aldolase C, a protein that has a catalytic activity over fructose 1,6- (bis)phosphate and fructose 1-phosphate (287), has been suggested to be a potential biomarker associated with obesity, decreasing after weight loss induced by a very low calorie diet, in overweight/obese subjects (288).

Taking all the results, globally, whereas study 1 presented evidences about the need of deeply studying dog obesity, since its prevalence is considerably high, studies 2 and 3 allowed increasing the knowledge about dog saliva characteristics, useful for solving methodological difficulties in dog salivary proteome analysis. In this context, the optimization of protocols and the clarification about the main proteins affected by factors such as saliva stimulation, breed and gender was needed. These first 3 studies allowed us to go to studies 4 and 5, where obese dogs were studied, either for ORMD or for the effects of weight loss, respectively. These two studies, together, allowed the observation that several of the salivary proteins related with the studied conditions are proteins mainly present in circulation and already reported to be associated with metabolism or obesity related complications. This highlights the potential of saliva, as a non-invasive fluid, for the study of dog obesity.

One of the limitations in working with dogs, in proteomic studies, comparatively to working with humans is that protein databases for *Canis lupus familiaris* is scarce compare to protein databases for humans. As such, it's possible that some proteins present in dog saliva and involved in the situations studied could not be identified.

CHAPTER IV

CONCLUSIONS

The five studies performed with the aim of using the salivary proteome in the study of dog obesity allowed to draw the following main conclusions:

a) Dogs obesity prevalence is high, in Portugal; although it is not positively correlated with owners obesity, in this country;

b) Dog saliva has its own particularities, thus proteomic protocols used in saliva samples of humans and other animals, used as models, are not always adapted to dogs' saliva samples. Among the several precipitation methods studied in the present thesis, precipitation using 20% (w/v) TCA showed best results once, although resulting in considerable total protein loss, the protein profiles are highly correlated with the ones from the original samples;

c) Influence factors such as dogs breed and saliva stimulation with acid should be considered when results from different labs are compared, since dog saliva proteome was observed to be affected by them;

d) Saliva proteome in dogs has the potential of improving the knowledge of obesity-related diseases, namely on ORMD, for which some salivary proteins have been observed to present different abundance levels. These proteins are LTA4H, CAI, BVRB, VDPB, Allergen Feld 4-like precursor and PK proteins, which are proteins already reported to be related to obesity and/or type 2 diabetes. Their functions suggest the implication of inflammation and prothrombotic state in dogs with ORMD;

e) Experimentally-induced weight loss has resulted in physiological improvements in obese dogs, as expected. The abundance of salivary proteins related with immune system/inflammation, oxidative stress and glucose metabolism changed after weight loss, supporting these improvements. These were the proteins peptidyl-prolyl cis-trans isomerase, fructose-bisphosphate aldolase C and 78kDa glucose regulated protein;

f) Interestingly, there were some salivary proteins whose levels were positively correlated with serum triglycerides concentration in both studies 4 and 5 such as matrix metalloproteinase, phosphoglycerate mutase, histone H2A, rho GDP dissociation inhibitor beta, purine nucleoside phosphorylase, actinin alpha 1, and S100 calcium binding protein P. This relation, observed in two different experiments, performed in different dog populations, reinforces that these are salivary proteins that can act as markers of serum triglycerides levels and can be relevant in the study of obesity. Further studies are warranted to elucidate this relationship.

Overall, the experimental studies presented in this thesis emphasize the importance of salivary proteome for studying obesity in dogs and improving the understanding of obesity-related diseases, namely obesity-related metabolic dysfunction. There are few works on dogs' salivary proteome, and from our knowledge, this is the first studying obesity. In this work, an integrated perspective of salivary proteome is highlighted and opens new possibilities for future studies focused in evaluating the value of some potential biomarkers for the diagnosis and prognosis of obesity-related metabolic

dysfunction in dogs. Although, this biological fluid is more studied in humans, this thesis reinforces its potential to be used in veterinary health care. Nonetheless, for the translational of those potential targets to clinics, a deeper high-throughput analysis, in a large scale population, is required. Moreover, to disclose the functional relation of some identified salivary proteins will contribute to a deeper knowledge in obesity pathophysiology, as well in obesity-related diseases.

FUTURE PERSPECTIVES

The present thesis allowed to optimize procedures for using in dog saliva studies and allowed to conclude about the potential of saliva in the context of dog obesity. This was the first study where the prevalence of dog obesity in Portugal was assessed and also the first study where the salivary proteome of obese dogs was studied. The results obtained support future perspectives, such as:

- To deeply study why dog obesity is not positively correlated with owner obesity, in Portugal, by opposition to some different countries;
- To validate the proteins identified in the ORMD study as related with this condition, by using a large population of obese dogs with ORMD;
- To compare the salivary proteome of obese dogs with the one of normal weight ones. In the present thesis, dog salivary proteome was studied in obese dogs, under different situations (with or without ORMD and before and after weight loss), but no comparisons were made with normal weight dogs and this might be interesting.

1. Sandøe P, Palmer C, Corr S, Astrup A, Bjørnvad CR. Canine and feline obesity: a One Health perspective. Vet Rec. 2014 Dec 20;175(24):610–6.

2. German AJ. The growing problem of obesity in dogs and cats. J Nutr. 2006;136(7):1940S–1946S.

3. Chang ML, Terrill RL, Bautista MM, Carlson EJ, Dyer DJ, Overall KL, et al. Large-Scale SNP Genotyping with Canine Buccal Swab DNA. J Hered. 2007 Jul 23;98(5):428–37.

4. Bjornvad CR, Nielsen DH, Armstrong PJ, McEvoy F, Hoelmkjaer KM, Jensen KS, et al. Evaluation of a nine-point body condition scoring system in physically inactive pet cats. Am J Vet Res. 2011 Apr;72(4):433–7.

5. German AJ, Holden SL, Moxham GL, Holmes KL, Hackett RM, Rawlings JM. A Simple, Reliable Tool for Owners to Assess the Body Condition of Their Dog or Cat. J Nutr. 2006 Jul 1;136(7):2031S-2033S.

6. Witzel AL, Kirk CA, Henry GA, Toll PW, Brejda JJ, Paetau-Robinson I. Use of a novel morphometric method and body fat index system for estimation of body composition in overweight and obese dogs. J Am Vet Med Assoc. 2014;244(11):1279–1284.

7. Sagawa MM, Nakadomo F, Honjoh T, Ishioka K, Saito M. Correlation between plasma leptin concentration and body fat content in dogs. Am J Vet Res. 2002;63(1):7–10.

8. Tvarijonaviciute A, Ceron JJ, Holden SL, Cuthbertson DJ, Biourge V, Morris PJ, et al. Obesity-related metabolic dysfunction in dogs: a comparison with human metabolic syndrome. BMC Vet Res. 2012 Aug 28;8:147.

9. Lund EM, Armstrong PJ, Kirk CA, Klausner JS. Prevalence and risk factors for obesity in adult dogs from private US veterinary practices. Int J Appl Res Vet Med. 2006;4(2):177.

10. McGreevy PD, Thomson PC, Pride C, Fawcett A, Grassi T, Jones B. Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. Vet Rec-Engl Ed. 2005;156(22):695–701.

11. Jeusette I, Greco D, Aquino F, Detilleux J, Peterson M, Romano V, et al. Effect of breed on body composition and comparison between various methods to estimate body composition in dogs. Res Vet Sci. 2010 Apr;88(2):227–32.

12. Kobayashi T, Koie H, Kusumi A, Kitagawa M, Kanayama K, Otsuji K. Comparative Investigation of Body Composition in Male Dogs Using CT and Body Fat Analysis Software. J Vet Med Sci. 2014;76(3):439–46.

13. Mawby DI, Bartges JW, d'Avignon A, Laflamme DP, Moyers TD, Cottrell T. Comparison of Various Methods for Estimating Body Fat in Dogs. J Am Anim Hosp Assoc. 2004 Mar 1;40(2):109–14.

14. German AJ, Holden SL, Morris PJ, Biourge V. Comparison of a bioimpedance monitor with dual-energy x-ray absorptiometry for noninvasive estimation of percentage body fat in dogs. Am J Vet Res. 2010 Apr 1;71(4):393–8.

15. Jeusette I, Greco D, Aquino F, Detilleux J, Peterson M, Romano V, et al. Effect of breed on body composition and comparison between various methods to estimate body composition in dogs. Res Vet Sci. 2010 Apr 1;88(2):227–32.

16. Day MJ. One Health: the small animal dimension. Vet Rec. 2010 Nov 27;167(22):847–9.

17. Courcier EA, Thomson RM, Mellor DJ, Yam PS. An epidemiological study of environmental factors associated with canine obesity. J Small Anim Pract. 2010 Jul 1;51(7):362–7.

18. Laflamme DP. Companion animals symposium: obesity in dogs and cats: what is wrong with being fat? J Anim Sci. 2012;90(5):1653–1662.

19. Muñoz-Prieto A, Nielsen LR, Dąbrowski R, Bjørnvad CR, Söder J, Lamy E, et al. European dog owner perceptions of obesity and factors associated with human and canine obesity. Sci Rep. 2018 Sep 6;8(1):13353.

20. Park H-J, Lee S-E, Kim H-B, Isaacson RE, Seo K-W, Song K-H. Association of Obesity with Serum Leptin, Adiponectin, and Serotonin and Gut Microflora in Beagle Dogs. J Vet Intern Med. 2015 Jan;29(1):43–50.

21. Zoran DL. Obesity in Dogs and Cats: A Metabolic and Endocrine Disorder. Vet Clin North Am Small Anim Pract. 2010 Mar;40(2):221–39.

22. Kealy RD, Lawler DF, Ballam JM, Mantz SL, Biery DN, Greeley EH, et al. Effects of diet restriction on life span and age-related changes in dogs. J Am Vet Med Assoc. 2002 May 1;220(9):1315–20.

23. Larson BT, Lawler DF, Spitznagel EL, Kealy RD. Improved Glucose Tolerance with Lifetime Diet Restriction Favorably Affects Disease and Survival in Dogs. J Nutr. 2003 Sep 1;133(9):2887–92.

24. Lawler DF, Evans RH, Larson BT, Spitznagel EL, Ellersieck MR, Kealy RD. Influence of lifetime food restriction on causes, time, and predictors of death in dogs. J Am Vet Med Assoc. 2005 Jan 15;226(2):225–31.

25. Colliard L, Ancel J, Benet J-J, Paragon B-M, Blanchard G. Risk factors for obesity in dogs in France. J Nutr. 2006;136(7):1951S–1954S.

26. Usui S, Yasuda H, Koketsu Y. Characteristics of obese or overweight dogs visiting private Japanese veterinary clinics. Asian Pac J Trop Biomed. 2016 Apr;6(4):338–43.

27. Edney AT, Smith PM. Study of obesity in dogs visiting veterinary practices in the United Kingdom. Vet Rec. 1986 Apr 5;118(14):391–6.

28. Switonski M, Mankowska M. Dog obesity – The need for identifying predisposing genetic markers. Res Vet Sci. 2013 Dec;95(3):831–6.

29. Laflamme DP. Understanding and Managing Obesity in Dogs and Cats. Vet Clin North Am Small Anim Pract. 2006 Nov;36(6):1283–95.

30. Mao J, Xia Z, Chen J, Yu J. Prevalence and risk factors for canine obesity surveyed in veterinary practices in Beijing, China. Prev Vet Med. 2013 Nov 1;112(3–4):438–42.

31. Sallander M, Hagberg M, Hedhammar Å, Rundgren M, Lindberg JE. Energy-intake and activity risk factors for owner-perceived obesity in a defined population of Swedish dogs. Prev Vet Med. 2010 Aug;96(1–2):132–41.

32. Robertson ID. The association of exercise, diet and other factors with owner-perceived obesity in privately owned dogs from metropolitan Perth, WA. Prev Vet Med. 2003 Apr 30;58(1–2):75–83.

33. Marshall WG, Bockstahler BA, Hulse DA, Carmichael S. A review of osteoarthritis and obesity: current understanding of the relationship and benefit of obesity treatment and prevention in the dog. Vet Comp Orthop Traumatol. 2009;22(05):339–45.

34. Hess RS, Kass PH, Van Winkle TJ. Association between Diabetes Mellitus, Hypothyroidism or Hyperadrenocorticism, and Atherosclerosis in Dogs. J Vet Intern Med. 2003 Jul 1;17(4):489–94.

35. Hess RS, Kass PH, Shofer FS, Van TW, Washabau RJ. Evaluation of risk factors for fatal acute pancreatitis in dogs. J Am Vet Med Assoc. 1999 Jan;214(1):46–51.

36. Kienzle E, Bergler R, Mandernach A. A Comparison of the Feeding Behavior and the Human–Animal Relationship in Owners of Normal and Obese Dogs. J Nutr. 1998 Jan 12;128(12):2779S-2782S.

37. Heuberger R, Wakshlag J. The relationship of feeding patterns and obesity in dogs: Feeding patterns and obesity in dogs. J Anim Physiol Anim Nutr. 2011 Feb;95(1):98–105.

38. Bland IM, Guthrie-Jones A, Taylor RD, Hill J. Dog obesity: Owner attitudes and behaviour. Prev Vet Med. 2009 Dec;92(4):333–40.

39. German AJ, Ryan VH, German AC, Wood IS, Trayhurn P. Obesity, its associated disorders and the role of inflammatory adipokines in companion animals. Vet J. 2010 Jul;185(1):4–9.

40. German AJ, Holden SL, Morris PJ, Biourge V. Long-term follow-up after weight management in obese dogs: The role of diet in preventing regain. Vet J. 2012 Apr;192(1):65–70.

41. White GA, Hobson-West P, Cobb K, Craigon J, Hammond R, Millar KM. Canine obesity: is there a difference between veterinarian and owner perception? J Small Anim Pract. 2011 Dec;52(12):622–6.

42. Rohlf VI, Toukhsati S, Coleman GJ, Bennett PC. Dog Obesity: Can Dog Caregivers' (Owners') Feeding and Exercise Intentions and Behaviors Be Predicted From Attitudes? J Appl Anim Welf Sci. 2010 Jun 21;13(3):213–36.

43. White GA, Ward L, Pink C, Craigon J, Millar KM. "Who's been a good dog?" – Owner perceptions and motivations for treat giving. Prev Vet Med. 2016 Sep;132:14–9.

44. Yam PS, Naughton G, Butowski CF, Root AL. Inaccurate Assessment of Canine Body Condition Score, Bodyweight, and Pet Food Labels: A Potential Cause of Inaccurate Feeding. Vet Sci. 2017 Jun 9;4(4):30.

45. Daminet S, Jeusette I, Duchateau L, Diez M, Maele IV de, Rick AD. Evaluation of Thyroid Function in Obese Dogs and in Dogs Undergoing a Weight Loss Protocol. J Vet Med Ser A. 2003 May 1;50(4):213–8.

46. Tvarijonaviciute A, Ceron JJ, de Torre C, Ljubić BB, Holden SL, Queau Y, et al. Obese dogs with and without obesity-related metabolic dysfunction – a proteomic approach. BMC Vet Res [Internet]. 2016 Sep 20;12. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5028949/

47. Verkest KR. Is the metabolic syndrome a useful clinical concept in dogs? A review of the evidence. Vet J. 2014 Jan;199(1):24–30.

48. Simmons RK, Alberti KGMM, Gale EAM, Colagiuri S, Tuomilehto J, Qiao Q, et al. The metabolic syndrome: useful concept or clinical tool? Report of a WHO Expert Consultation. Diabetologia. 2010 Apr;53(4):600–5.

49. Verkest KR, Fleeman LM, Morton JM, Ishioka K, Rand JS. Compensation for obesity-induced insulin resistance in dogs: assessment of the effects of leptin, adiponectin, and glucagon-like peptide-1 using path analysis. Domest Anim Endocrinol. 2011 Jul 1;41(1):24–34.

50. Verkest KR, Fleeman LM, Rand JS, Morton JM. Evaluation of beta-cell sensitivity to glucose and first-phase insulin secretion in obese dogs. Am J Vet Res. 2011 Feb 28;72(3):357–66.

51. Tropf M, Nelson OL, Lee PM, Weng HY. Cardiac and Metabolic Variables in Obese Dogs. J Vet Intern Med. 2017;31(4):1000–7.

52. Alberti KGMM, Eckel RH, Grundy SM, Zimmet PZ, Cleeman JI, Donato KA, et al. Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. Circulation. 2009 Oct 20;120(16):1640–5.

53. Chikamune T, Katamoto H, Ohashi F, Shimada Y. Serum Lipid and Lipoprotein Concentrations in Obese Dogs. J Vet Med Sci. 1995 Aug 15;57(4):595–8.

54. Veiga APM, Price CA, Oliveira ST de, Santos AP dos, Campos R, Barbosa PR, et al. Association of canine obesity with reduced serum levels of C-reactive protein. J Vet Diagn Invest. 2008 Mar 1;20(2):224–8.

55. José Lahm Cardoso M, Fagnani R, Zaghi Cavalcante C, de Souza Zanutto M, Júnior AZ, Holsback da Silveira Fertonani L, et al. Blood Pressure, Serum Glucose, Cholesterol, and Triglycerides in Dogs with Different Body Scores. Vet Med Int [Internet]. 2016. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5183795/

56. Xenoulis PG, Steiner JM. Canine hyperlipidaemia. J Small Anim Pract. 2015 Oct 1;56(10):595–605.

57. Verkest KR, Rand JS, Fleeman LM, Morton JM. Spontaneously obese dogs exhibit greater postprandial glucose, triglyceride, and insulin concentrations than lean dogs. Domest Anim Endocrinol. 2012 Feb 1;42(2):103–12.

58. German AJ, Hervera M, Hunter L, Holden SL, Morris PJ, Biourge V, et al. Improvement in insulin resistance and reduction in plasma inflammatory adipokines after weight loss in obese dogs. Domest Anim Endocrinol. 2009 Nov;37(4):214–26.

59. Tvarijonaviciute A, Ceron JJ, Holden SL, Morris PJ, Biourge V, German AJ. Effects of weight loss in obese cats on biochemical analytes related to inflammation and glucose homeostasis. Domest Anim Endocrinol. 2012 Apr 1;42(3):129–41.

60. White R a. S, Williams JM. Tracheal collapse in the dog - is there really a role for surgery? A survey of 100 cases. J Small Anim Pract. 1994 Apr 1;35(4):191–6.

61. Bodey AR, Michell AR. Epidemiological study of blood Sressure in omestic dogs. J Small Anim Pract. 1996 Mar 1;37(3):116–25.

62. Mehlman E, Bright J m., Jeckel K, Porsche C, Veeramachaneni D n. r., Frye M. Echocardiographic Evidence of Left Ventricular Hypertrophy in Obese Dogs. J Vet Intern Med. 2013 Jan 1;27(1):62–8.

63. Holt PE. Studies on the control of urinary continence in the bitch [Internet] [Ph.D.]. University of Bristol; 1987. Available from: http://ethos.bl.uk/OrderDetails.do?uin=uk.bl.ethos.380164

64. Henegar JR, Bigler SA, Henegar LK, Tyagi SC, Hall JE. Functional and Structural Changes in the Kidney in the Early Stages of Obesity. J Am Soc Nephrol. 2001 Jan 6;12(6):1211–7.

65. Lekcharoensuk C, Lulich JP, Osborne CA, Pusoonthornthum R, Allen TA, Koehler LA, et al. Patient and environmental factors associated with calcium oxalate urolithiasis in dogs. J Am Vet Med Assoc. 2000 Aug 1;217(4):515–9.

66. Tvarijonaviciute A, Ceron JJ, Holden SL, Biourge V, Morris PJ, German AJ. Effect of Weight Loss in Obese Dogs on Indicators of Renal Function or Disease. J Vet Intern Med. 27(1):31–8.

67. Sonnenschein EG, Glickman LT, Goldschmidt MH, McKee LJ. Body Conformation, Diet, and Risk of Breast Cancer in Pet Dogs: A Case-Control Study. Am J Epidemiol. 1991 Apr 1;133(7):694–703.

68. Glickman LT, Schofer FS, McKee LJ, Reif JS, Goldschmidt MH. Epidemiologic study of insecticide exposures, obesity, and risk of bladder cancer in household dogs. J Toxicol Environ Health. 1989 Dec 1;28(4):407–14.

69. Gibbs EPJ. The evolution of One Health: a decade of progress and challenges for the future. Vet Rec. 2014 Jan 25;174(4):85–91.

70. Cunningham AA, Daszak P, Wood JLN. One Health, emerging infectious diseases and wildlife: two decades of progress? Philos Trans R Soc B Biol Sci. 2017 Jul 19;372(1725):20160167.

71. Bartges J, Kushner RF, Michel KE, Sallis R, Day MJ. One Health Solutions to Obesity in People and Their Pets. J Comp Pathol [Internet]. [cited 2017 May 10]; Available from: http://www.sciencedirect.com/science/article/pii/S002199751730124X

72. Chandler M, Cunningham S, Lund EM, Khanna C, Naramore R, Patel A, et al. Obesity and Associated Comorbidities in People and Companion Animals: A One Health Perspective. J Comp Pathol. 2017 May;156(4):296–309.

73. Day MJ. One Health Approach to Preventing Obesity in People and Their Pets. J Comp Pathol. 2017 May;156(4):293–5.

74. McDermott MP, Tischler VA, Cobb MA, Robbé IJ, Dean RS. Veterinarian-Client Communication Skills: Current State, Relevance, and Opportunities for Improvement. J Vet Med Educ. 2015;42(4):305–14.

75. Churchill J, Ward E. Communicating with Pet Owners About Obesity: Roles of the Veterinary Health Care Team. Vet Clin North Am Small Anim Pract. 2016 Sep;46(5):899–911.

76. Bauer UE, Briss PA, Goodman RA, Bowman BA. Prevention of chronic disease in the 21st century: elimination of the leading preventable causes of premature death and disability in the USA. The Lancet. 2014 Jul 5;384(9937):45–52.

77. Estrada E, Eneli I, Hampl S, Mietus-Snyder M, Mirza N, Rhodes E, et al. Children's Hospital Association Consensus Statements for Comorbidities of Childhood Obesity. Child Obes. 2014 Jul 14;10(4):304–17.

78. Paeratakul S, Lovejoy JC, Ryan DH, Bray GA. The relation of gender, race and socioeconomic status to obesity and obesity comorbidities in a sample of US adults. Int J Obes. 2002 Sep;26(9):1205–10.

79. Bomberg E, Birch L, Endenburg N, German AJ, Neilson J, Seligman H, et al. The Financial Costs, Behaviour and Psychology of Obesity: A One Health Analysis. J Comp Pathol. 2017 May;156(4):310–25.

80. Wang Y, Beydoun MA. The Obesity Epidemic in the United States—Gender, Age, Socioeconomic, Racial/Ethnic, and Geographic Characteristics: A Systematic Review and Meta-Regression Analysis. Epidemiol Rev. 2007 Jan 1;29(1):6–28.

81. Hedley AA, Ogden CL, Johnson CL, Carroll MD, Curtin LR, Flegal KM. Prevalence of Overweight and Obesity Among US Children, Adolescents, and Adults, 1999-2002. JAMA. 2004 Jun 16;291(23):2847–50.

82. You W, Henneberg M. Relaxed natural selection contributes to global obesity increase more in males than in females due to more environmental modifications in female body mass. PLOS ONE. 2018 Jul 18;13(7):e0199594.

83. Darmon N, Drewnowski A. Contribution of food prices and diet cost to socioeconomic disparities in diet quality and health: a systematic review and analysis. Nutr Rev. 2015 Oct 1;73(10):643–60.

84. Ogden CL, Flegal KM. No Statistically Significant Change Since 2003-2004. 2007;(1):8.

85. Beechy L, Galpern J, Petrone A, Das SK. Assessment tools in obesity — Psychological measures, diet, activity, and body composition. Physiol Behav. 2012 Aug;107(1):154–71.

86. Nijland ML, Stam F, Seidell JC. Overweight in dogs, but not in cats, is related to overweight in their owners. Public Health Nutr. 2010 Jan;13(01):102–106.

87. Christakis NA, Fowler JH. The Spread of Obesity in a Large Social Network over 32 Years. N Engl J Med. 2007 Jul 26;357(4):370–9.

88. German AJ. Style over substance: what can parenting styles tell us about ownership styles and obesity in companion animals? Br J Nutr. 2015 Jan;113(S1):S72–7.

89. Pretlow RA, Corbee RJ. Similarities between obesity in pets and children: the addiction model. Br J Nutr. 2016 Sep;116(05):944–9.

90. Weber M, Bissot T, Servet E, Sergheraert R, Biourge V, German AJ. A High-Protein, High-Fiber Diet Designed for Weight Loss Improves Satiety in Dogs. J Vet Intern Med. 21(6):1203–8.

91. Wood CT, Skinner AC, Yin HS, Rothman RL, Sanders LM, Delamater AM, et al. Bottle Size and Weight Gain in Formula-Fed Infants. Pediatrics. 2016 Jul 1;138(1):e20154538.

92. Rollins BY, Savage JS, Fisher JO, Birch LL. Alternatives to restrictive feeding practices to promote self-regulation in childhood: a developmental perspective. Pediatr Obes. 2016 Oct 1;11(5):326–32.

93. Cutt H, Giles-Corti B, Knuiman M, Timperio A, Bull F. Understanding Dog Owners' Increased Levels of Physical Activity: Results From RESIDE. Am J Public Health. 2008 Jan 1;98(1):66–9.

94. Christian H, Trapp G, Lauritsen C, Wright K, Giles-Corti B. Understanding the relationship between dog ownership and children's physical activity and sedentary behaviour. Pediatr Obes. 2013 Oct;8(5):392–403.

95. Engelberg JK, Carlson JA, Conway TL, Cain KL, Saelens BE, Glanz K, et al. Dog walking among adolescents: Correlates and contribution to physical activity. Prev Med. 2016 Jan;82:65–72.

96. Owen CG, Nightingale CM, Rudnicka AR, Ekelund U, McMinn AM, van Sluijs EMF, et al. Family Dog Ownership and Levels of Physical Activity in Childhood: Findings From the Child Heart and Health Study in England. Am J Public Health. 2010 Sep;100(9):1669–71.

97. German AJ. Obesity prevention and weight maintenance after loss. Vet Clin North Am Small Anim Pract. 2016;46(5):913–929.

98. Bove CF, Olson CM. Obesity in low-income rural women: qualitative insights about physical activity and eating patterns. Women Health. 2006;44(1):57–78.

99. McLaren L. Socioeconomic Status and Obesity. Epidemiol Rev. 2007 Jan 1;29(1):29–48.

100.   Robert SA, Reither EN. A multilevel analysis of race, community disadvantage, and body mass index among adults in the US. Soc Sci Med. 2004 Dec;59(12):2421–34.

101.   Silva FA do N, Quessada AM, Lima DASD, Lima WC, Silva JDC, Camapum JLR. The profiles of cat and dog tutors participating in a pet neutering campaign. Acta Vet Bras. 2017 Dec 22;11(4):184–6.

102.   Shearer P. Literature Review – Canine, Feline and Human Overweight and Obesity. :8.

103.   Etelson D, Brand DA, Patrick PA, Shirali A. Childhood Obesity: Do Parents Recognize This Health Risk? Obes Res. 2003 Nov 1;11(11):1362–8.

104.   Mathieu M-E, Drapeau V, Tremblay A. Parental Misperception of Their Child's Body Weight Status Impedes the Assessment of the Child's Lifestyle Behaviors. Int J Pediatr [Internet]. 2010. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2939384/

105.   Courcier EA, Mellor DJ, Thomson RM, Yam PS. A cross sectional study of the prevalence and risk factors for owner misperception of canine body shape in first opinion practice in Glasgow. Prev Vet Med. 2011 Oct 1;102(1):66–74.

106.   Eastland-Jones RC, German AJ, Holden SL, Biourge V, Pickavance LC. Owner misperception of canine body condition persists despite use of a body condition score chart. J Nutr Sci [Internet]. 2014 Oct 8;3. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4473163/

107.   German AJ, Holden SL, Wiseman-Orr ML, Reid J, Nolan AM, Biourge V, et al. Quality of life is reduced in obese dogs but improves after successful weight loss. Vet J. 2012 Jun;192(3):428–34.

108.   German AJ, Blackwell E, Evans M, Westgarth C. Overweight dogs are more likely to display undesirable behaviours: results of a large online survey of dog owners in the UK. J Nutr Sci [Internet]. 2017 ed;6. Available from: https://www.cambridge.org/core/journals/journal-of-nutritional-science/article/overweight-dogs-are-more-likely-to-display-undesirable-behaviours-results-of-a-large-online-survey-of-dog-owners-in-the-uk/933AD361252C0F31F33ECC4D52412D41

109.   Strauss RS. Childhood Obesity and Self-Esteem. Pediatrics. 2000 Jan 1;105(1):e15–e15.

110.   Gariepy G, Nitka D, Schmitz N. The association between obesity and anxiety disorders in the population: a systematic review and meta-analysis. Int J Obes. 2010 Mar;34(3):407–19.

111.   Vandendriessche VL, Picavet P, Hesta M. First detailed nutritional survey in a referral companion animal population. J Anim Physiol Anim Nutr. 101(S1):4–14.

112.   German AJ, Holden SL, Gernon LJ, Morris PJ, Biourge V. Do feeding practices of obese dogs, before weight loss, affect the success of weight management? Br J Nutr. 2011 Oct;106(S1):S97–100.

113.   Thomson RM, Hammond J, Ternent HE, Yam PS. Feeding practices and the use of supplements for dogs kept by owners in different socioeconomic groups. Vet Rec. 2008 Nov;163(21):621–4.

114.   Lund EM, Armstrong PJ, Kirk CA, Kolar LM, Klausnor JS. Health status and population characteristics of dogs and cats examined at private veterinary practices in the United States. J-Am Vet Med Assoc. 1999;214:1336–1341.

115.   Bland IM, Guthrie-Jones A, Taylor RD, Hill J. Dog obesity: Veterinary practices' and owners' opinions on cause and management. Prev Vet Med. 2010 May 1;94(3–4):310–5.

116.   Kobelt AJ, Hemsworth PH, Barnett JL, Coleman GJ. A survey of dog ownership in suburban Australia—conditions and behaviour problems. Appl Anim Behav Sci. 2003 Jun 24;82(2):137–48.

117.   McArthur ML, Fitzgerald JR. Companion animal veterinarians' use of clinical communication skills. Aust Vet J. 2013 Sep;91(9):374–80.

118.   Banks: Digestive system II—extramural organs - Google Académico [Internet]. [cited 2018 Sep 12]. Available from: https://scholar.google.com/scholar_lookup?title=Digestive%20system%20II%20-%20extramural%20organs&author=WJ.%20Banks&pages=360-373&publication_year=1993a

119.   Dellmann HD, Eurell JA. Textbook of veterinary histology. Textb Vet Histol [Internet]. 1998;(Ed. 5). Available from: https://www.cabdirect.org/cabdirect/abstract/19992213118

120.   Paula F de, Teshima THN, Hsieh R, Souza MM, Nico MMS, Lourenco SV. Overview of Human Salivary Glands: Highlights of Morphology and Developing Processes. Anat Rec. 2017 Jul 1;300(7):1180–8.

121.   Textbook of Veterinary Histology, 1e by Don A. Samuelson | 9780721681740 | Get Textbooks | New Textbooks | Used Textbooks | College Textbooks - GetTextbooks.com [Internet]. [cited 2018 Sep 12]. Available from: https://www.gettextbooks.com/isbn/9780721681740/

122.   Holsinger FC, Bui DT. Anatomy, Function, and Evaluation of the Salivary Glands. In: Myers EN, Ferris RL, editors. Salivary Gland Disorders [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2007. p. 1–16. Available from: https://doi.org/10.1007/978-3-540-47072-4_1

123.   Rao RS, Patil S, Amrutha N, Sanketh D, Agarwal A. The ambiguous salivary myoepithelial cells. J Contemp Dent Pract. 2014;15(4):523–8.

124.   Shah AAK, Mulla AF, Mayank M. Pathophysiology of myoepithelial cells in salivary glands. J Oral Maxillofac Pathol JOMFP. 2016;20(3):480–90.

125.   Chitturi RT, Veeravarmal V, Nirmal RM, Reddy BVR. Myoepithelial Cells (MEC) of the Salivary Glands in Health and Tumours. J Clin Diagn Res JCDR. 2015 Mar;9(3):ZE14–8.

126.   Durand A, Finck M, Sullivan M, Hammond G. Computed tomography and magnetic resonance diagnosis of variations in the anatomical location of the major salivary glands in 1680 dogs and 187 cats. Vet J. 2016 Mar;209:156–62.

127.   Gil F, Arencibia A, García V, Ramírez G, Vázquez JM. Anatomic and magnetic resonance imaging features of the salivary glands in the dog. Anat Histol Embryol [Internet]. 2018 Aug 14; Available from: http://doi.wiley.com/10.1111/ahe.12396

128.   E. Sales Baptista, E. Lamy, M. Mau, F. Capela E Silva, A.V. Coelho. VARIATION IN SALIVARY PROTEIN COMPOSITION RELATED TO FEEDING BEHAVIOR AND ITS ECOLOGICAL IMPLICATIONS, pp. 115-136 [Internet]. [cited 2018 Sep 14]. Available from: https://www.novapublishers.com/catalog/product_info.php?products_id=14473

129.   Pereira KF, Souza DR de, Ferreira LS, Chela PR, Helrigle C, Araújo EG de. Morphological aspects of the salivary glands of Crab-eating racoon (Procyon cancrivorus). Aspectos morfológicos das glândulas salivares do Mão-pelada (Procyon cancrivorus) [Internet]. 2013 Mar; Available from: http://repositorio.bc.ufg.br/handle/ri/12569

130.   Eliasson L, Carlén A. An update on minor salivary gland secretions. Eur J Oral Sci. 2010 Oct 1;118(5):435–42.

131.   Carpenter GH. The Secretion, Components, and Properties of Saliva. Annu Rev Food Sci Technol. 2013;4(1):267–76.

132.   Hand AR, Pathmanathan D, Field RB. Morphological features of the minor salivary glands. Arch Oral Biol. 1999 May 1;44:S3–10.

133.   Martinez-Madrigal F, Micheau C. Histology of the major salivary glands. Am J Surg Pathol. 1989 Oct;13(10):879–99.

134.   Dinnella C. Prédiction de l'astringence via l'essai in vitro de réactivité tanins/protéines. Rev Oenologues Tech Vitivinic Oenologicques Mag Trimest Inf Prof. 2010;37(134):25–7.

135.   Cabras T, Melis M, Castagnola M, Padiglia A, Tepper BJ, Messana I, et al. Responsiveness to 6-n-Propylthiouracil (PROP) Is Associated with Salivary Levels of Two Specific Basic Proline-Rich Proteins in Humans. PLOS ONE. 2012 Feb 1;7(2):e30962.

136.   Aps JKM, Martens LC. Review: The physiology of saliva and transfer of drugs into saliva. Forensic Sci Int. 2005 Jun 10;150(2):119–31.

137.   Amerongen A van N, Bolscher JGM, Veerman ECI. Salivary Proteins: Protective and Diagnostic Value in Cariology? Caries Res. 2004;38(3):247–53.

138.   Dodds MWJ, Johnson DA, Yeh C-K. Health benefits of saliva: a review. J Dent. 2005 Mar 1;33(3):223–33.

139.   Emmelin N. Nervous control of mammalian salivary glands. Phil Trans R Soc Lond B. 1981 Dec 18;296(1080):27–35.

140.   Lamy E, Neves S, Ferreira J, Rodrigues L, da Costa G, Cordeiro C, et al. Effects of hyperleptinemia in rat saliva composition, histology and ultrastructure of the major salivary glands. Arch Oral Biol. 2018 Dec 1;96:1–12.

141.   Voutetakis A, Kok MR, Zheng C, Bossis I, Wang J, Cotrim AP, et al. Reengineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics. Proc Natl Acad Sci. 2004 Mar 2;101(9):3053–8.

142.   McDougall EI. Studies on ruminant saliva. 1. The composition and output of sheep's saliva. Biochem J. 1948;43(1):99–109.

143.   Lensen CMM, Moons CPH, Diederich C. Saliva sampling in dogs: How to select the most appropriate procedure for your study. J Vet Behav Clin Appl Res. 2015 Nov;10(6):504–12.

144.   Watanabe S, Dawes C. The effects of different foods and concentrations of citric acid on the flow rate of whole saliva in man. Arch Oral Biol. 1988;33(1):1–5.

145. DAWES C. Factors influencing salivary flow rate and composition. Br Dent J. 1996;27–41.

146. Xiao X, Liu Y, Guo Z, Liu X, Sun H, Li Q, et al. Comparative proteomic analysis of the influence of gender and acid stimulation on normal human saliva using LC/MS/MS. PROTEOMICS - Clin Appl. 2017 Jul;11(7–8):1600142.

147. Graves PR, Haystead TAJ. Molecular Biologist's Guide to Proteomics. Microbiol Mol Biol Rev. 2002 Mar 1;66(1):39–63.

148. Carrera M, Gallardo JM, Pascual S, González ÁF, Medina I. Protein biomarker discovery and fast monitoring for the identification and detection of Anisakids by parallel reaction monitoring (PRM) mass spectrometry. J Proteomics. 2016 Jun 16;142:130–7.

149. Lamy E, Simões C, Rodrigues L, Costa AR, Vitorino R, Amado F, et al. Changes in the salivary protein profile of morbidly obese women either previously subjected to bariatric surgery or not. J Physiol Biochem. 2015 Dec;71(4):691–702.

150. Desai GS, Mathews ST. Saliva as a non-invasive diagnostic tool for inflammation and insulin-resistance. World J Diabetes. 2014 Dec 15;5(6):730–8.

151. Kikkawa A, Uchida Y, Suwa Y, Taguchi K. A Novel Method for Estimating the Adaptive Ability of Guide Dogs Using Salivary sIgA. J Vet Med Sci. 2005;67(7):707–12.

152. Kikkawa A, Uchida Y, Nakade T, Taguchi K. Salivary Secretory IgA Concentrations in Beagle Dogs. J Vet Med Sci. 2003;65(6):689–93.

153. Neyraud E, Sayd T, Morzel M, Dransfield E. Proteomic Analysis of Human Whole and Parotid Salivas Following Stimulation by Different Tastes. J Proteome Res. 2006 Sep;5(9):2474–80.

154. Amado FML, Ferreira RP, Vitorino R. One decade of salivary proteomics: Current approaches and outstanding challenges. Clin Biochem. 2013 Apr 1;46(6):506–17.

155. Lamy E, Mau M. Saliva proteomics as an emerging, non-invasive tool to study livestock physiology, nutrition and diseases. J Proteomics. 2012 Jul;75(14):4251–8.

156. Rodrigues L, Costa G, Cordeiro C, Pinheiro C, Amado F, Lamy E. Salivary proteome and glucose levels are related with sweet taste sensitivity in young adults. Food Nutr Res. 2017 Jan 1;61(1):1389208.

157. Lamy E, Morzel M, Rodrigues L, Pinheiro C, Costa AR, Antunes CM, et al. The Use of Electrophoresis for the Study of Saliva Involvement in Ingestive Behavior. In: Ghowsi K, editor. Field Effect Electroosmosis - A Novel Phenomenon in Electrokinetics and its Applications in Capillary Electrophoresis [Internet]. InTech; 2015. Available from: http://www.intechopen.com/books/field-effect-electroosmosis-a-novel-phenomenon-in-electrokinetics-and-its-applications-in-capillary-electrophoresis/the-use-of-electrophoresis-for-the-study-of-saliva-involvement-in-ingestive-behavior

158. Fic E, Kedracka-Krok S, Jankowska U, Pirog A, Dziedzicka-Wasylewska M. Comparison of protein precipitation methods for various rat brain structures prior to proteomic analysis. ELECTROPHORESIS. 2010 Oct 1;31(21):3573–9.

159.   Rodríguez-Suárez E, Whetton AD. The application of quantification techniques in proteomics for biomedical research. Mass Spectrom Rev. 2013 Jan 1;32(1):1–26.

160.   Sullivan AG, Russell S, Brzeski H, Somiari RI, Shriver CD. Serum or Plasma Sample Preparation for Two-Dimensional Gel Electrophoresis. In: Walker JM, editor. The Proteomics Protocols Handbook [Internet]. Totowa, NJ: Humana Press; 2005. p. 49–54. Available from: https://doi.org/10.1385/1-59259-890-0:049

161.   Lamy E, Costa AR, Antunes CM, Vitorino R, Amado F. Protein Electrophoresis in Saliva Study [Internet]. 2012. Available from: http://dspace.uevora.pt/rdpc/handle/10174/5827

162.   Guo Y, Fu Z, Van Eyk JE. A Proteomic Primer for the Clinician. Proc Am Thorac Soc. 2007 Jan 1;4(1):9–17.

163.   Matt P, Fu Z, Fu Q, Van Eyk JE. Biomarker discovery: proteome fractionation and separation in biological samples. Physiol Genomics. 2008 Mar;33(1):12–7.

164.   Lin Y, Zhou J, Bi D, Chen P, Wang X, Liang S. Sodium-deoxycholate-assisted tryptic digestion and identification of proteolytically resistant proteins. Anal Biochem. 2008 Jun 15;377(2):259–66.

165.   Henzel WJ, Watanabe C, Stults JT. Protein identification: the origins of peptide mass fingerprinting. J Am Soc Mass Spectrom. 2003 Sep 1;14(9):931–42.

166.   Probability-based protein identification by searching sequence databases using mass spectrometry data - Perkins - 1999 - ELECTROPHORESIS - Wiley Online Library [Internet]. Available from: https://onlinelibrary.wiley.com/doi/pdf/10.1002/%28SICI%291522-2683%2819991201%2920%3A18%3C3551%3A%3AAID-ELPS3551%3E3.0.CO%3B2-2

167.   Dakna M, He Z, Yu WC, Mischak H, Kolch W. Technical, bioinformatical and statistical aspects of liquid chromatography–mass spectrometry (LC–MS) and capillary electrophoresis-mass spectrometry (CE-MS) based clinical proteomics: A critical assessment. J Chromatogr B. 2009 May 1;877(13):1250–8.

168.   Csősz É, Kalló G, Márkus B, Deák E, Csutak A, Tőzsér J. Quantitative body fluid proteomics in medicine — A focus on minimal invasiveness. J Proteomics. 2017 Feb 5;153:30–43.

169.   Torres SMF, Furrow E, Souza CP, Granick JL, de Jong EP, Griffin TJ, et al. Salivary proteomics of healthy dogs: An in depth catalog. Guo X, editor. PLOS ONE. 2018 Jan 12;13(1):e0191307.

170.   Oyama D, Hyodo M, Doi H, Kurachi T, Takata M, Koyama S, et al. Saliva collection by using filter paper for measuring cortisol levels in dogs. Domest Anim Endocrinol. 2014 Jan;46:20–5.

171.   Cobb ML, Iskandarani K, Chinchilli VM, Dreschel NA. A systematic review and meta-analysis of salivary cortisol measurement in domestic canines. Domest Anim Endocrinol. 2016;57:31–42.

172.   Dreschel NA, Cobb M, Iskandarani K, Chinchilli VM. A meta-analysis of salivary cortisol measurement in domestic canines. J Vet Behav Clin Appl Res. 2014 Nov 1;9(6):e11–2.

173.   Sandri M, Colussi A, Perrotta MG, Stefanon B. Salivary cortisol concentration in healthy dogs is affected by size, sex, and housing context. J Vet Behav Clin Appl Res. 2015 Jul;10(4):302–6.

174.   Dreschel NA, Granger DA. Methods of collection for salivary cortisol measurement in dogs. Horm Behav. 2009 Jan 1;55(1):163–8.

175.   Svobodová I, Chaloupková H, Končel R, Bartoš L, Hradecká L, Jebavý L. Cortisol and Secretory Immunoglobulin A Response to Stress in German Shepherd Dogs. Suchodolski JS, editor. PLoS ONE. 2014 Mar 17;9(3):e90820.

176.   Diverio S, Guelfi G, Barbato O, Di Mari W, Egidi MG, Santoro MM. Non-invasive assessment of animal exercise stress: real-time PCR of GLUT4, COX2, SOD1 and HSP70 in avalanche military dog saliva. animal. 2015 Jan;9(01):104–9.

177.   Reece EA, Wiznitzer A, Le E, Homko CJ, Behrman H, Spencer EM. The relation between human fetal growth and fetal blood levels of insulin-like growth factors I and II, their binding proteins, and receptors. Obstet Gynecol. 1994 Jul;84(1):88–95.

178.   Scannapieco FA. Saliva-Bacterium Interactions in Oral Microbial Ecology. Crit Rev Oral Biol Med. 1994 Sep 1;5(3):203–48.

179.   de Sousa-Pereira P, Cova M, Abrantes J, Ferreira R, Trindade F, Barros A, et al. Cross-species comparison of mammalian saliva using an LC–MALDI based proteomic approach. PROTEOMICS. 2015 May 1;15(9):1598–607.

180.   Vieths S, Scheurer S, Reindl J, Lüttkopf D, Wangorsch A, Kästner M, et al. Optimized allergen extracts and recombinant allergens in diagnostic applications. Allergy. 2001 Apr 1;56:78–82.

181.   Polovic N, Wadén K, Binnmyr J, Hamsten C, Grönneberg R, Palmberg C, et al. Dog saliva – an important source of dog allergens. Allergy. 2013 May 1;68(5):585–92.

182.   Breitenbuecher C, Belanger JM, Levy K, Mundell P, Fates V, Gershony L, et al. Protein expression and genetic variability of canine Can f 1 in golden and Labrador retriever service dogs. Canine Genet Epidemiol. 2016;3:3.

183.   Kawas SA, Rahim ZHA, Ferguson DB. Potential uses of human salivary protein and peptide analysis in the diagnosis of disease. Arch Oral Biol. 2012 Jan;57(1):1–9.

184.   Oudhoff MJ, Bolscher JGM, Nazmi K, Kalay H, van 't Hof W, Amerongen AVN, et al. Histatins are the major wound-closure stimulating factors in human saliva as identified in a cell culture assay. FASEB J. 2008 Jul 23;22(11):3805–12.

185.   Brand HS, Ligtenberg AJM, Veerman ECI. Saliva and Wound Healing. Saliva Secret Funct. 2014;24:52–60.

186.   Tvarijonaviciute A, Barranco T, Rubio M, Carrillo JM, Martinez-Subiela S, Tecles F, et al. Measurement of Creatine kinase and Aspartate aminotransferase in saliva of dogs: a pilot study. BMC Vet Res [Internet]. 2017 Jun 9;13. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5466776/

187.   Pasha S, Inui T, Chapple I, Harris S, Holcombe L, Grant MM. The Saliva Proteome of Dogs: Variations Within and between Breeds and between Species. PROTEOMICS. 2018 Jan 12;1700293.

188.   Fleissig Y, Reichenberg E, Redlich M, Zaks B, Deutsch O, Aframian DJ, et al. Comparative proteomic analysis of human oral fluids according to gender and age. Oral Dis. 2010 Nov 1;16(8):831–8.

189.   Sun S, Zhao F, Wang Q, Zhong Y, Cai T, Wu P, et al. Analysis of age and gender associated N-glycoproteome in human whole saliva. Clin Proteomics. 2014 Jun 5;11(1):25.

190.   Mitsouras K, Faulhaber EA. Saliva as an alternative source of high yield canine genomic DNA for genotyping studies. BMC Res Notes. 2009;2(1):219.

191.   Nakanishi H, Ohmori T, Hara M, Yoneyama K, Takada A, Saito K. Identification of canine saliva using mRNA-based assay. Int J Legal Med. 2017 Jan;131(1):39–43.

192.   Castagnola M, Scarano E, Passali GC, Messana I, Cabras T, Iavarone F, et al. Salivary biomarkers and proteomics: future diagnostic and clinical utilities. Acta Otorhinolaryngol Ital. 2017 Apr;37(2):94–101.

193.   Scarano E, Fiorita A, Picciotti P, Passali G, Calò L, Cabras T, et al. Proteomics of saliva: personal experience. Acta Otorhinolaryngol Ital. 2010 Jun;30(3):125–30.

194.   Alberti KGMM, Zimmet P, Shaw J. Metabolic syndrome—a new world-wide definition. A Consensus Statement from the International Diabetes Federation. Diabet Med. 2006 May 1;23(5):469–80.

195.   Alberti KGMM, Zimmet P, Shaw J, IDF Epidemiology Task Force Consensus Group. The metabolic syndrome--a new worldwide definition. Lancet Lond Engl. 2005 Sep 24;366(9491):1059–62.

196.   Reaven GM. Role of Insulin Resistance in Human Disease. Diabetes. 1988 Dec 1;37(12):1595–607.

197.   Reaven GM. Insulin Resistance: the Link Between Obesity and Cardiovascular Disease. Med Clin North Am. 2011 Sep 1;95(5):875–92.

198.   Zalewska A, Knaś M, Żendzian-Piotrowska M, Waszkiewicz N, Szulimowska J, Prokopiuk S, et al. Antioxidant profile of salivary glands in high fat diet-induced insulin resistance rats. Oral Dis. 2014 Sep 1;20(6):560–6.

199.   Nigro E, Piombino P, Scudiero O, Monaco ML, Schettino P, Chambery A, et al. Evaluation of salivary adiponectin profile in obese patients. Peptides. 2015 Jan 1;63:150–5.

200.   Goodson JM, Kantarci A, Hartman M-L, Denis GV, Stephens D, Hasturk H, et al. Metabolic Disease Risk in Children by Salivary Biomarker Analysis. PLoS ONE [Internet]. 2014 Jun 10;9(6). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4051609/

201.   Chauhan A, Yadav SS, Dwivedi P, Lal N, Usman K, Khattri S. Correlation of Serum and Salivary Cytokines Level With Clinical Parameters in Metabolic Syndrome With Periodontitis. J Clin Lab Anal. 2016 Sep 1;30(5):649–55.

202.   Shi P, Goodson JM, Hartman M-L, Hasturk H, Yaskell T, Vargas J, et al. Continuous Metabolic Syndrome Scores for Children Using Salivary Biomarkers. PLoS ONE [Internet]. 2015 Sep 29;10(9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4587796/

203.   Oberbach A, Blüher M, Wirth H, Till H, Kovacs P, Kullnick Y, et al. Combined Proteomic and Metabolomic Profiling of Serum Reveals Association of the Complement System with Obesity and Identifies Novel Markers of Body Fat Mass Changes. J Proteome Res. 2011 Oct 7;10(10):4769–88.

204.   Kelesidis T, Kelesidis I, Chou S, Mantzoros CS. Narrative Review: The Role of Leptin in Human Physiology: Emerging Clinical Applications. Ann Intern Med. 2010 Jan 19;152(2):93–100.

205.   Yin J, Gao H, Yang J, Xu L, Li M. Measurement of Salivary Resistin Level in Patients with Type 2 Diabetes [Internet]. International Journal of Endocrinology. 2012 [cited 2018 Aug 20]. Available from: https://www.hindawi.com/journals/ije/2012/359724/

206.   Jamaluddin MS, Weakley SM, Yao Q, Chen C. Resistin: functional roles and therapeutic considerations for cardiovascular disease. Br J Pharmacol. 2012 Feb;165(3):622–32.

207.   Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science. 2005 Jan 21;307(5708):426–30.

208.   Du Clos TW. Function of C-reactive protein. Ann Med. 2000 May;32(4):274–8.

209.   Stasi C, Milani S. Functions of Ghrelin in Brain, Gut and Liver. CNS Neurol Disord Drug Targets. 2016;15(8):956–63.

210.   Williamson S, Munro C, Pickler R, Grap MJ, Elswick RK. Comparison of Biomarkers in Blood and Saliva in Healthy Adults. Nurs Res Pract [Internet]. 2012. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3350846/

211.   Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism [Internet]. Nature. 2001. Available from: https://www.nature.com/articles/414799a

212.   Monea A, Gruber R, Elod N, Bereşescu G, Moldovan C, Monea M. SALIVA AND SERUM LEVELS OF TNF-α AND IL- 6 IN A SAMPLE OF ROMANIAN ADULT SUBJECTS WITH TYPE 2 DIABETES MELLITUS AND PERIODONTAL DISEASE. Eur Sci J ESJ [Internet]. 2014 Mar 31;10(9). Available from: https://eujournal.org/index.php/esj/article/view/3067

213.   Malathi L, Masthan KMK, Balachander N, Babu NA, Rajesh E. Estimation of Salivary Amylase in Diabetic Patients and Saliva as a Diagnostic Tool in Early Diabetic Patients. J Clin Diagn Res JCDR. 2013 Nov;7(11):2634–6.

214.   Nater UM, Rohleder N. Salivary alpha-amylase as a non-invasive biomarker for the sympathetic nervous system: Current state of research. Psychoneuroendocrinology. 2009 May 1;34(4):486–96.

215.   Rao PV, Reddy AP, Lu X, Dasari S, Krishnaprasad A, Biggs E, et al. Proteomic Identification of Salivary Biomarkers of Type-2 Diabetes. J Proteome Res. 2009 Jan 2;8(1):239–45.

216.   Rodrigues L, da Costa G, Cordeiro C, Pinheiro CC, Amado F, Lamy E. Relationship between saliva protein composition and 6- *n* -Propylthiouracil bitter taste responsiveness in young adults: RODRIGUES et al. J Sens Stud. 2017 Jul 21;e12275.

217.   Qvarnstrom M, Janket S-J, Jones JA, Jethwani K, Nuutinen P, Garcia RI, et al. Association of salivary lysozyme and C-reactive protein with metabolic syndrome. J Clin Periodontol. 2010 Sep;37(9):805–11.

218.   Adam TC, Hasson RE, Ventura EE, Toledo-Corral C, Le K-A, Mahurkar S, et al. Cortisol Is Negatively Associated with Insulin Sensitivity in Overweight Latino Youth. J Clin Endocrinol Metab. 2010 Oct 1;95(10):4729–35.

219.   Jessop DS, Turner-Cobb JM. Measurement and meaning of salivary cortisol: a focus on health and disease in children. Stress Amst Neth. 2008 Jan;11(1):1–14.

220.   Guo Y, Guo L-N, Zhu J-F, Tang C-Y, Feng Y-Z, Zhou H-D. Associations of Salivary BPIFA1 Protein in Chronic Periodontitis Patients with Type 2 Diabetes Mellitus. Int J Endocrinol [Internet]. 2017. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5646319/

221.   Prats-Puig A, Gispert-Saüch M, Carreras-Badosa G, Osiniri I, Soriano-Rodríguez P, Planella-Colomer M, et al. α-Defensins and bacterial/permeability-increasing protein as new markers of childhood obesity. Pediatr Obes. 2016 Mar 1;12(2):e10–3.

222.   Hoenig M. Comparative Aspects of Human, Canine, and Feline Obesity and Factors Predicting Progression to Diabetes. Vet Sci. 2014 Aug 21;1(2):121–35.

223.   Osto M, Lutz TA. Translational value of animal models of obesity—Focus on dogs and cats. Eur J Pharmacol. 2015 Jul;759:240–52.

224.   Hospital BP. Banfield Pet Hospital - State of Pet Health Report 2011. :38.

225.   Parra MD, Tecles F, Martínez-Subiela S, Cerón JJ. C-reactive protein measurement in canine saliva. J Vet Diagn Invest Off Publ Am Assoc Vet Lab Diagn Inc. 2005 Mar;17(2):139–44.

226.   Tvarijonaviciute A, Carrillo-Sanchez JD, García-Martinez JD, Tecles F, Martinez-Subiela S, German AJ, et al. Measurement of salivary adiponectin concentrations in dogs. Vet Clin Pathol. 2014 Sep 1;43(3):416–21.

227.   Piantedosi D, Di Loria A, Guccione J, De Rosa A, Fabbri S, Cortese L, et al. Serum biochemistry profile, inflammatory cytokines, adipokines and cardiovascular findings in obese dogs. Vet J. 2016 Oct;216:72–8.

228.   Ishioka K, Soliman MM, Sagawa M, Nakadomo F, Shibata H, Honjoh T, et al. Experimental and Clinical Studies on Plasma Leptin in Obese Dogs. J Vet Med Sci. 2002;64(4):349–53.

229. Ishioka K, Hosoya K, Kitagawa H, Shibata H, Honjoh T, Kimura K, et al. Plasma leptin concentration in dogs: Effects of body condition score, age, gender and breeds. Res Vet Sci. 2007 Feb;82(1):11–5.

230. Ishioka K, Omachi A, Sagawa M, Shibata H, Honjoh T, Kimura K, et al. Canine adiponectin: cDNA structure, mRNA expression in adipose tissues and reduced plasma levels in obesity. Res Vet Sci. 2006 Apr;80(2):127–32.

231. Barić Rafaj R, Kuleš J, Marinculić A, Tvarijonaviciute A, Ceron J, Mihaljević ž., et al. Plasma markers of inflammation and hemostatic and endothelial activity in naturally overweight and obese dogs. BMC Vet Res [Internet]. 2016 Dec;13(1). Available from: http://bmcvetres.biomedcentral.com/articles/10.1186/s12917-016-0929-8

232. Mori N, Lee P, Kondo K, Kido T, Saito T, Arai T. Potential use of cholesterol lipoprotein profile to confirm obesity status in dogs. Vet Res Commun. 2011 Feb 15;35(4):223–35.

233. Peña C, Suárez L, Bautista I, Montoya JA, Juste MC. Relationship between analytic values and canine obesity. J Anim Physiol Anim Nutr. 2008 Jun 1;92(3):324–5.

234. Xenoulis PG, Levinski MD, Suchodolski JS, Steiner JM. Association of hypertriglyceridemia with insulin resistance in healthy Miniature Schnauzers. J Am Vet Med Assoc. 2011 Apr 15;238(8):1011–6.

235. Tvarijonaviciute A, Tecles F, Ceron JJ. Relationship between serum butyrylcholinesterase and obesity in dogs: A preliminary report. Vet J. 2010 Nov 1;186(2):197–200.

236. German AJ. Weight management in obese pets: the tailoring concept and how it can improve results. Acta Vet Scand [Internet]. 2016 Oct;58(S1). Available from: http://actavetscand.biomedcentral.com/articles/10.1186/s13028-016-0238-z

237. German A j., Titcomb J m., Holden S l., Queau Y, Morris P j., Biourge V. Cohort Study of the Success of Controlled Weight Loss Programs for Obese Dogs. J Vet Intern Med. 2015 Nov 1;29(6):1547–55.

238. Yamka RM, Friesen KG, Frantz NZ. Identification of canine markers related to obesity and the effects of weight loss on the markers of interest. Int J Appl Res Vet Med. 2006;4(4):282.

239. Tvarijonaviciute A, Tecles F, Martínez-Subiela S, Cerón JJ. Effect of weight loss on inflammatory biomarkers in obese dogs. Vet J. 2012 Aug;193(2):570–2.

240. Jeusette IC, Lhoest ET, Istasse LP, Diez MO. Influence of obesity on plasma lipid and lipoprotein concentrations in dogs. Am J Vet Res. 2005 Jan 1;66(1):81–6.

241. Diez M, Michaux C, Jeusette I, Baldwin P, Istasse L, Biourge V. Evolution of blood parameters during weight loss in experimental obese Beagle dogs. J Anim Physiol Anim Nutr. 88(3–4):166–71.

242. Blanchard G, Nguyen P, Gayet C, Leriche I, Siliart B, Paragon B-M. Rapid Weight Loss with a High-Protein Low-Energy Diet Allows the Recovery of Ideal Body Composition and Insulin Sensitivity in Obese Dogs. J Nutr. 2004 Aug 1;134(8):2148S-2150S.

243.   Tvarijonaviciute A, Gutiérrez AM, Miller I, Razzazi-Fazeli E, Tecles F, Ceron JJ. A proteomic analysis of serum from dogs before and after a controlled weight-loss program. Domest Anim Endocrinol. 2012 Nov 1;43(4):271–7.

244.   Jeusette IC, Detilleux J, Shibata H, Saito M, Honjoh T, Delobel A, et al. Effects of chronic obesity and weight loss on plasma ghrelin and leptin concentrations in dogs. Res Vet Sci. 2005 Oct;79(2):169–75.

245.   Chapman MJ. Comparative analysis of mammalian plasma lipoproteins. Methods Enzymol. 1986;128:70–143.

246.   Rodrigues L, Mouta R, Costa AR, Pereira A, Capela e Silva F, Amado F, et al. Effects of high-fat diet on salivary α-amylase, serum parameters and food consumption in rats. Arch Oral Biol. 2015 Jun 1;60(6):854–62.

247.   Deconte SR, Oliveira RJ da S, Calábria LK, Oliveira VN de, Gouveia NM de, Moraes A da S, et al. Alterations of antioxidant biomarkers and type I collagen deposition in the parotid gland of streptozotocin-induced diabetic rats. Arch Oral Biol. 2011 Aug 1;56(8):744–51.

248.   Carda C, Carranza M, Arriaga A, Díaz A, Peydró A, Gomez M de F. Structural differences between alcoholic and diabetic parotid sialosis. Med Oral Patol Oral Cir Bucal. 2005;10(4):309–14.

249.   Parlak S, Tatar A, Keles O, Selli J, Can I, Unal B. Effects of menopause and diabetes on the rat parotid glands: A histopathological and stereological study. Int J Med Sci Public Health. 2014;3(6):749.

250.   Bailey BJ, Johnson JT, Newlands SD. Head & Neck Surgery--otolaryngology. Lippincott Williams & Wilkins; 2006. 3053 p.

251.   Bozzato A, Burger P, Zenk J, Uter W, Iro H. Salivary gland biometry in female patients with eating disorders. Eur Arch Oto-Rhino-Laryngol Off J Eur Fed Oto-Rhino-Laryngol Soc EUFOS Affil Ger Soc Oto-Rhino-Laryngol - Head Neck Surg. 2008 Sep;265(9):1095–102.

252.   Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec 15;112(12):1796–808.

253.   Modéer T, Blomberg CC, Wondimu B, Julihn A, Marcus C. Association Between Obesity, Flow Rate of Whole Saliva, and Dental Caries in Adolescents. Obesity. 2010 Dec 1;18(12):2367–73.

254.   Brydon L, Wright CE, O'Donnell K, Zachary I, Wardle J, Steptoe A. Stress-induced cytokine responses and central adiposity in young women. Int J Obes. 2008 Mar;32(3):443–50.

255.   Johnson EO, Kostandi M, Moutsopoulos HM. Hypothalamic-pituitary-adrenal axis function in Sjögren's syndrome: mechanisms of neuroendocrine and immune system homeostasis. Ann N Y Acad Sci. 2006 Nov;1088:41–51.

256.   Flink H, Bergdahl M, Tegelberg A, Rosenblad A, Lagerlöf F. Prevalence of hyposalivation in relation to general health, body mass index and remaining teeth in different age groups of adults. Community Dent Oral Epidemiol. 2008 Dec;36(6):523–31.

257.   Corbee RJ. Obesity in show dogs. J Anim Physiol Anim Nutr. 2013 Oct 1;97(5):904–10.

258.   Laflamme DP. Understanding and Managing Obesity in Dogs and Cats. Vet Clin North Am Small Anim Pract. 2006 Nov;36(6):1283–95.

259.   Miller I, Preßlmayer-Hartler A, Wait R, Hummel K, Sensi C, Eberini I, et al. In between - Proteomics of dog biological fluids. J Proteomics. 2014 Jun 25;106:30–45.

260.   Nowroozilarki N, Jamshidi S, Salehi TZ, Kolahian S. Identification of Helicobacter and Wolinella spp. in Oral Cavity of Toy Breed Dogs With Periodontal Disease. Top Companion Anim Med [Internet]. 2017 Jul 15;0(0). Available from: http://www.companimalmed.com/article/S1938-9736(16)30085-X/abstract

261.   Delmonico L, Bravo M, Silvestre RT, Ornellas MHF, De Azevedo CM, Alves G. Proteomic profile of saliva and plasma from women with impalpable breast lesions. Oncol Lett. 2016 Sep;12(3):2145–52.

262.   Tvarijonaviciute A, Castillo C, Ceron JJ, Martinez-Subiela S, Tecles F, López-Jornet P. Leptin and NGF in saliva of patients with diabetes mellitus type 2: A pilot study. J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol. 2017 Oct;46(9):853–5.

263.   Kang D, Moon MH. Development of non-gel-based two-dimensional separation of intact proteins by an on-line hyphenation of capillary isoelectric focusing and hollow fiber flow field-flow fractionation. Anal Chem. 2006;78(16):5789–5798.

264.   San José C, Cárcel MJ, Tejedor MT, Monteagudo LV. Microsatellite DNA markers applied to the classification of the Podenco Valenciano canine breed. Ital J Anim Sci. 2018 Jan 2;17(1):49–52.

265.   Ostrander EA, Wayne RK. The canine genome. Genome Res. 2005 Jan 12;15(12):1706–16.

266.   Kobayashi K, Ogata H, Morikawa M, Iijima S, Harada N, Yoshida T, et al. Distribution and partial characterisation of IgG Fc binding protein in various mucin producing cells and body fluids. Gut. 2002 Aug 1;51(2):169–76.

267.   Kobelt AJ, Hemsworth PH, Barnett JL, Butler KL. Sources of sampling variation in saliva cortisol in dogs. Res Vet Sci. 2003 Oct;75(2):157–61.

268.   Hebert AS, Richards AL, Bailey DJ, Ulbrich A, Coughlin EE, Westphall MS, et al. The One Hour Yeast Proteome. Mol Cell Proteomics. 2014 Jan 1;13(1):339–47.

269.   Dollery CM. Neutrophil Elastase in Human Atherosclerotic Plaques: Production by Macrophages. Circulation. 2003 Jun 10;107(22):2829–36.

270.   Harris IS, Treloar AE, Inoue S, Sasaki M, Gorrini C, Lee KC, et al. Glutathione and Thioredoxin Antioxidant Pathways Synergize to Drive Cancer Initiation and Progression. Cancer Cell. 2015 Feb 9;27(2):211–22.

271.   Oesterle A, Bowman MAH. S100A12 and the S100/CalgranulinsSignificance: Emerging Biomarkers for Atherosclerosis and Possibly Therapeutic Targets. Arterioscler Thromb Vasc Biol. 2015 Dec 1;35(12):2496–507.

272.   Uchida T, Furumai K, Fukuda T, Akiyama H, Takezawa M, Asano T, et al. Prolyl Isomerase Pin1 Regulates Mouse Embryonic Fibroblast Differentiation into Adipose Cells. Rota R, editor. PLoS ONE. 2012 Mar 7;7(3):e31823.

273.   Khim S-K, Bauman J, Evans J, Freeman B, King B, Kirkland T, et al. Discovery of novel and potent aryl diamines as leukotriene A4 hydrolase inhibitors. Bioorg Med Chem Lett. 2008 Jul 15;18(14):3895–8.

274.   Ghosh C, Banik GD, Maity A, Som S, Chakraborty A, Selvan C, et al. Oxygen-18 isotope of breath CO_2 linking to erythrocytes carbonic anhydrase activity: a biomarker for pre-diabetes and type 2 diabetes. Sci Rep [Internet]. 2015 Jan 30;5. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4311236/

275.   Nagasawa H, Uto Y, Sasaki H, Okamura N, Murakami A, Kubo S, et al. Gc Protein (Vitamin D-binding Protein): Gc Genotyping and GcMAF Precursor Activity. Anticancer Res. 2005 Jan 11;25(6A):3689–95.

276.   Schmaier AH. The contact activation and kallikrein/kinin systems: pathophysiologic and physiologic activities. J Thromb Haemost. 2016 Jan 1;14(1):28–39.

277.   Chee CS, Chang KM, Loke MF, Angela Loo VP, Subrayan V. Association of potential salivary biomarkers with diabetic retinopathy and its severity in type-2 diabetes mellitus: a proteomic analysis by mass spectrometry. PeerJ [Internet]. 2016 May 12

;4. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4893325/

278.   Stavrou EX, Fang C, Merkulova A, Alhalabi O, Grobe N, Antoniak S, et al. Reduced thrombosis in Klkb1−/− mice is mediated by increased Mas receptor, prostacyclin, Sirt1, and KLF4 and decreased tissue factor. Blood. 2015 Jan 22;125(4):710–9.

279.   Jain SK, Kahlon G, Bass P, Levine SN, Warden C. Can l-Cysteine and Vitamin D Rescue Vitamin D and Vitamin D Binding Protein Levels in Blood Plasma of African American Type 2 Diabetic Patients? Antioxid Redox Signal. 2015 Sep 10;23(8):688–93.

280.   Qiu H, Gabrielsen A, Agardh HE, Wan M, Wetterholm A, Wong C-H, et al. Expression of 5-lipoxygenase and leukotriene A4 hydrolase in human atherosclerotic lesions correlates with symptoms of plaque instability. Proc Natl Acad Sci U S A. 2006 May 23;103(21):8161–6.

281.   Zhang L, Li Z, Zhang B, He H, Bai Y. PPIA is a novel adipogenic factor implicated in obesity. Obesity. 23(10):2093–100.

282.   Roumans NJ, Vink RG, Fazelzadeh P, Baak V, A M, Mariman EC. A role for leukocyte integrins and extracellular matrix remodeling of adipose tissue in the risk of weight regain after weight loss. Am J Clin Nutr. 2017 May 1;105(5):1054–62.

283.   Karuwanarint P, Phonrat B, Tungtrongchitr A, Suriyaprom K, Chuengsamarn S, Schweigert FJ, et al. Vitamin D-binding protein and its polymorphisms as a predictor for metabolic syndrome. Biomark Med. 2018 Mar 5;12(5):465–73.

284.   Speeckaert MM, Taes YE, De Buyzere ML, Christophe AB, Kaufman J-M, Delanghe JR. Investigation of the potential association of vitamin D binding protein with lipoproteins. Ann Clin Biochem. 2010 Mar;47(Pt 2):143–50.

285.   Li J, Ni M, Lee B, Barron E, Hinton DR, Lee AS. The unfolded protein response regulator GRP78/BiP is required for endoplasmic reticulum integrity and stress-induced autophagy in mammalian cells. Cell Death Differ. 2008 Sep;15(9):1460–71.

286.   Gregor MF, Yang L, Fabbrini E, Mohammed BS, Eagon JC, Hotamisligil GS, et al. Endoplasmic Reticulum Stress Is Reduced in Tissues of Obese Subjects After Weight Loss. Diabetes. 2009 Mar 1;58(3):693–700.

287.   Arakaki TL, Pezza JA, Cronin MA, Hopkins CE, Zimmer DB, Tolan DR, et al. Structure of human brain fructose 1,6-(bis)phosphate aldolase: Linking isozyme structure with function. Protein Sci. 2004 Dec 1;13(12):3077–84.

288.   Bouwman FG, Claessens M, van Baak MA, Noben J-P, Wang P, Saris WHM, et al. The Physiologic Effects of Caloric Restriction Are Reflected in the in Vivo Adipocyte-Enriched Proteome of Overweight/Obese Subjects. J Proteome Res. 2009 Dec 4;8(12):5532–40.

APPENDIX

SUPPLEMENTARY DATA

Supplementary material 1: Global final version of the questionnaire used in the study and criteria for organization of data for statistical treatment [a].

Obesity among pet-owners and pets

Number	Question	Criteria [a]

1	Where did you hear about this study?
	   1 Through a researcher involved in the study
	   2 Through the veterinary clinic I go to with my pet
	   3 Through somebody else who went to a veterinary clinic
	   4 Through a dog activity or dog breeding organization
	   5 Through a colleague
	   6 Through a friend or family member
	   7 Through social media (e.g. Facebook, Twitter)
	   8 Other	

2	In what part of the country do you currently live?
	   1 Southeast
	   2 Northeast
	   3 South-west
	   4 Northwest
	   5 Central
	   6 Madeira
	   7 Azores
		1 North (2+4)
		 2 South (1+3)
		 3 Centre
		 4 Islands (6+7)

	Owner data Criteria: - If more than one person is taking care of the same pet, the form must be filled in by only one individual	
3	Age (years)	1 18-40 2 41-55 3 >55

4	Gender
	     1 Woman
	     2 Man	

5	Height (cm)	BMI index
6	Weight (kg)	
7	Number of family members living with pet | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |	1 =1 2 2 - 4 3 > 4

8	Educational level of person responding to questionnaire
	   1 Primary
	   2 Secondary/High School
	   3 Vocational training
	   4 University degree
	   5 Postgraduate qualifications
	   6 Other
		1 Primary
		 2 Secondary/ vocational
		 3 University/ Postgraduate
		 Other was eliminated

9	Employment
	   1 Student
	   2 Employed
	   3 Retired
	   4 Unemployed
		1 Student
		 2 Employed/ retired
		 3 Unemployed

10	Monthly Family income	
	1 =< 500 EUR 2 500-1000 EUR 3 1000-2000 EUR 4 > 2000 EUR	1 =<500 EUR 2 500-1000 EUR 3 >1000 EUR
11-17	**Attitude towards physical activity** Please respond to the following statements with a score of 1-5 where 1 = total disagreement and 5 = total agreement. Please tick only one box in each row; if it is difficult to choose only one answer, choose the option that reflects your opinion *most of the time* Doing sport makes me feel good	Questions 14 and 17 were eliminated. The responses for questions 11, 13 and 15 were summed. For question 11 the sum was made from 1 to 5 and for questions 13 and 15 the sum was made from 5 to 1. Then a score was made: 1 => 10 (positive attitude) 2 1-9 (negative attitude) Questions 12 and 14 were considered separately. Question 12: 1 No 2 Yes Question 14: 1 Disagree 2 Agree

11-17 (continued) Attitude towards physical activity

Doing sport makes me feel good

□1□2□3□4□5□□T 1	2	3	4	5
Total disagreement			Total agreement	

I like doing one or more of the following activities on a regular basis (at least once a week): walking, running, sports, etc.

□1□2□3□4□5□□T 1	2	3	4	5
Total disagreement			Total agreement	

I get bored doing sports activities

□1□2□3□4□5□□T 1	2	3	4	5
Total disagreement			Total agreement	

Physical activity relieves stress

□1□2□3□4□5□□T 1	2	3	4	5
Total disagreement			Total agreement	

I do not like physical activity because I feel very tired

□1□2□3□4□5□□T 1	2	3	4	5
Total disagreement			Total agreement	

I do not have the self-discipline necessary to do exercise

□1□2□3□4□5□□T 1	2	3	4	5
Total disagreement			Total agreement	

I like physical activity because it is beneficial to health

□1□2□3□4□5□□T 1	2	3	4	5
Total disagreement			Total agreement	

18	During the past 30 days (1 month) on how many days did you smoke? 1 Every day or almost every day 2 Some days 3 Not smoker	This question was not evaluated.
19	Do you suffer from any disease? 1 No 2 Yes	
20	Which disease(s) do you suffer from?	
21	Are you receiving chronic treatment (> 1 month duration)? 1 Yes 2 No	
22	What treatment(s) do you receive?	
23-32	**Attitude towards diet** Please respond to the following statements with a score of 1-5 where 1 = total disagreement and 5 = total agreement. Please tick only one box in each row; if it is difficult to choose only one answer, choose the option that reflects your opinion *most of the time* I believe eating a healthy diet is important for my overall health ☐1☐2☐3☐4☐5☐☐T 2 3 4 5 Total disagreement / Total agreement Home-made food is better than fast-food or ready-prepared food ☐1☐2☐3☐4☐5☐☐T 2 3 4 5 Total disagreement / Total agreement I feel better eating a healthy diet ☐1☐2☐3☐4☐5☐☐T 2 3 4 5 Total disagreement / Total agreement Healthy food is boring ☐1☐2☐3☐4☐5☐☐T 2 3 4 5 Total disagreement / Total agreement Healthy meals are laborious (more difficult to shop for, prepare, transport, etc.) ☐1☐2☐3☐4☐5☐☐T 2 3 4 5 Total disagreement / Total agreement It is normal to skip breakfast ☐1☐2☐3☐4☐5☐☐T 2 3 4 5 Total disagreement / Total agreement	Questions 23 to 27, 29 and 31 were not evaluated. Question 28: 1 Disagree (1+2) 2 Agree (3+4+5)

	How food tastes is more important than its health benefit 	☐1☐2☐3☐4☐5☐☐T	2	3	4	5
---	---	---	---	---	---
1				
Total disagreement|	|Total agreement| | I do not have the self-discipline to follow a healthy diet | ☐1☐2☐3☐4☐5☐☐T |2|3|4|5
---	---	---	---	---	---
1				
Total disagreement|	|Total agreement| | A healthy diet includes a variety of foods (cereals, vegetables, meat, etc.) | ☐1☐2☐3☐4☐5☐☐T |2|3|4|5
---	---	---	---	---	---
1				
Total disagreement|	|Total agreement| | Do you consider important eating healthy food rather than feeling rapidly satiated? 1 I try to eat healthy food even it takes more time preparing it. 2 I eat everything what I have to eliminate hunger.	Question 30: 1 Disagree (1+2) 2 Agree (3+4+5)
	Pet data Criteria: - If you have more than one pet, you must apply the questionnaire to just one animal.	
33	Breed (Please chose one of the list)	The owners were provided by the list of dog breeds and the option "other" was also available.
34	Age (years)	1 - ≤ 1 2 - 1 a 7 3 - > 7

35	Sex
	                  1 Female
	                  2 Male	

36	Reproductive status
	    1 Intact
	    2 Neutered at <6 months
	    3 Neutered at 6-11 months
	    4 Neutered at 1 year
	    5 Neutered at 1-2 years
	    6 Neutered at 2-8 years
	    7 Neutered at >8 years
	    8 Not sure
		1 Intact
		2 Neutered

37	Weight (kg)	
38	On the basis of the images below: what score corresponds to your pet? 	☐1☐2☐3☐	2	3	4	5
---	---	---	---	---
1				
Very thin			Obese			The BCS chart was included to help owners to score dogs body condition: 1 < 3 (1+2) 2 =3 3 > 3 (4+5)

39	Given the image below: what body fat index corresponds to your pet?	The Hill´s body fat index (BFI) risk chart, was included to help owners to assess his/her dogs BFI: 1 - 20% 2 - 30 % 3 - > 30%
	20% | 30% | 40% | 50% | 60% | 70%	

40	Household
	     1 Rural zone; the dog spends almost the whole day outside
	     2 Rural zone; the dog spends almost the whole day in the house
	     3 House/apartment without access to garden
	     4 House/apartment with access to garden
	     5 Other
		1 House with backyard
		 2 House without backyard
		 Other was eliminated

41	Diet
	     1 Home-made food
	     2 Food scraps
	     3 Commercial pet food
	     4 Mixed
	     5 Other
		1 Home-made/ Food scraps
		 2 Commercial
		 3 Mixed/Other

42	Number of meals per day
	     1 1
	     2 2
	     3 3
	     4 > 3
		1 =1
		 2 =2
		 3 >2

43	How do you calculate the amount of food for your pet? 1 Following recommendations provided by a vet 2 According to specifications on food packaging 3 Dog fed until it stops eating 4 A constant food supply is made available 5 Other	1 By a vet 2 Commercial 3 Until it stops/*ad libitum* Other was eliminated

44	Feeding schedule
	     1 Fixed (at the same time every day)
	     2 Random
	     3 Other
		1 Fixed
		 2 Random/ other

45	Do you give your pet food supplements?
	     1 Yes
	     2 No	

46	What type of supplement (vitamins, fatty acids, etc.)	

47	Do you give your pet food rewards?
	     1 Yes
	     2 No	

48	Physical activity
	     1 None
	     2 Walking
	     3 Running	

49	Daily exercise (time) 　　　　　1 None 　　　　　2 < 30 min 　　　　　3 30 min - 1 hour 　　　　　4 > 1 hour 　　　　　5 Other:	1 - None 2 - ≤30 m 3 - > 30 m
50	Does your pet suffer from any disease(s)? 　　1　No 　　2　Yes	
51	What disease?	
52	What treatment(s) does your pet receive?	
53	How many times have you had to take your pet to the vet due to health problems during the last year?	1 =1 2 1 a 2 3 > 2
54	My pet gets sick easily? <table><tr><td>☐1☐2☐3☐4☐5☐☐T 1</td><td>2</td><td>3</td><td>4</td><td>5</td></tr><tr><td>Total disagreement</td><td></td><td></td><td>Total agreement</td><td></td></tr></table>	1 Disagree (1+2) 2 Agree (3+4+5)
55	Do you believe your pet is happy? <table><tr><td>☐1☐2☐3☐4☐5☐☐N 1</td><td>2</td><td>3</td><td>4</td><td>5</td></tr><tr><td>Not happy</td><td></td><td></td><td>Happy</td><td></td></tr></table>	1 Disagree (1+2+3) 2 Agree (4+5)
	Owner-pet relationship	
56	Who is responsible for caring for the animal? 　　1　The person filling out this questionnaire 　　2　Another member of the household 　　3　Several people 　　4　Other	1… 2… 3 Several/ other
57	How many other animals live with the pet? 　　1　None 　　2　1 　　3　2 　　4　3 　　5　4 　　6　5 　　7　> 5	1 0 2 1 a 2 3 >2
58	How long do you spend with your pet each day? You should indicate the active time. Excluding the hours of sleep. 　　1　< 2 hours 　　2　2 - 4 hours 　　3　4 - 6 hours 　　4　6 - 8 hours 　　5　> 8 hours	1 < 2 2 2 a 6 3 > 6
59	Do you share food with your pet while eating? <table><tr><td>☐1☐2 1</td><td>2</td><td>3</td><td>4</td><td>5</td></tr><tr><td>Never</td><td></td><td></td><td>Always</td><td></td></tr></table>	1 None (1+2) 2 Sometimes 3 Always (4+5)
60	Are you with your animal while he/she is eating? <table><tr><td>☐1☐2 1</td><td>2</td><td>3</td><td>4</td><td>5</td></tr><tr><td>Never</td><td></td><td></td><td>Always</td><td></td></tr></table>	1 None (1+2) 2 Sometimes 3 Always (4+5)

61	Do you think that giving treats or gifts to your dog makes him happier? □1□2 | 1 | 2 | 3 | 4 | 5 Never | | | | Always	1 No (1+2) 2 Yes (3+4+5)
62	Do you usually sleep with your pet? □1□2 | 1 | 2 | 3 | 4 | 5 Never | | | | Always	1 No (1+2) 2 Yes (3+4+5)

63	Do you consider your pet a member of the family?
	     1 Yes
	     2 No	

64	Does taking care of a pet have an influence on your physical activity?
	     1 Yes, I do more exercise
	     2 Yes, I do less exercise
	     3 It does not influence the amount of physical activity I do
		1 Yes (1+2)
		 2 No (3)

65	Do you regularly exercise with your pet?
	     1 No
	     2 Always
	     3 Sometimes
	     4 Occasionally
	     5 Hardly ever
		1 No/ occasionally/ hardly ever
		 2 Always
		 3 Sometimes

66	What benefits do you attribute to having a pet?	
67	What (if any) problems do your pet cause?	
	Obesity background	

68	Do you consider obesity in people a disease?
	     1 Yes
	     2 No
	     3 Other
		Other was eliminated

69	Do you consider obesity in animals a disease?
	     1 Yes
	     2 No
	     3 Other
		Other was eliminated

70	Obesity is increasing in society (among both people and pets). What do you think are the three main reasons?
	     1….
	     2….
	     3….	

71	What would you recommend to stop this increase?	
72	Do you think that collaboration between healthcare services and vets could be important for combating obesity? Why? 1… 2… 3…	
	Satisfaction	
73	Difficulty of the survey □1□2 | 1 | 2 | 3 | 4 | 5 Easy | | | | Tricky	

74	Length							
		☐1☐2 1	2	3	4	5		
		Short				Long		
75	Have you found the questionnaire interesting?							
		☐1☐2 1	2	3	4	5		
		No				Yes		
	Thank you very much for your help!							

Supplementary material 2: Descriptive data obtained from questionnaires.

Owner Data

Man/Woman	52 (16,7%)/259 (83,3%)
Age, years	18 - 72 (n=312)
	18-40 (234/75.7%), 41-55 (57/18.4%), >55 (19/6.1%)

BMI

o Underweight (BMI=<18,5)	10 (3.2%)
o Normal weght (18,5<BMI<25)	233 (74.9%)
o Overweight (25=>BMI<30)	49 (15.7%)
o Obese (BMI=>30)	19 (6.1%)

Number of family members living with pet	2-3 (1 - 8)
1	23 (7.3%)
2	91 (29.1%)
3	87 (27.8%)
4	82 (26.2%)
5	26 (8.3%)
6	1 (0.3%)
7	2 (0.6%)
8	1 (0.3%)
9	0 (0.0%)
10	0 (0.0%)

Educational level of person responding to questionnaire

o Primary	3 (0,7%)
o Secondary/High School	54 (17.3%)
o Vocational training	2 (0,7%)
o University degree	192 (61.5%)
o Postgraduate qualifications	63 (21,1%)
o Other:	0 (0%)

Employment

o Student	108 (34,6%)
o Employed	178 (57,1%)
o Retired	8 (2,6%)
o Unemployed	18 (5,8%)
o Other:	0

Monthly Family income

o =< 500	83 (27.0%)
o 500-1000	111 (35.8%)
o 1000-2000	77 (25,1%)
o >2000	35 (12.1%)

During the past 30 days (1 month) on how many days did you smoke?

o Every day or almost every day	53 (17%)
o Some days	26 (8,3%)
o Not smoker	233 (74.7%)

Attitude towards physical activity

Doing sport makes me feel good

1 Total disagreement	4 (1.3%9
2	14 (4.5%9
3	46 (14.7%)
4	89 (28.5%)
5 Total agreement	159 (51%)

I like doing one or more of the following activities on a regular basis (at least once a week): walking, running, sports, etc

1 Total disagreement	9 (2.9%)
2	24 (7.7%)
3	83 (26.6%)
4	91 (29.2%)

5 Total agreement	105 (33.7%)

I get bored doing sports activities

1 Total disagreement	105 (33.8%)
2	71 (22.8%)
3	84 (27%)
4	41 (13.2%)
5 Total agreement	10 (3.2%)

Physical activity relieves stress

1 Total disagreement	2 (0.6%)
2	7 (2.2%)
3	36 (11.5%)
4	82 (26.3)
5 Total agreement	185 (59.3)

I do not like physical activity because I feel very tired

1 Total disagreement	120 (38.6%)
2	94 (30.2%)
3	64 (20.6%)
4	27 (8.7%)
5 Total agreement	6 (1.9%)

I do not have the self-discipline necessary to do exercise

1 Total disagreement	73 (23.5%)
2	55 (17.7%)
3	84 (27%)
4	56 (18%)
5 Total agreement	43 (13.8%)

I like physical activity because it is beneficial to health

1 Total disagreement	6 (1.9%)
2	8 (2.6%)
3	49 (15.7%)
4	108 (34.6)
5 Total agreement	141 (45.2%)

Disease, Yes/No	66 (21.1%)/ 246 (78.8%)
Treatment, Yes/No	70 (22.3%)/ 243 (77.7%)

Attitude towards diet

I believe eating a healthy diet is important for my overall health

1 Total disagreement	0 (0%)
2	1 (0.3)
3	9 (2.9%)
4	70 (22.4%)
5 Total agreement	233 (74.4%)

Home-made food is better than fast-food or ready-prepared food

1 Total disagreement	0 (0%)
2	1 (0.3%)
3	7 (2.2%)
4	27 (8.6%)
5 Total agreement	278 (88.8%)

I feel better eating a healthy diet

1 Total disagreement	0 (0%)
2	6 (1.9%)
3	20 (6.4%)
4	63 (20.1%)
5 Total agreement	224 (71.6%)

Healthy food is boring

1 Total disagreement	148 (47.3%)
2	87 (27.8%)
3	52 (16.6%)
4	23 (7.3%)

5 Total agreement	3 (1%)

Healthy meals are laborious (more difficult to shop for, prepare, transport, etc.)

1 Total disagreement	75 (24%)
2	67 (21.4%)
3	82 (26.2%)
4	74 (23.6%)
5 Total agreement	15 (4.8%)

It is normal to skip breakfast

1 Total disagreement	209 (66.8%)
2	34 (10.9%)
3	28 (8.9%)
4	20 (6.4%)
5 Total agreement	22 (7%)

How food tastes is more important than its health benefit

1 Total disagreement	52 (16.6%)
2	106 (33.9%)
3	122 (39%)
4	30 (9.6%)
5 Total agreement	3 (1%)

I do not have the self-discipline to follow a healthy diet

1 Total disagreement	97 (31%)
2	98 (31.3%)
3	72 (23%)
4	33 (10.5%)
5 Total agreement	13 (4.2%)

A healthy diet includes a variety of foods (cereals, vegetables, meat, etc.)

1 Total disagreement	2 (0.6%)
2	6 (1.9%)
3	16 (5.1%)
4	73 (23.3%)
5 Total agreement	216 (69%)

Do you consider important eating healthy food rather than feeling rapidly satiated?

1 I try to eat healthy food even it takes more time preparing it.	247 (79.7%)
2 I eat everything what I have to eliminate hunger.	63 (20.3%)

Dog Data

Age, years	1 - 22 (n=312)
	< 1 (49, 15.8%), 1-7 (169,54.5%), >7 (92, 29.7%)
Sex, Female/Male	153 (49,4%)/ 157 (50,6%)
Breed (5 breeds most repeated)	Labrador Retriever (n=32), German shepherd (n=10), Golden Retriever (n=8), Jack Russell Terrier (n=8), Yorkshire terrier (n=7)

Reproductive status:

o Intact	161 (52,0%)
o Neutered at <6 months	23 (7,4%)
o Neutered at 6-11 months	38 (12,2%)
o Neutered at 1 year	13 (4,2%)
o Neutered at 1-2 years	17 (5,4%)
o Neutered at 2-8 years	39 (12,5%)
o Neutered at >8 years	15 (4,8%)
o Not sure	6 (2,0%)

On the basis of the images below: what score corresponds to your pet? BCS

o	1	2 (0.6%)
o	2	27 (8.7%)
o	3	186 (59.6%)
o	4	81 (26.1%)
o	5	15 (4.8%)

Given the image below: what body fat index corresponds to your pet? BFI

o	20%	130 (41.8%)
o	30%	104 (33.4%)
o	40%	44 (14.1%)
o	50%	24 (7.7%)
o	60%	9 (2.9%)
o	70%	0 (0%)

Household

1	Rural zone; the dog spends almost the whole day outside	10 (32.6%)
2	Rural zone; the dog spends almost the whole day in the house	30 (6.8%)
3	House/apartment without access to garden	80 (25.8%)
4	House/apartment with access to garden	114 (35.2%)
5	Other	0 (0.0%)

Diet

1	Home-made food	6 (1.9%)
2	Food scraps	0 (0%)
3	Commercial pet food	223 (71.5%)
4	Mixed	78 (25.0%)
5	Other	5 (1.6%)

Number of meals per day

1	44 (14.1%)
2	194 (62.4%)
3	36 (11.5%)
>3	17 (5.1%)
Other	20 (6.4%)

How do you calculate the amount of food for your pet?

1 Following recommendations provided by a vet	70 (22.4%)
2 According to specifications on food packaging	148 (47.4%)
3 Dog fed until it stops eating	13 (4.7%)
4 A constant food supply is made available	63 (20.9%)
5 Other	18 (5.8%)

Feeding schedule

1	Fixed (at the same time every day)	223 (71.7%)
2	Random	82 (26.4%)
3	Other	6 (1.9%)

Do you give your pet food supplements?

1 Yes	43 (13.8%)
2 No	268 (86.2%)

Do you give your pet food rewards?

1 Yes	160 (51.4%)
2 No	151 (48.6%)

Physical activity

1 None	34 (11%)
2 Walking	180 (58.1%)
3 Running	96 (31%)

Daily exercise (time)

1 None	56 (18.0%)
2 < 30 min	87 (28.0%)
3 30 min - 1 hour	93 (29.9%)
4 > 1 hour	45 (14.5%)
5 Other:	30 (9.6%)

Does your pet suffer from any disease(s)?

1 No	53 (17%)
2 Yes	258 (83%)

Visits to vet during last year, because of health problems   0-48

0	21 (7.0%)
1	91 (30.5%)
2	65 (21.8%)
>2	121 (40.6%)

My pet gets sick easily?

1 Total disagreement	186 (62.6%)
2	82 (27.6%)
3	18 (6.1%)
4	11 (3.7%)
5 Total agreement	13 (4.4%)

Do you believe your pet is happy?

1 Total disagreement	1 (0.3%)
2	2 (0.6%)
3	30 (9.7%)
4	98 (31.6%)
5 Total agreement	179 (57.7%)

Who is responsible for caring for the animal?

1	The person filling out this questionnaire	148 (47.4%)
2	Another member of the household	44 (14.1%)
3	Several people	116 (37.2%)
4	Other	3 (1%)

How many other animals live with the pet?

1	None	101 (32.4%)
2	1	91 (29.3%)
3	2	47 (15.1%)
4	3	24 (7.7%)
5	4	13 (4.1%)
6	5	12 3.9%)
7	> 5	23 (7.4%)

How long do you spend with your pet each day?
You should indicate the active time. Excluding the hours of sleep.

1	< 2 hours	48 (15.4%)
2	2 - 4 hours	80 (25.7%)
3	4 - 6 hours	76 (24.4%)
4	6 - 8 hours	49 (15.8%)
5	> 8 hours	58 (18.6%)

Do you share food with your pet while eating?

1 Never	158 (50.8%)
2	83 (26.7%)
3	47 (15.1%)
4	17 (5.5%)
5 Always	6 (1.9%)

Are you with your animal while he/she is eating?

1 Never	69 (21.9%)
2	69 (22.2%)
3	104 (33.8%)
4	38 (12.2%)
5 Always	32 (10.0%)

Do you think that giving treats or gifts to your dog makes him happier?

1 Never	39 (12.5%)
2	37 (11.9%)
3	104 (33.4%)
4	83 (26.7%)
5 Always	49 (15.4%)

Do you usually sleep with your pet?

1 Never	162 (52.1%)
2	27 (8.7%)
3	37 (11.9%)
4	24 (7.7%)
5 Always	61 (19.6%)

Do you consider your pet a member of the family?

1 Yes	300 (96.5%)
2 No	11 (3.5%)

Does taking care of a pet have an influence on your physical activity?

1 Yes, I do more exercise	149 (47.6%)
2 Yes, I do less exercise	2 (0.6%)
3 It does not influence the amount of physical activity I do	162 (51.8%)

Do you regularly exercise with your pet?

1 No	84 (26.7%)
2 Always	37 (11.9%)
3 Sometimes	115 (37.0%)
4 Occasionally	59 (19.0%)
5 Hardly ever	17 (5.5%)

Do you consider obesity in people a disease?

1 Yes	302 (97.1%)
2 No	10 (2.9%)
3 Other	0

Do you consider obesity in animals a disease?

1 Yes	304 (97.7%)
2 No	7 (2.3%)
3 Other	0
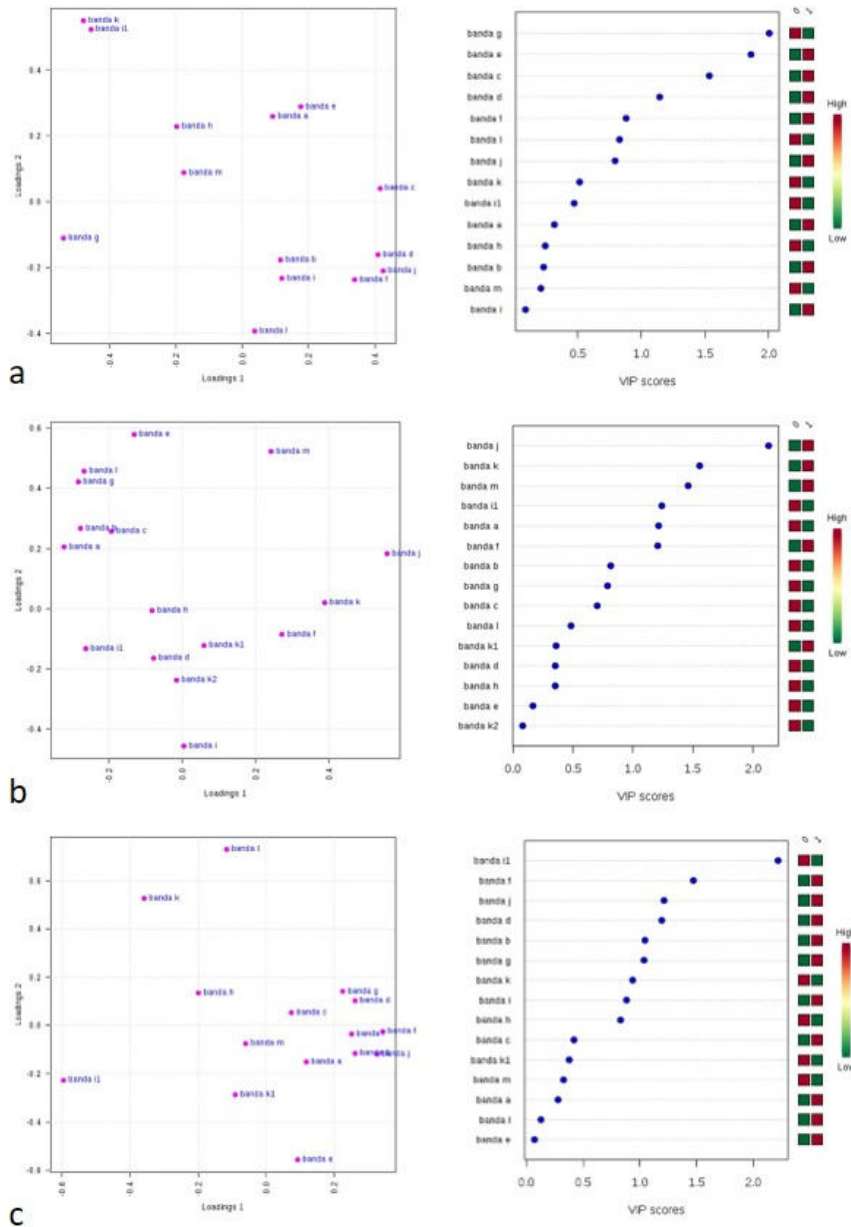
Supplementary Figure 1: PLS-DA loading plots (left) of the first two components for analysis of SDS-PAGE bands of profiles from saliva collected with and without acid stimulation in Beagles (a), Greyhound (b), and Portuguese Podengo (c); for each case, variable importance in the projection (VIP) is presented, with 1.5 score considered as thresholder (right). 0 – with acid stimulation; 1 – without acid stimulation.
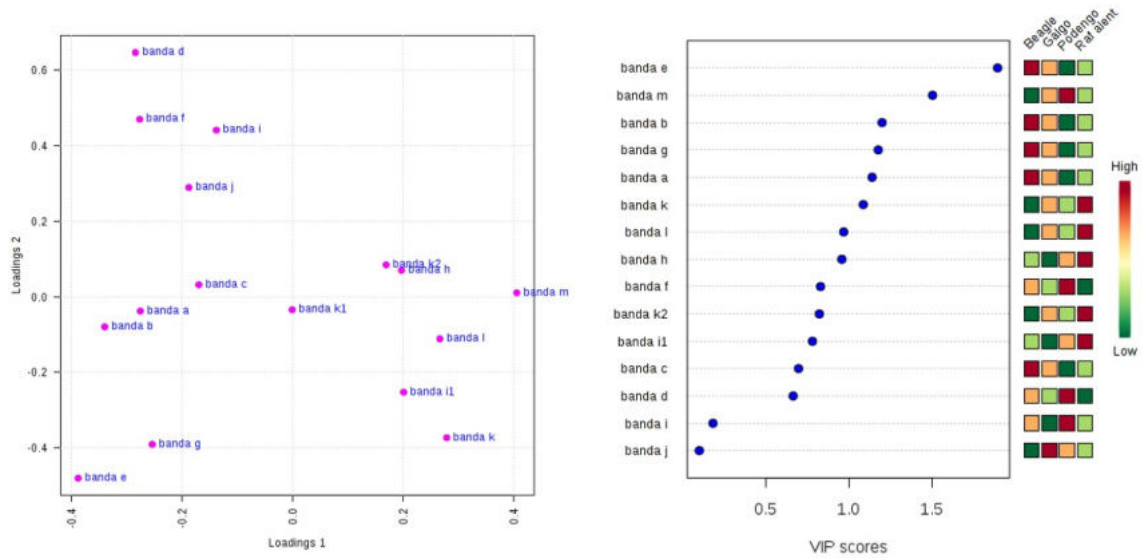
Supplementary Figure 2: PLS-DA loading plots (left) of the first two components for analysis of protein bands of salivary profiles from the different dog breeds. Variable importance in the projection (VIP) is presented (right).
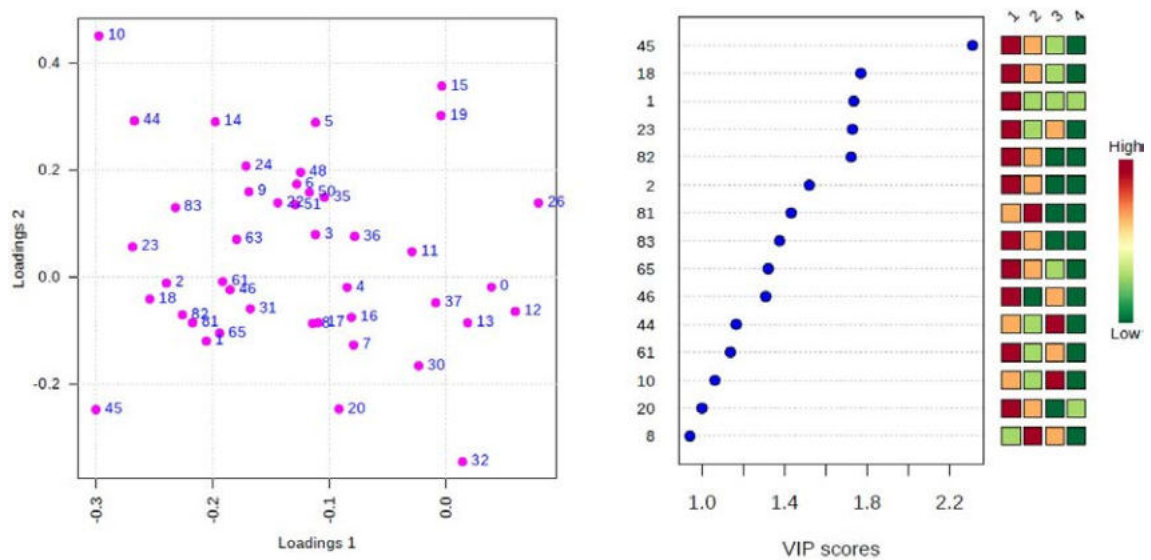
Supplementary Figure 3: PLS-DA loading plots (left) of the first two components for analysis of protein spots of salivary profiles from the different dog breeds. Variable importance in the projection (VIP) is presented (right). 1 – Portuguese Podengo; 2- Greyhound; 3- Rafeiro Alentejano; 4- Beagle.

Supplementary Table 1 - List of the proteins identified, by LC-MS/MS in obese dog saliva

N	Accession	Name	Species
1	sp|P49822|ALBU_CANLF	Serum albumin	CANLF
2	tr|F1P6A9|F1P6A9_CANLF	Uncharacterized protein	CANLF
3	tr|E2RH46|E2RH46_CANLF	Uncharacterized protein	CANLF
4	sp|I00001|MALE_GFP	IS	GFP
5	tr|J9JHJ8|J9JHJ8_CANLF	Mucin 5B, oligomeric mucus/gel-forming	CANLF
6	tr|F6USN4|F6USN4_CANLF	Uncharacterized protein	CANLF
7	tr|J9P9J6|J9P9J6_CANLF	Uncharacterized protein	CANLF
8	tr|J9NXL3|J9NXL3_CANLF	Uncharacterized protein	CANLF
9	tr|F1PR54|F1PR54_CANLF	Uncharacterized protein	CANLF
10	tr|E2QXJ0|E2QXJ0_CANLF	BPI fold containing family A member 2	CANLF
11	tr|J9P430|J9P430_CANLF	Transferrin	CANLF
12	tr|F6Y6T8|F6Y6T8_CANLF	Uncharacterized protein	CANLF
13	tr|E2R2B9|E2R2B9_CANLF	BPI fold containing family B member 2	CANLF
14	sp|O18874|ALL2_CANLF	Minor allergen Can f 2	CANLF
15	tr|F1PCG9|F1PCG9_CANLF	Carbonic anhydrase 6	CANLF
16	tr|E2QWJ3|E2QWJ3_CANLF	BPI fold containing family B member 1	CANLF
17	tr|F1PIX8|F1PIX8_CANLF	Complement C3	CANLF
18	tr|E2QWN7|E2QWN7_CANLF	Lymphocyte cytosolic protein 1	CANLF
19	sp|P60524|HBB_CANLF	Hemoglobin subunit beta	CANLF
20	tr|F1P931|F1P931_CANLF	Uncharacterized protein	CANLF
21	tr|F1PR78|F1PR78_CANLF	Uncharacterized protein	CANLF
22	tr|E2RFI9|E2RFI9_CANLF	Lactoperoxidase	CANLF
23	tr|F1PBS8|F1PBS8_CANLF	Lipocalin 1	CANLF
24	tr|F1PDJ5|F1PDJ5_CANLF	Apolipoprotein A-I	CANLF
25	tr|F1Q3K7|F1Q3K7_CANLF	Uncharacterized protein	CANLF
26	tr|F1PE28|F1PE28_CANLF	Transketolase	CANLF
27	tr|J9NXE2|J9NXE2_CANLF	Actin, cytoplasmic 1 Actin, cytoplasmic 1, N-terminally processed	CANLF
28	tr|E2QWD8|E2QWD8_CANLF	Uncharacterized protein	CANLF
29	tr|L7N0F2|L7N0F2_CANLF	Uncharacterized protein	CANLF
30	tr|J9P0R6|J9P0R6_CANLF	Myeloperoxidase	CANLF
31	tr|G1K265|G1K265_CANLF	Lysozyme	CANLF
32	tr|J9NSZ4|J9NSZ4_CANLF	Uncharacterized protein	CANLF
33	tr|G1K2D9|G1K2D9_CANLF	Haptoglobin	CANLF
34	tr|E2RCC8|E2RCC8_CANLF	Immunoglobulin heavy constant mu	CANLF
35	tr|J9PAQ5|J9PAQ5_CANLF	Protein S100	CANLF
36	tr|L7N071|L7N071_CANLF	Actinin alpha 4	CANLF
37	tr|E2R0H6|E2R0H6_CANLF	Prolactin induced protein	CANLF
38	tr|J9P950|J9P950_CANLF	Uncharacterized protein	CANLF
39	tr|F1PTY1|F1PTY1_CANLF	Keratin, type II cytoskeletal 1	CANLF
40	tr|F1PRV8|F1PRV8_CANLF	Deleted in malignant brain tumors 1	CANLF
41	tr|E2R0T6|E2R0T6_CANLF	Uncharacterized protein	CANLF
42	tr|F1PCH3|F1PCH3_CANLF	Enolase 1	CANLF
43	tr|E2R2C3|E2R2C3_CANLF	Glucose-6-phosphate isomerase	CANLF
44	tr|F1Q2Y5|F1Q2Y5_CANLF	Zymogen granule protein 16B	CANLF
45	tr|J9P7B6|J9P7B6_CANLF	Uncharacterized protein	CANLF
46	tr|F1PBZ4|F1PBZ4_CANLF	NAD(P)H quinone dehydrogenase 1	CANLF
47	tr|F1PYU9|F1PYU9_CANLF	Keratin, type I cytoskeletal 10	CANLF
48	tr|Q8MJD1|Q8MJD1_CANLF	Elastase, neutrophil expressed	CANLF
49	tr|A0A0A0MPD0|A0A0A0MPD0_CANLF	Triosephosphate isomerase	CANLF
50	tr|E2R0A4|E2R0A4_CANLF	Secretoglobin family 1A member 1	CANLF
51	tr|F1PZR4|F1PZR4_CANLF	Hemopexin	CANLF
52	tr|F1PVW0|F1PVW0_CANLF	L-lactate dehydrogenase	CANLF
53	tr|F1P841|F1P841_CANLF	GC, vitamin D binding protein	CANLF
54	tr|F1PQ93|F1PQ93_CANLF	Stratifin	CANLF
55	tr|F1Q0B9|F1Q0B9_CANLF	Uncharacterized protein	CANLF
56	tr|J9NV93|J9NV93_CANLF	Peptidyl-prolyl cis-trans isomerase	CANLF
57	tr|E2R413|E2R413_CANLF	Capping actin protein, gelsolin like	CANLF
58	tr|F1PK60|F1PK60_CANLF	Transglutaminase 3	CANLF
59	tr|J9JHH5|J9JHH5_CANLF	Joining chain of multimeric IgA and IgM	CANLF
60	tr|F6Y3P9|F6Y3P9_CANLF	Gelsolin	CANLF
61	tr|F1PE09|F1PE09_CANLF	6-phosphogluconate dehydrogenase, decarboxylating	CANLF
62	tr|J9P6A9|J9P6A9_CANLF	Desmocollin 2	CANLF
63	tr|F1PYF5|F1PYF5_CANLF	Matrix metalloproteinase	CANLF
64	tr|E2R002|E2R002_CANLF	Prostaglandin reductase 1	CANLF
65	tr|E2QXE7|E2QXE7_CANLF	BPI fold containing family A member 1	CANLF
66	tr|F1PAX2|F1PAX2_CANLF	Ceruloplasmin	CANLF
67	tr|E2R8Z5|E2R8Z5_CANLF	Keratin 5	CANLF
68	tr|J9P2K8|J9P2K8_CANLF	Mucin 7, secreted	CANLF
69	tr|E2RT65|E2RT65_CANLF	Phosphoglycerate mutase	CANLF
70	tr|J9P839|J9P839_CANLF	Uncharacterized protein	CANLF
71	tr|L7N0D9|L7N0D9_CANLF	Histone H2A	CANLF
72	tr|E2RSI6|E2RSI6_CANLF	Ezrin	CANLF
73	tr|J9P7D5|J9P7D5_CANLF	Glutathione S-transferase mu 4	CANLF
74	tr|F1PQN5|F1PQN5_CANLF	Cofilin 1	CANLF
75	tr|E2RAL0|E2RAL0_CANLF	Rho GDP dissociation inhibitor beta	CANLF
76	tr|F1PIC7|F1PIC7_CANLF	Uncharacterized protein	CANLF
77	tr|A0A077S9R2|A0A077S9R2_CANLF	Lysozyme	CANLF
78	tr|F1PB77|F1PB77_CANLF	Alpha-1,4 glucan phosphorylase	CANLF

79	tr|J9NS47|J9NS47_CANLF	Histone H2B	CANLF
80	tr|F1PHR2|F1PHR2_CANLF	Pyruvate kinase	CANLF
81	tr|E2RD02|E2RD02_CANLF	Chromosome 6 open reading frame 58	CANLF
82	tr|J9PBN6|J9PBN6_CANLF	Uncharacterized protein	CANLF
83	tr|F1P9J3|F1P9J3_CANLF	Myosin-9	CANLF
84	tr|F1PSX9|F1PSX9_CANLF	Hyaluronidase	CANLF
85	tr|F1P8G0|F1P8G0_CANLF	Fibrinogen gamma chain	CANLF
86	tr|J9NSS7|J9NSS7_CANLF	Leucine rich alpha-2-glycoprotein 1	CANLF
87	tr|F1PGY1|F1PGY1_CANLF	Heat shock protein 90 alpha family class A member 1	CANLF
88	tr|F1PQM1|F1PQM1_CANLF	Purine nucleoside phosphorylase	CANLF
89	tr|J9P9E9|J9P9E9_CANLF	Glucose-6-phosphate 1-dehydrogenase	CANLF
90	tr|E2QZ50|E2QZ50_CANLF	Adenylyl cyclase-associated protein	CANLF
91	tr|J9NY79|J9NY79_CANLF	Malate dehydrogenase	CANLF
92	tr|J9P4H9|J9P4H9_CANLF	Keratin 13	CANLF
93	tr|E2R9B6|E2R9B6_CANLF	Fetuin B	CANLF
94	sp|Q8WNN6|SODC_CANLF	Superoxide dismutase [Cu-Zn]	CANLF
95	tr|F1Q3Y0|F1Q3Y0_CANLF	Profilin	CANLF
96	tr|Q95N05|Q95N05_CANLF	Carboxylic ester hydrolase	CANLF
97	tr|F1PB68|F1PB68_CANLF	Olfactomedin 4	CANLF
98	tr|E2RPK8|E2RPK8_CANLF	Phosphatidylethanolamine binding protein 4	CANLF
99	tr|E2RHG2|E2RHG2_CANLF	Uncharacterized protein	CANLF
100	tr|F1PBL1|F1PBL1_CANLF	Uncharacterized protein	CANLF
101	tr|F1PBL4|F1PBL4_CANLF	Fibrinogen alpha chain	CANLF
102	tr|F1PDJ7|F1PDJ7_CANLF	Uncharacterized protein	CANLF
103	tr|F1P6P2|F1P6P2_CANLF	Transgelin	CANLF
104	tr|J9PAL7|J9PAL7_CANLF	Alpha-amylase	CANLF
105	tr|F1Q421|F1Q421_CANLF	Plasminogen	CANLF
106	tr|E2RLF1|E2RLF1_CANLF	Prosaposin	CANLF
107	tr|F1PBT3|F1PBT3_CANLF	Fructose-bisphosphate aldolase	CANLF
108	tr|F6UYJ9|F6UYJ9_CANLF	Calreticulin	CANLF
109	tr|E2RB38|E2RB38_CANLF	Tropomyosin 3	CANLF
110	tr|J9NVC6|J9NVC6_CANLF	Immunoglobulin heavy constant mu	CANLF
111	tr|E2R735|E2R735_CANLF	Amine oxidase	CANLF
112	tr|F6V234|F6V234_CANLF	Plastin 3	CANLF
113	tr|J9NVM0|J9NVM0_CANLF	L-lactate dehydrogenase	CANLF
114	tr|F1PW60|F1PW60_CANLF	Desmoglein-1	CANLF
115	tr|F1PK62|F1PK62_CANLF	Peptidyl-prolyl cis-trans isomerase	CANLF
116	tr|F2Z4Q6|F2Z4Q6_CANLF	Serum albumin	CANLF
117	tr|E2RFK4|E2RFK4_CANLF	CD177 molecule	CANLF
118	tr|L7N0L3|L7N0L3_CANLF	Histone H4	CANLF
119	tr|F1PZC6|F1PZC6_CANLF	Histidine rich glycoprotein	CANLF
120	tr|J9P4E8|J9P4E8_CANLF	Uncharacterized protein	CANLF
121	tr|E2R0V4|E2R0V4_CANLF	Caspase 14	CANLF
122	tr|F1PTZ9|F1PTZ9_CANLF	Glyceraldehyde-3-phosphate dehydrogenase	CANLF
123	tr|F1PLS4|F1PLS4_CANLF	Uncharacterized protein	CANLF
124	tr|F1PAL5|F1PAL5_CANLF	Angiotensinogen	CANLF
125	tr|F1PW65|F1PW65_CANLF	Fibrinogen beta chain	CANLF
126	tr|J9PAK3|J9PAK3_CANLF	ARP3 actin related protein 3 homolog	CANLF
127	tr|Q6TN20|Q6TN20_CANLF	Cathelicidin	CANLF
128	tr|E2QXS7|E2QXS7_CANLF	Adenosylhomocysteinase	CANLF
129	tr|E2QY08|E2QY08_CANLF	Actinin alpha 1	CANLF
130	tr|F1PC59|F1PC59_CANLF	Peroxiredoxin 6	CANLF
131	tr|E2QUU4|E2QUU4_CANLF	Keratin 4	CANLF
132	tr|F1PSF3|F1PSF3_CANLF	Cysteine rich secretory protein 2	CANLF
133	tr|F1PQ97|F1PQ97_CANLF	Alpha-2-macroglobulin like 1	CANLF
134	tr|F1PYE3|F1PYE3_CANLF	Heat shock protein 27 kDa beta-1	CANLF
135	tr|E2QYU2|E2QYU2_CANLF	Clusterin	CANLF
136	tr|F1PFI3|F1PFI3_CANLF	Phospholipase C beta 3	CANLF
137	tr|F1PL97|F1PL97_CANLF	Protein disulfide-isomerase	CANLF
138	tr|J9NWJ5|J9NWJ5_CANLF	Thioredoxin	CANLF
139	tr|J9P732|J9P732_CANLF	Uncharacterized protein	CANLF
140	tr|F1PCK2|F1PCK2_CANLF	Alpha-1-B glycoprotein	CANLF
141	tr|E2R5P5|E2R5P5_CANLF	Protein S100	CANLF
142	sp|Q6TEQ7|ANXA2_CANLF	Annexin A2	CANLF
143	tr|F1PWR2|F1PWR2_CANLF	Uncharacterized protein	CANLF
144	tr|J9NTL7|J9NTL7_CANLF	Uncharacterized protein	CANLF
145	tr|F6XF05|F6XF05_CANLF	Peptidyl arginine deiminase 4	CANLF
146	tr|E2R8C2|E2R8C2_CANLF	Hexokinase 3	CANLF
147	tr|E2RLS3|E2RLS3_CANLF	Heat shock protein 90 alpha family class B member 1	CANLF
148	tr|E2QVU9|E2QVU9_CANLF	Biliverdin reductase B	CANLF
149	tr|C0LQL0|C0LQL0_CANLF	Protein S100	CANLF
150	tr|F1P6B7|F1P6B7_CANLF	Annexin	CANLF
151	tr|F1PL93|F1PL93_CANLF	Rho GDP dissociation inhibitor alpha	CANLF
152	tr|G1K268|G1K268_CANLF	Uncharacterized protein	CANLF
153	tr|F1PUM3|F1PUM3_CANLF	Glutathione S-transferase omega 1	CANLF
154	tr|F6XRY2|F6XRY2_CANLF	Uncharacterized protein	CANLF
155	tr|F1PAQ3|F1PAQ3_CANLF	Maltase-glucoamylase	CANLF
156	tr|F1PVR0|F1PVR0_CANLF	Matrix metalloproteinase	CANLF
157	tr|E2QUV3|E2QUV3_CANLF	Alpha 2-HS glycoprotein	CANLF
158	tr|J9P4Y2|J9P4Y2_CANLF	Protein S100	CANLF

159	tr|E2RSF2|E2RSF2_CANLF	Superoxide dismutase	CANLF
160	tr|J9JHQ2|J9JHQ2_CANLF	Xanthine dehydrogenase	CANLF
161	tr|F1Q1R1|F1Q1R1_CANLF	Malate dehydrogenase	CANLF
162	tr|E2QUN9|E2QUN9_CANLF	Uncharacterized protein	CANLF
163	tr|J9PAD4|J9PAD4_CANLF	Uncharacterized protein	CANLF
164	tr|A8QWU1|A8QWU1_CANLF	Protease inhibitor	CANLF
165	tr|L7N095|L7N095_CANLF	Keratin 5	CANLF
166	tr|F1PPU5|F1PPU5_CANLF	Trefoil factor 3	CANLF
167	tr|L7N0G4|L7N0G4_CANLF	Tubulin alpha chain	CANLF
168	tr|J9NY67|J9NY67_CANLF	Uncharacterized protein	CANLF
169	tr|F1PAA4|F1PAA4_CANLF	Cadherin-1	CANLF
170	tr|J9NRF9|J9NRF9_CANLF	LY6/PLAUR domain containing 3	CANLF
171	tr|F1PTX4|F1PTX4_CANLF	Keratin, type II cytoskeletal 2 epidermal	CANLF
172	tr|E2RD86|E2RD86_CANLF	Protein disulfide-isomerase	CANLF
173	tr|F1PKQ1|F1PKQ1_CANLF	Uncharacterized protein	CANLF
174	tr|F1PBK6|F1PBK6_CANLF	Carbonic anhydrase 1	CANLF
175	sp|P63050|RL40_CANLF	Ubiquitin-60S ribosomal protein L40	CANLF
176	tr|F1PLT8|F1PLT8_CANLF	Sulfhydryl oxidase	CANLF
177	tr|J9P0X7|J9P0X7_CANLF	Coactosin like F-actin binding protein 1	CANLF
178	tr|J9JHZ3|J9JHZ3_CANLF	Uncharacterized protein	CANLF
179	tr|F1PGM9|F1PGM9_CANLF	Complement component 4 binding protein alpha	CANLF
180	tr|E2QZQ1|E2QZQ1_CANLF	Uncharacterized protein	CANLF
181	tr|F6UME0|F6UME0_CANLF	Alpha-2-macroglobulin	CANLF
182	tr|F1PF95|F1PF95_CANLF	Alkaline phosphatase	CANLF
183	tr|E2QSZ5|E2QSZ5_CANLF	Coronin	CANLF
184	tr|E2RES2|E2RES2_CANLF	Serpin family C member 1	CANLF
185	tr|J9P758|J9P758_CANLF	Uncharacterized protein	CANLF
186	tr|H9GW87|H9GW87_CANLF	Transaldolase	CANLF
187	tr|E2RCI8|E2RCI8_CANLF	Annexin	CANLF
188	tr|J9P8M2|J9P8M2_CANLF	Fibronectin	CANLF
189	tr|F1P658|F1P658_CANLF	Arginase	CANLF
190	tr|F1PCE8|F1PCE8_CANLF	Uncharacterized protein	CANLF
191	tr|E2RBA5|E2RBA5_CANLF	Chloride channel accessory 1	CANLF
192	tr|J9P284|J9P284_CANLF	Family with sequence similarity 3 member B	CANLF
193	tr|F1PCG4|F1PCG4_CANLF	Peroxiredoxin 2	CANLF
194	tr|F1PJ65|F1PJ65_CANLF	IQ motif containing GTPase activating protein 1	CANLF
195	tr|E2RN10|E2RN10_CANLF	Beta-2-microglobulin	CANLF
196	tr|F1PAR9|F1PAR9_CANLF	Epididymal secretory protein E1	CANLF
197	tr|F1PS73|F1PS73_CANLF	Cystatin B	CANLF
198	tr|E2RC20|E2RC20_CANLF	Nucleoside diphosphate kinase	CANLF
199	tr|H9GWA3|H9GWA3_CANLF	Uncharacterized protein	CANLF
200	sp|Q4LAL9|CATD_CANLF	Cathepsin D	CANLF
201	tr|J9P5V6|J9P5V6_CANLF	Talin 1	CANLF
202	tr|E2QUV2|E2QUV2_CANLF	X-prolyl aminopeptidase 2	CANLF
203	sp|P05124|KCRB_CANLF	Creatine kinase B-type	CANLF
204	tr|F1PTQ7|F1PTQ7_CANLF	Alpha-lactalbumin	CANLF
205	tr|D6BR72|D6BR72_CANLF	Keratin 4	CANLF
206	tr|F1PLV2|F1PLV2_CANLF	Peptidyl-prolyl cis-trans isomerase	CANLF
207	tr|E2RB37|E2RB37_CANLF	Uncharacterized protein	CANLF
208	tr|F1PYR5|F1PYR5_CANLF	Uncharacterized protein	CANLF
209	tr|F1PRB0|F1PRB0_CANLF	Uncharacterized protein	CANLF
210	tr|E2R5B9|E2R5B9_CANLF	Glutathione S-transferase	CANLF
211	tr|E2QWU0|E2QWU0_CANLF	Actin-related protein 2/3 complex subunit 4	CANLF
212	tr|J9P028|J9P028_CANLF	Glutathione peroxidase	CANLF
213	tr|F1P8L7|F1P8L7_CANLF	Rab GDP dissociation inhibitor	CANLF
214	tr|E2RLQ9|E2RLQ9_CANLF	Valosin containing protein	CANLF
215	tr|J9P730|J9P730_CANLF	Uncharacterized protein	CANLF
216	tr|E2R886|E2R886_CANLF	Uncharacterized protein	CANLF
217	tr|E2QVW0|E2QVW0_CANLF	TNF alpha induced protein 1	CANLF
218	tr|J9NTI8|J9NTI8_CANLF	Uncharacterized protein	CANLF
219	tr|E2RMJ8|E2RMJ8_CANLF	Dynein axonemal heavy chain 10	CANLF
220	tr|F1PBC8|F1PBC8_CANLF	Copper transport protein ATOX1	CANLF

221	tr|J9P9V0|J9P9V0_CANLF	Uncharacterized protein	CANLF
222	tr|J9P0D9|J9P0D9_CANLF	Solute carrier family 44 member 2	CANLF
223	tr|J9NVT2|J9NVT2_CANLF	Hypoxanthine phosphoribosyltransferase	CANLF
224	tr|J9NUV0|J9NUV0_CANLF	Ras-related protein Rab-10	CANLF
225	tr|F6Y290|F6Y290_CANLF	Leukotriene A(4) hydrolase	CANLF
226	tr|F1PUA2|F1PUA2_CANLF	Acetyl-coenzyme A synthetase	CANLF
227	tr|F1PDY8|F1PDY8_CANLF	Carbonic anhydrase 2	CANLF
228	tr|F1P9N2|F1P9N2_CANLF	Phospholipase B domain containing 1	CANLF
229	tr|J9P0I1|J9P0I1_CANLF	Keratin, type I cytoskeletal 9	CANLF
230	sp|O18873|ALL1_CANLF	Major allergen Can f 1	CANLF
231	tr|F2Z4N7|F2Z4N7_CANLF	Actin, alpha, cardiac muscle 1	CANLF
232	tr|L7N094|L7N094_CANLF	Keratin 3	CANLF
233	tr|F1Q462|F1Q462_CANLF	Superoxide dismutase [Cu-Zn]	Cu-Zn
234	tr|J9NUN7|J9NUN7_CANLF	Uncharacterized protein	CANLF
235	tr|E2R7F1|E2R7F1_CANLF	Moesin	CANLF
236	tr|J9P2G7|J9P2G7_CANLF	Cofilin 2	CANLF
237	tr|F1PYZ1|F1PYZ1_CANLF	Uncharacterized protein	CANLF
238	tr|E2R150|E2R150_CANLF	Keratin 24	CANLF
239	tr|J9NRH5|J9NRH5_CANLF	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma	CANLF
240	sp|Q50KA9|NDKA_CANLF	Nucleoside diphosphate kinase A	CANLF
241	tr|J9P1V4|J9P1V4_CANLF	Rab GDP dissociation inhibitor	CANLF
242	tr|J9P716|J9P716_CANLF	Tubulin beta 1 class VI	CANLF
243	tr|F1Q1V6|F1Q1V6_CANLF	Tudor domain containing 1	CANLF
244	tr|F1PBI6|F1PBI6_CANLF	Thrombospondin 1	CANLF
245	tr|E2RQ18|E2RQ18_CANLF	Uncharacterized protein	CANLF
246	tr|E2RJE4|E2RJE4_CANLF	Cystatin	CANLF
247	sp|P33703|APOH_CANLF	Beta-2-glycoprotein 1	CANLF
248	tr|J9P8J3|J9P8J3_CANLF	Uncharacterized protein	CANLF
249	tr|J9P7I7|J9P7I7_CANLF	SKI proto-oncogene	CANLF
250	tr|J9P6I6|J9P6I6_CANLF	Oligodendrocyte transcription factor 2	CANLF
251	tr|J9P309|J9P309_CANLF	Actin-related protein 2/3 complex subunit 3	CANLF
252	tr|J9P2V6|J9P2V6_CANLF	Caspase activity and apoptosis inhibitor 1	CANLF
253	tr|J9P1D5|J9P1D5_CANLF	Ankyrin repeat domain 13B	CANLF
254	tr|J9NV09|J9NV09_CANLF	PML-RARA regulated adaptor molecule 1	CANLF
255	tr|H9GWR3|H9GWR3_CANLF	Uncharacterized protein	CANLF
256	tr|F1PXE7|F1PXE7_CANLF	Aspartic peptidase retroviral like 1	CANLF
257	tr|F1PV95|F1PV95_CANLF	Ankyrin repeat domain 52	CANLF
258	tr|F1PUR5|F1PUR5_CANLF	Anterior gradient 2, protein disulphide isomerase family member	CANLF
259	tr|F1PU81|F1PU81_CANLF	Tyrosine-protein phosphatase non-receptor type	CANLF
260	tr|F1PU44|F1PU44_CANLF	Resistin	CANLF
261	tr|F1PRR4|F1PRR4_CANLF	Vasodilator-stimulated phosphoprotein	CANLF
262	tr|F1PGD2|F1PGD2_CANLF	Golgi associated, gamma adaptin ear containing, ARF binding protein 1	CANLF
263	tr|F1PCN7|F1PCN7_CANLF	Uncharacterized protein	CANLF
264	tr|F1PAF0|F1PAF0_CANLF	Tissue alpha-L-fucosidase	CANLF
265	tr|F1P9E5|F1P9E5_CANLF	Platelet-activating factor acetylhydrolase	CANLF
266	tr|E2RSV9|E2RSV9_CANLF	Uncharacterized protein	CANLF
267	tr|E2RSQ9|E2RSQ9_CANLF	Dihydropyrimidine dehydrogenase [NADP(+)]	NADP(+)
268	tr|E2RNX5|E2RNX5_CANLF	Family with sequence similarity 234 member B	CANLF
269	tr|E2RN02|E2RN02_CANLF	Platelet activating factor acetylhydrolase 1b catalytic subunit 2	CANLF
270	tr|E2RLZ9|E2RLZ9_CANLF	Abhydrolase domain containing 14B	CANLF
271	tr|E2RJY0|E2RJY0_CANLF	Potassium channel tetramerization domain containing 12	CANLF
272	tr|E2RH59|E2RH59_CANLF	Glucosamine-6-phosphate isomerase	CANLF
273	tr|E2RGK7|E2RGK7_CANLF	Phospholipase A2 inhibitor and LY6/PLAUR domain containing	CANLF
274	tr|E2RBC6|E2RBC6_CANLF	Uncharacterized protein	CANLF
275	tr|E2R830|E2R830_CANLF	Carboxypeptidase D	CANLF
276	tr|E2R7R1|E2R7R1_CANLF	ISG15 ubiquitin-like modifier	CANLF
277	tr|E2R7A4|E2R7A4_CANLF	Involucrin	CANLF
278	tr|E2R5Q3|E2R5Q3_CANLF	MLX interacting protein	CANLF
279	tr|E2R4V3|E2R4V3_CANLF	Serpin family F member 2	CANLF
280	tr|E2QXD6|E2QXD6_CANLF	Uncharacterized protein	CANLF
281	tr|E2QUP2|E2QUP2_CANLF	Uncharacterized protein	CANLF
282	tr|E2QTL3|E2QTL3_CANLF	Mitochondrial fission 1 protein	CANLF
285	tr|E2R6E0|E2R6E0_CANLF	Lipocalin like 1	CANLF
286	tr|F1PUY9|F1PUY9_CANLF	Uncharacterized protein	CANLF
287	tr|E2RPW3|E2RPW3_CANLF	Paraoxonase 1	CANLF
288	tr|F1PPQ4|F1PPQ4_CANLF	G protein subunit alpha L	CANLF
289	tr|F6Y478|F6Y478_CANLF	Uncharacterized protein	CANLF
290	tr|E2QUU5|E2QUU5_CANLF	60 kDa heat shock protein, mitochondrial	CANLF
291	tr|F1Q418|F1Q418_CANLF	Inter-alpha-trypsin inhibitor heavy chain 1	CANLF
292	tr|E2R7Q1|E2R7Q1_CANLF	Uncharacterized protein	CANLF
293	sp|Q7YRU7|DSG3_CANLF	Desmoglein-3	CANLF
294	tr|F1P778|F1P778_CANLF	S100 calcium binding protein P	CANLF
295	tr|E2R446|E2R446_CANLF	Coiled-coil domain containing 91	CANLF
296	tr|J9P4F3|J9P4F3_CANLF	Vinculin	CANLF
297	tr|F1PYQ2|F1PYQ2_CANLF	Peptidylprolyl isomerase	CANLF
298	tr|F6XY66|F6XY66_CANLF	Transforming protein RhoA	CANLF
299	tr|E2RFC0|E2RFC0_CANLF	Amine oxidase	CANLF
300	tr|F1PT44|F1PT44_CANLF	Replication timing regulatory factor 1	CANLF
301	tr|J9NXY1|J9NXY1_CANLF	Eukaryotic translation initiation factor 5A	CANLF
302	tr|F1PWW0|F1PWW0_CANLF	Filamin A	CANLF

PANTHER GO-Slim Molecular Function
Total # Genes: 211   Total # function hits: 156

- Antioxidant activity (4.8%)
- Binding (37.2%)
- Catalytic activity (47.3%)
- Receptor activity 1.1%)
- Signal transducer activity 1.6%)
- Structural molecule activity (6.9%)
- Translation regulator activity (1.1%)

PANTHER GO-Slim Biological Process
Total # Genes: 211   Total # process hits: 282

- Biological adhesion (1.8%)
- Biological regulation (8.9%)
- Cellular component organization or biogenesis (9.6%)
- Cellular process (27.7%)
- Developmental process (3.5%)
- Growth (0.4%)
- Immune system process (2.8%)
- Localization (6%)
- Locomotion (0.7%)
- Metabolic process (23.8%)
- Multicellular organismal process (2.8%)
- Reproduction (1.1%)
- Response to stimulus (11%)

Supplementary Figure 1- Protein Analysis Through Gene List Analysis (PANTHER, PCS) for molecular functions (left) and biological process (right) of all identified salivary proteins by LC-MS/MS analysis in the present study.

Supplementary Table 2 - List of the proteins identified, by LC-MS/MS in obese dog saliva

Max. no. peptides	Accession number (Uniprot)	Protein Name	Species
1	J9P1D5_CANLF	Ankyrin repeat domain 13B	CANLF
4	F1PSF3_CANLF	Cysteine rich secretory protein 2	CANLF
3	F1PL93_CANLF	Rho GDP dissociation inhibitor alpha	CANLF
7	F1PK60_CANLF	Transglutaminase 3	CANLF
1	F1PV95_CANLF	Ankyrin repeat domain 52	CANLF
4	L7N071_CANLF	Actinin alpha 4	CANLF
4	E2R0V4_CANLF	Caspase 14	CANLF
1	J9P2V6_CANLF	Caspase activity and apoptosis inhibitor 1	CANLF
5	F1PQ93_CANLF	Stratifin	CANLF
4	F1PQN5_CANLF	Cofilin 1	CANLF
3	F1PC59_CANLF	Peroxiredoxin 6	CANLF
1	E2R7R1_CANLF	ISG15 ubiquitin-like modifier	CANLF
2	F1PYE3_CANLF	Heat shock protein 27 kDa beta-1	CANLF
1	J9NVT2_CANLF	Hypoxanthine phosphoribosyltransferase	CANLF
15	E2QWN7_CANLF	Lymphocyte cytosolic protein 1	CANLF
1	E2R8Z5_CANLF	Keratin 5	CANLF
1	E2QWU0_CANLF	Actin-related protein 2/3 complex subunit 4	CANLF
8	E2RAL0_CANLF	Rho GDP dissociation inhibitor beta	CANLF
3	E2RLF1_CANLF	Prosaposin	CANLF
1	J9NRH5_CANLF	Tyrosine 3-monooxygenase/tryptophan 5-monoox	CANLF
6	E2R413_CANLF	Capping actin protein, gelsolin like	CANLF
1	F1PBI6_CANLF	Thrombospondin 1	CANLF
1	F1P8L7_CANLF	Rab GDP dissociation inhibitor	CANLF
1	F1PFI3_CANLF	Phospholipase C beta 3	CANLF
4	J9PAK3_CANLF	ARP3 actin related protein 3 homolog	CANLF
4	J9NVC6_CANLF	Immunoglobulin heavy constant mu	CANLF
3	F1PZC6_CANLF	Histidine rich glycoprotein	CANLF
1	E2RN02_CANLF	Platelet activating factor acetylhydrolase 1b cataly	CANLF
1	F1PPQ4_CANLF	G protein subunit alpha L	CANLF
2	E2QY08_CANLF	Actinin alpha 1	CANLF
2	F6XF05_CANLF	Peptidyl arginine deiminase 4	CANLF
13	E2RCC8_CANLF	Immunoglobulin heavy constant mu	CANLF
2	F2Z4N7_CANLF	Actin, alpha, cardiac muscle 1	CANLF
3	J9NSS7_CANLF	Leucine rich alpha-2-glycoprotein 1	CANLF
1	E2RJY0_CANLF	Potassium channel tetramerization domain contaii	CANLF
3	RL40_CANLF	Ubiquitin-60S ribosomal protein L40	CANLF
1	E2RPW3_CANLF	Paraoxonase 1	CANLF
1	J9P6I6_CANLF	Oligodendrocyte transcription factor 2	CANLF
7	E2R0A4_CANLF	Secretoglobin family 1A member 1	CANLF
6	E2R9B6_CANLF	Fetuin B	CANLF
1	F1P778_CANLF	S100 calcium binding protein P	CANLF
1	J9P0X7_CANLF	Coactosin like F-actin binding protein 1	CANLF
1	E2R4V3_CANLF	Serpin family F member 2	CANLF
1	J9P284_CANLF	Family with sequence similarity 3 member B	CANLF
7	F1P841_CANLF	GC, vitamin D binding protein	CANLF
2	E2R8C2_CANLF	Hexokinase 3	CANLF
2	F1PGM9_CANLF	Complement component 4 binding protein alpha	CANLF
1	F1PGD2_CANLF	Golgi associated, gamma adaptin ear containing, A	CANLF
3	E2QVU9_CANLF	Biliverdin reductase B	CANLF
1	F1PCG4_CANLF	Peroxiredoxin 2	CANLF
1	E2R446_CANLF	Coiled-coil domain containing 91	CANLF

Table 3 - Correlations between the levels of salivary proteins and serum parameters (triglycerides, total cholesterol and glucose) concentrations.

Protein name	Accession Number (Uniprot)	Cholesterol			Triglycerides			Glucose		
		R	P	N	R	P	N	R	P	N
Uncharacterized protein	E2QUP2	0.83	**0.001**	12	-0.198	0.537	12	-0.174	0.589	12
Protein disulfide-isomerase	E2RD86	0.631	**0.028**	12	0.497	0.1	12	0.164	0.611	12
Joining chain of multimeric IgA and IgM	J9JHH5	0.604	**0.037**	12	0.027	0.933	12	-0.233	0.465	12
Uncharacterized protein	E2QUN9	0.748	**0.008**	11	-0.164	0.63	11	-0.23	0.497	11
Actin-related protein 2/3 complex subunit 4	E2QWU0	0.576	**0.05**	12	0.385	0.217	12	0.056	0.863	12
Rho GDP dissociation inhibitor beta	E2RAL0	0.583	**0.047**	12	0.649	**0.022**	12	0.267	0.401	12
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma	J9NRH5	0.6	**0.039**	12	-0.027	0.934	12	-0.018	0.956	12
Histone H4	L7N0L3	0.638	**0.026**	12	-0.067	0.835	12	-0.185	0.564	12
Histone H2A	L7N0D9	0.61	**0.035**	12	-0.024	0.94	12	-0.2	0.533	12
Superoxide dismutase	E2RSF2	-0.625	**0.04**	11	-0.321	0.336	11	0.502	0.116	11
Fibrinogen gamma chain	F1P8G0	0.622	**0.031**	12	0.041	0.899	12	-0.143	0.657	12
Uncharacterized protein	J9PBN6	0.588	**0.044**	12	0.007	0.982	12	-0.139	0.667	12
Uncharacterized protein	E2R886	-0.65	**0.03**	11	-0.343	0.302	11	0.468	0.147	11
Uncharacterized protein	J9NUN7	0.588	**0.044**	12	0.253	0.427	12	-0.028	0.932	12
Vinculin	J9P4F3	0.471	0.123	12	0.781	**0.003**	12	0.347	0.27	12
Uncharacterized protein	J9P732	0.356	0.256	12	0.879	**0.0**	12	0.366	0.243	12
Mucin 7, secreted	J9P2K8	0.578	0.08	10	0.695	**0.026**	10	-0.198	0.583	10
Rho GDP dissociation inhibitor alpha	F1PL93	0.267	0.401	12	0.802	**0.002**	12	0.386	0.215	12
Phosphoglycerate mutase	E2RT65	0.322	0.333	11	0.881	**0.0**	11	0.406	0.216	11
Actinin alpha 4	L7N071	0.001	0.999	11	0.632	**0.037**	11	0.051	0.882	11
Transgelin	F1P6P2	-0.209	0.514	12	0.617	**0.033**	12	0.055	0.864	12
Adenosylhomocysteinase	E2QXS7	0.044	0.893	12	0.721	**0.008**	12	0.233	0.467	12
Ras-related protein Rab-10	J9NUV0	0.166	0.626	11	0.7	**0.016**	11	0.318	0.34	11
Uncharacterized protein	J9P9V0	0.21	0.512	12	0.759	**0.004**	12	0.274	0.389	12
Protein S100	C0LQL0	0.176	0.585	12	0.919	**0.0**	12	0.309	0.328	12
Tropomyosin 3	E2RB38	0.368	0.239	12	0.621	**0.031**	12	0.293	0.356	12
Adenylyl cyclase-associated protein	E2QZ50	0.453	0.139	12	0.717	**0.009**	12	0.391	0.209	12
Fructose-bisphosphate aldolase	F1PBT3	0.373	0.233	12	0.655	**0.021**	12	0.149	0.644	12
Lymphocyte cytosolic protein 1	E2QWN7	0.539	0.071	12	0.602	**0.038**	12	0.308	0.33	12
Glucose-6-phosphate isomerase	E2R2C3	0.52	0.083	12	0.692	**0.013**	12	0.154	0.632	12
Thioredoxin	J9NWJ5	0.165	0.627	11	0.862	**0.001**	11	0.211	0.533	11
Peptidyl-prolyl cis-trans isomerase	J9NV93	0.092	0.788	11	0.867	**0.001**	11	0.186	0.584	11
Uncharacterized protein	E2R0T6	0.553	0.062	12	0.609	**0.036**	12	0.24	0.452	12
Enolase 1	F1PCH3	0.419	0.176	12	0.663	**0.019**	12	0.241	0.45	12
Peptidyl-prolyl cis-trans isomerase	F1PK62	0.408	0.188	12	0.775	**0.003**	12	0.234	0.464	12
Purine nucleoside phosphorylase	F1PQM1	0.006	0.986	12	0.846	**0.001**	12	0.358	0.253	12
Ceruloplasmin	F1PAX2	-0.155	0.63	12	0.631	**0.028**	12	0.016	0.961	12
Transketolase	F1PE28	0.198	0.56	11	0.734	**0.01**	11	0.251	0.456	11
Thrombospondin 1	F1PBI6	-0.067	0.845	11	0.663	**0.026**	11	-0.045	0.896	11
Platelet activating factor acetylhydrolase 1b catalytic subunit 2	E2RN02	0.012	0.972	12	0.633	**0.027**	12	0.469	0.124	12
Actinin alpha 1	E2QY08	0.29	0.36	12	0.731	**0.007**	12	0.463	0.129	12
Matrix metalloproteinase	F1PYF5	0.163	0.613	12	0.601	**0.039**	12	0.592	**0.042**	12
Angiotensinogen	F1PAL5	-0.228	0.5	11	0.609	**0.047**	11	0.088	0.798	11
Uncharacterized protein	H9GWR3	0.028	0.936	11	0.772	**0.005**	11	0.214	0.527	11
Elastase, neutrophil expressed	Q8MJD1	0.118	0.714	12	0.852	**0.0**	12	0.243	0.447	12
Haptoglobin	G1K2D9	0.372	0.233	12	0.636	**0.026**	12	0.403	0.195	12
Uncharacterized protein	E2RB37	-0.114	0.724	12	0.758	**0.004**	12	0.317	0.315	12
Paraoxonase 1	E2RPW3	-0.175	0.607	11	0.778	**0.005**	11	0.14	0.681	11
IQ motif containing GTPase activating protein 1	F1PJ65	0.501	0.097	12	0.697	**0.012**	12	0.202	0.53	12
S100 calcium binding protein P	F1P778	0.188	0.559	12	0.818	**0.001**	12	0.423	0.17	12
Capping actin protein, gelsolin like	E2R413	-0.219	0.518	11	0.17	0.618	11	0.807	**0.003**	11
Profilin	F1Q3Y0	-0.242	0.473	11	-0.119	0.727	11	0.672	**0.024**	11

PANTHER GO-Slim Molecular Function
Total # Genes: 12   Total # function hits: 12
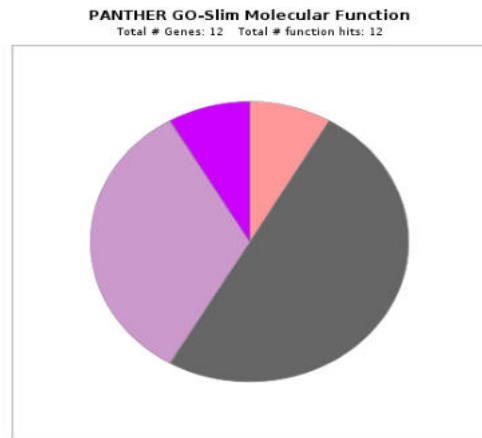
Antioxidant activity (9.1%)
Binding (45.5%)
Catalytic activity (36.4%)
Structural molecule activity (9.1%)

PANTHER GO-Slim Molecular Function
Total # Genes: 32   Total # function hits: 26
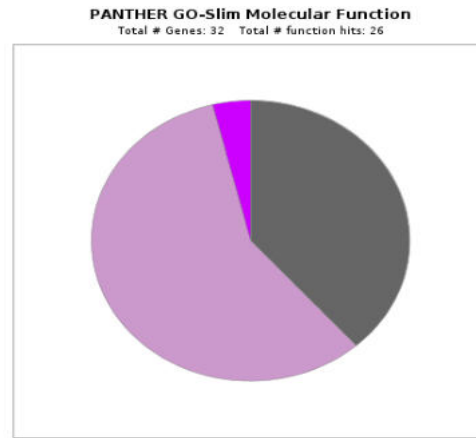
Binding (40%)
Catalytic activity (56%)
Structural molecule activity (4%)

Supplementary Figure 2 - Protein Analysis Through Gene List Analysis (PANTHER) for molecular functions of the salivary proteins correlated with cholesterol (left) and triglycerides (right).

PANTHER GO-Slim Biological Process
Total # Genes: 12   Total # process hits: 14

- Biological adhesion (7.1%)
- Biological regulation (21.4%)
- Cellular component organization or biogenesis (14.3%)
- Cellular process (35.7%)
- Developmental process (7.1%)
- Metabolic process (7.1%)
- Response to stimulus (7.1%)

PANTHER GO-Slim Biological Process
Total # Genes: 32   Total # process hits: 32

- Biological regulation (6.7%)
- Cellular component organization or biogenesis (13.3%)
- Cellular process (33.3%)
- Localization (6.7%)
- Metabolic process (30%)
- Multicellular organismal process (3.3%)
- Response to stimulus (6.7%)
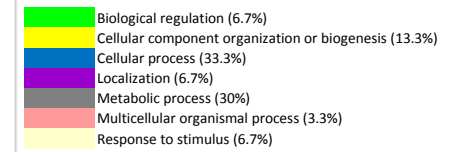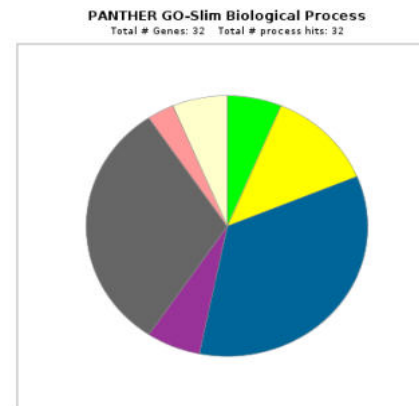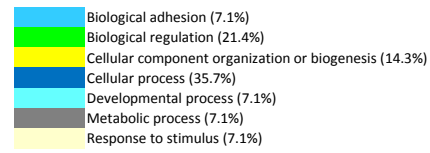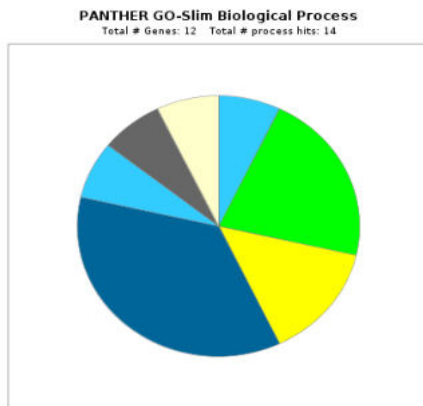
Supplementary Figure 3 - Protein Analysis Through Gene List Analysis (PANTHER) for biological processes of the salivary proteins correlated with cholesterol (left) and triglycerides (right).

Supplementary Table 1 - List of the proteins identified, by LC-MS/MS in obese dog saliva

Max. No. Peptides	Acession number (Uniprot)	Protein name	species
1	F1PCG4_CANLF	Peroxiredoxin 2	CANLF
1	J9NY67_CANLF	Uncharacterized protein	CANLF
1	E2RLQ9_CANLF	Valosin containing protein	CANLF
1	L7N0G4_CANLF	Tubulin alpha chain	CANLF
1	E2RQ18_CANLF	Uncharacterized protein	CANLF
1	E2RN02_CANLF	Platelet activating factor acetylhydrolase 1b c	CANLF
1	E2R735_CANLF	Amine oxidase	CANLF
1	F1PF95_CANLF	Alkaline phosphatase	CANLF
1	F1PLT8_CANLF	Sulfhydryl oxidase	CANLF
1	F6Y290_CANLF	Leukotriene A(4) hydrolase	CANLF
1	J9P9V0_CANLF	Uncharacterized protein	CANLF
1	E2RSQ9_CANLF	Dihydropyrimidine dehydrogenase [NADP(+)]	CANLF
1	F1PL97_CANLF	Protein disulfide-isomerase	CANLF
1	E2QTL3_CANLF	Mitochondrial fission 1 protein	CANLF
1	E2R886_CANLF	Uncharacterized protein	CANLF
1	F1PTX4_CANLF	Keratin, type II cytoskeletal 2 epidermal	CANLF
1	E2QVU9_CANLF	Biliverdin reductase B	CANLF
1	E2RCI8_CANLF	Annexin	CANLF
1	J9JHZ3_CANLF	Uncharacterized protein	CANLF
1	J9P2V6_CANLF	Caspase activity and apoptosis inhibitor 1	CANLF
1	J9P8J3_CANLF	Uncharacterized protein	CANLF
1	CATD_CANLF	Cathepsin D	CANLF
1	J9NVT2_CANLF	Hypoxanthine phosphoribosyltransferase	CANLF
1	J9P8M2_CANLF	Fibronectin	CANLF
1	J9P758_CANLF	Uncharacterized protein	CANLF
1	F1PFI3_CANLF	Phospholipase C beta 3	CANLF
1	E2RSF2_CANLF	Superoxide dismutase	CANLF
1	E2QXS7_CANLF	Adenosylhomocysteinase	CANLF
1	F1PXE7_CANLF	Aspartic peptidase retroviral like 1	CANLF
1	DSG3_CANLF	Desmoglein-3	CANLF
1	F1Q421_CANLF	Plasminogen	CANLF
1	J9P716_CANLF	Tubulin beta 1 class VI	CANLF
2	J9P0X7_CANLF	Coactosin like F-actin binding protein 1	CANLF
1	E2QZQ1_CANLF	Uncharacterized protein	CANLF
1	E2R0V4_CANLF	Caspase 14	CANLF
1	KCRB_CANLF	Creatine kinase B-type	CANLF
1	E2R5B9_CANLF	Glutathione S-transferase	CANLF
1	J9NRH5_CANLF	Tyrosine 3-monooxygenase/tryptophan 5-mo	CANLF
1	J9P1D5_CANLF	Ankyrin repeat domain 13B	CANLF
1	L7N094_CANLF	Keratin 3	CANLF
1	J9P0I1_CANLF	Keratin, type I cytoskeletal 9	CANLF
1	J9P4F3_CANLF	Vinculin	CANLF
1	E2RES2_CANLF	Serpin family C member 1	CANLF
1	J9P732_CANLF	Uncharacterized protein	CANLF
1	ANXA2_CANLF	Annexin A2	CANLF
1	F1PYR5_CANLF	Uncharacterized protein	CANLF
1	E2RBA5_CANLF	Chloride channel accessory 1	CANLF
1	F1PGM9_CANLF	Complement component 4 binding protein al	CANLF
1	NDKA_CANLF	Nucleoside diphosphate kinase A	CANLF
1	F1PLV2_CANLF	Peptidyl-prolyl cis-trans isomerase	CANLF
3	F1PGY1_CANLF	Heat shock protein 90 alpha family class A me	CANLF
1	J9P284_CANLF	Family with sequence similarity 3 member B	CANLF
2	E2R8C2_CANLF	Hexokinase 3	CANLF
1	J9NUV0_CANLF	Ras-related protein Rab-10	CANLF
1	F6Y478_CANLF	Uncharacterized protein	CANLF
1	J9P1V4_CANLF	Rab GDP dissociation inhibitor	CANLF
1	E2RD86_CANLF	Protein disulfide-isomerase	CANLF
1	H9GW87_CANLF	Transaldolase	CANLF
1	E2RHG2_CANLF	Uncharacterized protein	CANLF
2	F1PUM3_CANLF	Glutathione S-transferase omega 1	CANLF
1	E2QUN9_CANLF	Uncharacterized protein	CANLF
1	E2RB37_CANLF	Uncharacterized protein	CANLF
1	F1PJ65_CANLF	IQ motif containing GTPase activating protein	CANLF
1	E2RGK7_CANLF	Phospholipase A2 inhibitor and LY6/PLAUR do	CANLF
1	F1P9E5_CANLF	Platelet-activating factor acetylhydrolase	CANLF
1	E2QUV2_CANLF	X-prolyl aminopeptidase 2	CANLF
1	J9JHQ2_CANLF	Xanthine dehydrogenase	CANLF
1	E2RJY0_CANLF	Potassium channel tetramerization domain co	CANLF
2	J9P730_CANLF	Uncharacterized protein	CANLF
1	F1PBC8_CANLF	Copper transport protein ATOX1	CANLF
1	F1PBK6_CANLF	Carbonic anhydrase 1	CANLF
1	E2RBC6_CANLF	Uncharacterized protein	CANLF
1	F1PVR0_CANLF	Matrix metalloproteinase	CANLF
1	H9GWA3_CANLF	Uncharacterized protein	CANLF
3	E2QY08_CANLF	Actinin alpha 1	CANLF
2	F1PAL5_CANLF	Angiotensinogen	CANLF
2	J9NTL7_CANLF	Uncharacterized protein	CANLF
1	F1PBI6_CANLF	Thrombospondin 1	CANLF
1	F1PPQ4_CANLF	G protein subunit alpha L	CANLF
1	E2QUP2_CANLF	Uncharacterized protein	CANLF
1	F1PLS4_CANLF	Uncharacterized protein	CANLF
1	E2R6E0_CANLF	Lipocalin like 1	CANLF
1	F1PAF0_CANLF	Tissue alpha-L-fucosidase	CANLF
3	F1PZC6_CANLF	Histidine rich glycoprotein	CANLF

2	F1PTZ9_CANLF	Glyceraldehyde-3-phosphate dehydrogenase	CANLF
1	E2R446_CANLF	Coiled-coil domain containing 91	CANLF
1	E2QWU0_CANLF	Actin-related protein 2/3 complex subunit 4	CANLF
2	F6V234_CANLF	Plastin 3	CANLF
2	J9PAK3_CANLF	ARP3 actin related protein 3 homolog	CANLF
2	J9PAD4_CANLF	Uncharacterized protein	CANLF
1	J9P6I6_CANLF	Oligodendrocyte transcription factor 2	CANLF
2	F1PTQ7_CANLF	Alpha-lactalbumin	CANLF
4	F1PKQ1_CANLF	Uncharacterized protein	CANLF
2	F1PAR9_CANLF	Epididymal secretory protein E1	CANLF
3	E2QSZ5_CANLF	Coronin	CANLF
4	F1P9J3_CANLF	Myosin-9	CANLF
2	F1PWR2_CANLF	Uncharacterized protein	CANLF
1	F1P9N2_CANLF	Phospholipase B domain containing 1	CANLF
4	F6UME0_CANLF	Alpha-2-macroglobulin	CANLF
2	F1PQ97_CANLF	Alpha-2-macroglobulin like 1	CANLF
1	E2RB38_CANLF	Tropomyosin 3	CANLF
1	F1PU81_CANLF	Tyrosine-protein phosphatase non-receptor t	CANLF
2	G1K268_CANLF	Uncharacterized protein	CANLF
3	J9NTI8_CANLF	Uncharacterized protein	CANLF
2	E2RN10_CANLF	Beta-2-microglobulin	CANLF
1	J9P028_CANLF	Glutathione peroxidase	CANLF
1	F1P8L7_CANLF	Rab GDP dissociation inhibitor	CANLF
3	J9NV93_CANLF	Peptidyl-prolyl cis-trans isomerase	CANLF
2	F1Q1R1_CANLF	Malate dehydrogenase	CANLF
1	E2R8Z5_CANLF	Keratin 5	CANLF
3	J9PAL7_CANLF	Alpha-amylase	CANLF
1	E2RLS3_CANLF	Heat shock protein 90 alpha family class B me	CANLF
2	F6XF05_CANLF	Peptidyl arginine deiminase 4	CANLF
2	J9NRF9_CANLF	LY6/PLAUR domain containing 3	CANLF
4	J9NY79_CANLF	Malate dehydrogenase	CANLF
1	F1PV95_CANLF	Ankyrin repeat domain 52	CANLF
4	F1P841_CANLF	GC, vitamin D binding protein	CANLF
2	F1PAA4_CANLF	Cadherin-1	CANLF
2	E2QYU2_CANLF	Clusterin	CANLF
3	E2RSI6_CANLF	Ezrin	CANLF
3	F1PYE3_CANLF	Heat shock protein 27 kDa beta-1	CANLF
3	F1PQN5_CANLF	Cofilin 1	CANLF
1	F6XRY2_CANLF	Uncharacterized protein	CANLF
3	F1PK62_CANLF	Peptidyl-prolyl cis-trans isomerase	CANLF
3	E2R9B6_CANLF	Fetuin B	CANLF
2	F1PC59_CANLF	Peroxiredoxin 6	CANLF
1	RL40_CANLF	Ubiquitin-60S ribosomal protein L40	CANLF
2	E2QUU4_CANLF	Keratin 4	CANLF
2	F1PBT3_CANLF	Fructose-bisphosphate aldolase	CANLF
3	F1PL93_CANLF	Rho GDP dissociation inhibitor alpha	CANLF
4	F1PIC7_CANLF	Uncharacterized protein	CANLF
2	F1PAQ3_CANLF	Maltase-glucoamylase	CANLF
2	F1PCK2_CANLF	Alpha-1-B glycoprotein	CANLF
2	E2RJE4_CANLF	Cystatin	CANLF
4	F1PHR2_CANLF	Pyruvate kinase	CANLF
1	E2R830_CANLF	Carboxypeptidase D	CANLF
2	F1P8G0_CANLF	Fibrinogen gamma chain	CANLF
4	J9P4H9_CANLF	Keratin 13	CANLF
2	F1P6P2_CANLF	Transgelin	CANLF
3	F1PW60_CANLF	Desmoglein-1	CANLF
4	F1PUY9_CANLF	Uncharacterized protein	CANLF
3	E2RLF1_CANLF	Prosaposin	CANLF
5	F1PK60_CANLF	Transglutaminase 3	CANLF
4	F1PB68_CANLF	Olfactomedin 4	CANLF
6	E2R413_CANLF	Capping actin protein, gelsolin like	CANLF
2	F2Z4N7_CANLF	Actin, alpha, cardiac muscle 1	CANLF
4	L7N071_CANLF	Actinin alpha 4	CANLF
4	F1PW65_CANLF	Fibrinogen beta chain	CANLF
3	F1PBL4_CANLF	Fibrinogen alpha chain	CANLF
3	F1PBL1_CANLF	Uncharacterized protein	CANLF
5	J9PBN6_CANLF	Uncharacterized protein	CANLF
3	J9NWJ5_CANLF	Thioredoxin	CANLF
3	J9NSS7_CANLF	Leucine rich alpha-2-glycoprotein 1	CANLF
4	E2QZ50_CANLF	Adenylyl cyclase-associated protein	CANLF
5	F1PTY1_CANLF	Keratin, type II cytoskeletal 1	CANLF
3	J9NVM0_CANLF	L-lactate dehydrogenase	CANLF
4	F6UYJ9_CANLF	Calreticulin	CANLF
4	Q95N05_CANLF	Carboxylic ester hydrolase	CANLF
2	F1P6B7_CANLF	Annexin	CANLF
4	F1PDJ7_CANLF	Uncharacterized protein	CANLF

1	F1Q462_CANLF	Superoxide dismutase [Cu-Zn]	CANLF
2	F1Q3Y0_CANLF	Profilin	CANLF
2	L7N095_CANLF	Keratin 5	CANLF
3	F1PQM1_CANLF	Purine nucleoside phosphorylase	CANLF
4	F1PB77_CANLF	Alpha-1,4 glucan phosphorylase	CANLF
2	J9P4E8_CANLF	Uncharacterized protein	CANLF
5	F1PVW0_CANLF	L-lactate dehydrogenase	CANLF
2	J9NVC6_CANLF	Immunoglobulin heavy constant mu	CANLF
4	J9P7D5_CANLF	Glutathione S-transferase mu 4	CANLF
7	E2RT65_CANLF	Phosphoglycerate mutase	CANLF
6	F1PQ93_CANLF	Stratifin	CANLF
3	C0LQL0_CANLF	Protein S100	CANLF
5	J9P839_CANLF	Uncharacterized protein	CANLF
6	F1PAX2_CANLF	Ceruloplasmin	CANLF
2	J9P9E9_CANLF	Glucose-6-phosphate 1-dehydrogenase	CANLF
6	F1PZR4_CANLF	Hemopexin	CANLF
3	E2RPK8_CANLF	Phosphatidylethanolamine binding protein 4	CANLF
3	E2RFK4_CANLF	CD177 molecule	CANLF
6	F1PYU9_CANLF	Keratin, type I cytoskeletal 10	CANLF
3	J9P7B6_CANLF	Uncharacterized protein	CANLF
4	Q6TN20_CANLF	Cathelicidin	CANLF
2	F1PPU5_CANLF	Trefoil factor 3	CANLF
5	F1PE09_CANLF	6-phosphogluconate dehydrogenase, decarbo	CANLF
8	E2RAL0_CANLF	Rho GDP dissociation inhibitor beta	CANLF
7	J9P6A9_CANLF	Desmocollin 2	CANLF
4	F2Z4Q6_CANLF	Serum albumin	CANLF
1	J9NUN7_CANLF	Uncharacterized protein	CANLF
8	F6Y3P9_CANLF	Gelsolin	CANLF
1	F1P778_CANLF	S100 calcium binding protein P	CANLF
7	A0A0A0MPD0_CANLF	Triosephosphate isomerase	CANLF
8	E2RD02_CANLF	Chromosome 6 open reading frame 58	CANLF
4	ALBU_CANLF	Serum albumin	CANLF
5	E2R002_CANLF	Prostaglandin reductase 1	CANLF
7	E2R5P5_CANLF	Protein S100	CANLF
3	F1PCE8_CANLF	Uncharacterized protein	CANLF
5	F1PYF5_CANLF	Matrix metalloproteinase	CANLF
9	E2R2C3_CANLF	Glucose-6-phosphate isomerase	CANLF
15	F1PDJ5_CANLF	Apolipoprotein A-I	CANLF
8	J9P950_CANLF	Uncharacterized protein	CANLF
10	J9NXE2_CANLF	Actin, cytoplasmic 1 Actin, cytoplasmic 1, N-t	CANLF
6	E2R0T6_CANLF	Uncharacterized protein	CANLF
14	F1PIX8_CANLF	Complement C3	CANLF
7	L7N0D9_CANLF	Histone H2A	CANLF
4	F1PSF3_CANLF	Cysteine rich secretory protein 2	CANLF
9	J9P0R6_CANLF	Myeloperoxidase	CANLF
11	F1Q0B9_CANLF	Uncharacterized protein	CANLF
4	HBB_CANLF	Hemoglobin subunit beta	CANLF
10	Q8MJD1_CANLF	Elastase, neutrophil expressed	CANLF
13	E2QWD8_CANLF	Uncharacterized protein	CANLF
14	F1PE28_CANLF	Transketolase	CANLF
15	E2QWN7_CANLF	Lymphocyte cytosolic protein 1	CANLF
13	F1PCH3_CANLF	Enolase 1	CANLF
11	J9PAQ5_CANLF	Protein S100	CANLF
8	F1PSX9_CANLF	Hyaluronidase	CANLF
13	J9P430_CANLF	Transferrin	CANLF
10	F1PBZ4_CANLF	NAD(P)H quinone dehydrogenase 1	CANLF
8	J9P2K8_CANLF	Mucin 7, secreted	CANLF
3	L7N0L3_CANLF	Histone H4	CANLF
8	E2R0A4_CANLF	Secretoglobin family 1A member 1	CANLF
11	E2QXE7_CANLF	BPI fold containing family A member 1	CANLF
14	G1K2D9_CANLF	Haptoglobin	CANLF
5	J9NS47_CANLF	Histone H2B	CANLF
9	J9JHH5_CANLF	Joining chain of multimeric IgA and IgM	CANLF
10	F1PRV8_CANLF	Deleted in malignant brain tumors 1	CANLF
8	A0A077S9R2_CANLF	Lysozyme	CANLF
10	E2RCC8_CANLF	Immunoglobulin heavy constant mu	CANLF
11	F1Q2Y5_CANLF	Zymogen granule protein 16B	CANLF
14	J9NXL3_CANLF	Uncharacterized protein	CANLF
15	E2RFI9_CANLF	Lactoperoxidase	CANLF
14	F1PR78_CANLF	Uncharacterized protein	CANLF
13	F1PCG9_CANLF	Carbonic anhydrase 6	CANLF
15	E2QWJ3_CANLF	BPI fold containing family B member 1	CANLF
13	E2R0H6_CANLF	Prolactin induced protein	CANLF
15	F6USN4_CANLF	Uncharacterized protein	CANLF
14	J9NSZ4_CANLF	Uncharacterized protein	CANLF
14	ALL2_CANLF	Minor allergen Can f 2	CANLF
14	F1PR54_CANLF	Uncharacterized protein	CANLF
15	E2R2B9_CANLF	BPI fold containing family B member 2	CANLF
13	L7N0F2_CANLF	Uncharacterized protein	CANLF
15	J9JHJ8_CANLF	Mucin 5B, oligomeric mucus/gel-forming	CANLF
15	G1K265_CANLF	Lysozyme	CANLF
15	E2RH46_CANLF	Uncharacterized protein	CANLF
15	F1Q3K7_CANLF	Uncharacterized protein	CANLF
14	F6Y6T8_CANLF	Uncharacterized protein	CANLF
15	F1P931_CANLF	Uncharacterized protein	CANLF
14	F1P6A9_CANLF	Uncharacterized protein	CANLF
15	J9P9J6_CANLF	Uncharacterized protein	CANLF
15	E2QXJ0_CANLF	BPI fold containing family A member 2	CANLF

PANTHER GO-Slim Molecular Function
Total # Genes: 204   Total # function hits: 149
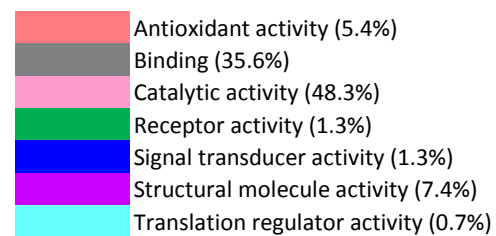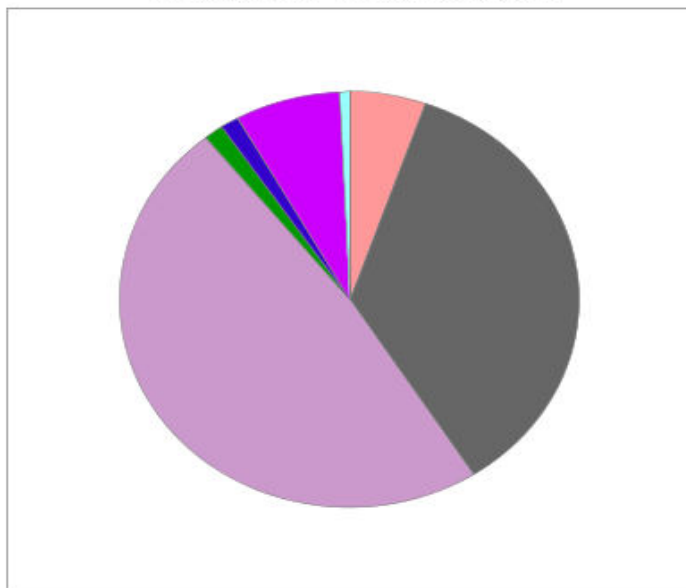
- Antioxidant activity (5.4%)
- Binding (35.6%)
- Catalytic activity (48.3%)
- Receptor activity (1.3%)
- Signal transducer activity (1.3%)
- Structural molecule activity (7.4%)
- Translation regulator activity (0.7%)

Supplementary Figure 1- Protein Analysis Through Gene List Analysis (PANTHER, PCS) for molecular functions of all identified salivary proteins by LC-MS/MS analysis in the present study.

PANTHER GO-Slim Biological Process
Total # Genes: 204   Total # process hits: 273
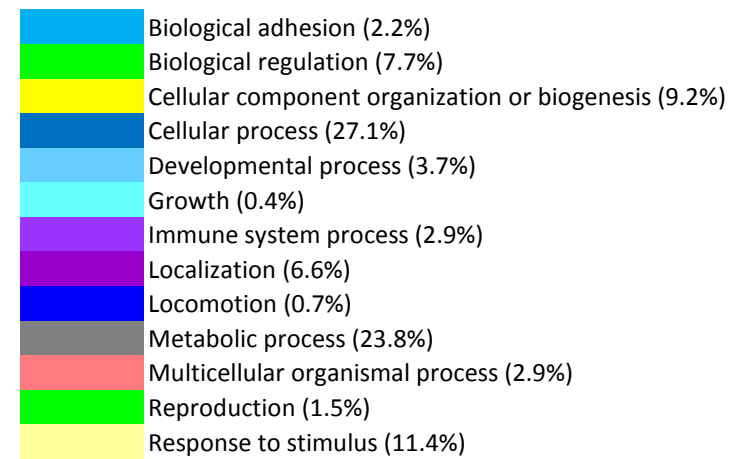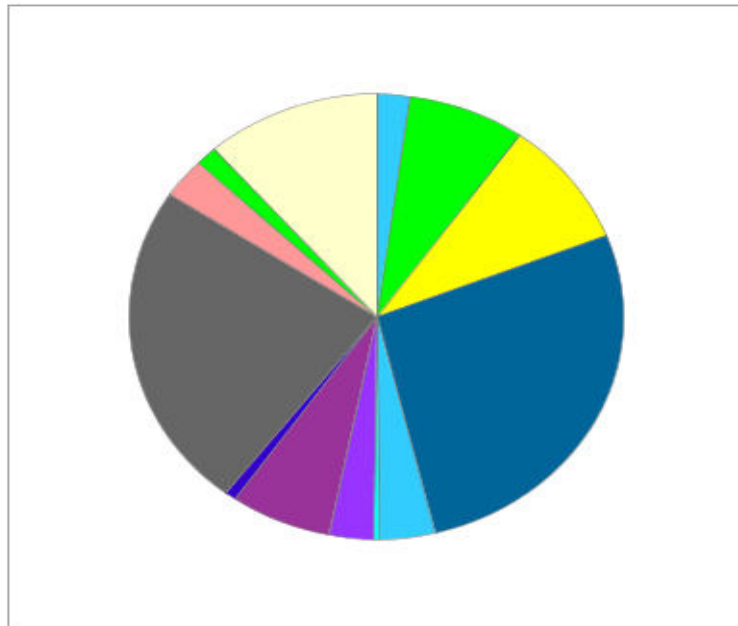
Biological adhesion (2.2%)
Biological regulation (7.7%)
Cellular component organization or biogenesis (9.2%)
Cellular process (27.1%)
Developmental process (3.7%)
Growth (0.4%)
Immune system process (2.9%)
Localization (6.6%)
Locomotion (0.7%)
Metabolic process (23.8%)
Multicellular organismal process (2.9%)
Reproduction (1.5%)
Response to stimulus (11.4%)

Supplementary Figure 2- Protein Analysis Through Gene List Analysis (PANTHER, PCS) for biological processes of all identified salivary proteins by LC-MS/MS analysis in the present study.
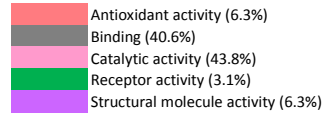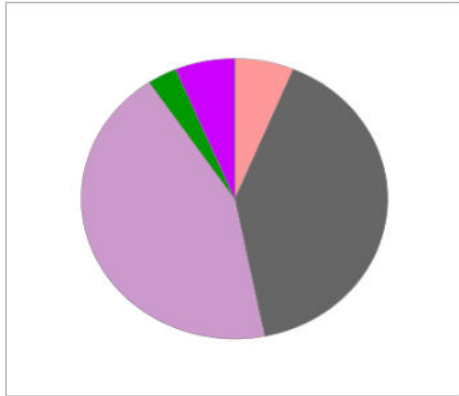
Table 2 - Correlations between the levels of salivary proteins and serum parameters (triglycerides, total cholesterol and glucose) concentrations.

Protein name	Accession Number (Uniprot)	Triglycerides			Cholesterol			Glucose		
		R	*P*	N	R	*P*	N	R	*P*	N
BPI fold containing family B member 1	E2QWJ3	-0.4	0.6	8	1	**0.0**	8	1	**0.0**	8
Uncharacterized protein	L7N0F2	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Myeloperoxidase	J9P0R6	0.5	0.667	9	-1	**0.0**	9	-1	**0.0**	9
Lysozyme	G1K265	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Uncharacterized protein	J9NSZ4	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Protein S100	J9PAQ5	0.5	0.667	8	-1	**0.0**	8	-1	**0.0**	8
Actinin alpha 4	L7N071	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Prolactin induced protein	E2R0H6	-1	**0.0**	8	0.5	0.667	8	0.5	0.667	8
Uncharacterized protein	J9P950	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Deleted in malignant brain tumors 1	F1PRV8	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Uncharacterized protein	E2R0T6	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Enolase 1	F1PCH3	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Glucose-6-phosphate isomerase	E2R2C3	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Zymogen granule protein 16B	F1Q2Y5	-0.5	0.667	9	1	**0.0**	9	1	**0.0**	9
Uncharacterized protein	J9P7B6	0.5	0.667	9	-1	**0.0**	9	-1	**0.0**	9
NAD(P)H quinone dehydrogenase 1	F1PBZ4	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Keratin, type I cytoskeletal 10	F1PYU9	-1	**0.0**	9	0.5	0.667	9	0.5	0.667	9
Elastase, neutrophil expressed	Q8MJD1	0.5	0.667	9	-1	**0.0**	9	-1	**0.0**	9
Triosephosphate isomerase	A0A0A0MPD0	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
GC, vitamin D binding protein	F1P841	-0.5	0.667	10	1	**0.0**	10	-1	**0.0**	10
Stratifin	F1PQ93	0.5	0.667	10	1	**0.0**	10	-1	**0.0**	10
Uncharacterized protein	F1Q0B9	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Peptidyl-prolyl cis-trans isomerase	J9NV93	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Capping actin protein, gelsolin like	E2R413	0.5	0.667	9	-1	**0.0**	9	-1	**0.0**	9
Transglutaminase 3	F1PK60	0.5	0.667	10	-1	**0.0**	10	-1	**0.0**	10
Joining chain of multimeric IgA and IgM	J9JHH5	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Gelsolin	F6Y3P9	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
6-phosphogluconate dehydrogenase, deca	F1PE09	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Desmocollin 2	J9P6A9	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
Matrix metalloproteinase	F1PYF5	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
Prostaglandin reductase 1	E2R002	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
Keratin 5	E2R8Z5	1	**0.0**	8	-1	**0.0**	8	-1	**0.0**	8
Mucin 7, secreted	J9P2K8	-1	**0.0**	8	1	**0.0**	8	1	**0.0**	8
Phosphoglycerate mutase	E2RT65	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
Histone H2A	L7N0D9	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Ezrin	E2RSI6	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Glutathione S-transferase mu 4	J9P7D5	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
Rho GDP dissociation inhibitor beta	E2RAL0	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
Uncharacterized protein	F1PIC7	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Alpha-1,4 glucan phosphorylase	F1PB77	1	**0.0**	9	-1	**0.0**	9	-1	**0.0**	9
Histone H2B	J9NS47	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Myosin-9	F1P9J3	1	**0.0**	8	-1	**0.0**	8	-1	**0.0**	8
Hyaluronidase	F1PSX9	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Fibrinogen gamma chain	F1P8G0	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Heat shock protein 90 alpha family class	F1PGY1	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Purine nucleoside phosphorylase	F1PQM1	1	**0.0**	10	-1	**0.0**	10	-1	**0.0**	10
Profilin	F1Q3Y0	0.143	0.787	10	-0.829	**0.042**	10	-0.829	**0.042**	10
Phosphatidylethanolamine binding protei	E2RPK8	0.886	**0.019**	8	-0.657	0.156	8	-0.429	0.397	8
Transgelin	F1P6P2	0.429	0.397	10	-0.943	**0.005**	10	-0.771	0.072	10
L-lactate dehydrogenase	J9NVM0	0.371	0.468	10	-0.657	0.156	10	-0.829	**0.042**	10
Peptidyl-prolyl cis-trans isomerase	F1PK62	0.086	0.872	10	-0.429	0.397	10	-0.829	**0.042**	10
CD177 molecule	E2RFK4	-0.829	**0.042**	10	-0.543	0.266	10	-0.486	0.329	10
Fibrinogen beta chain	F1PW65	0.086	0.872	9	-0.543	0.266	9	-0.886	**0.019**	9
Actinin alpha 1	E2QY08	0.943	**0.005**	9	-0.657	0.156	9	-0.486	0.329	9
Peroxiredoxin 6	F1PC59	0.314	0.544	9	-0.371	0.468	9	-0.886	**0.019**	9
Clusterin	E2QYU2	-0.657	0.156	10	0.886	**0.019**	10	0.771	0.072	10
Protein disulfide-isomerase	F1PL97	0.543	0.266	10	-1	**0.0**	10	-0.657	0.156	10
Thioredoxin	J9NWJ5	0.486	0.329	9	-0.886	**0.019**	9	-0.714	0.111	9
Protein S100	E2R5P5	0.771	0.072	10	-0.886	**0.019**	10	-0.771	0.072	10
Uncharacterized protein	F1PWR2	0.086	0.872	8	-0.543	0.266	8	-0.886	**0.019**	8
Hexokinase 3	E2R8C2	0.829	**0.042**	9	-0.829	0.042	9	-0.714	0.111	9
Annexin	F1P6B7	0.143	0.787	9	0.2	0.704	9	-0.943	**0.005**	9
Rho GDP dissociation inhibitor alpha	F1PL93	0.143	0.787	9	0.2	0.704	9	-0.943	**0.005**	9
Uncharacterized protein	G1K268	0.314	0.544	9	0.257	0.623	9	-0.886	**0.019**	9
Glutathione S-transferase omega 1	F1PUM3	0.143	0.787	10	0.486	0.329	10	-0.829	**0.042**	10
Uncharacterized protein	F6XRY2	0.2	0.704	10	0.029	0.957	10	-0.886	**0.019**	10
Superoxide dismutase	E2RSF2	0.829	**0.042**	9	-0.6	0.208	9	-0.371	0.468	9
Xanthine dehydrogenase	J9JHQ2	0.314	0.544	10	0.257	0.623	10	-0.886	**0.019**	10
Uncharacterized protein	J9NY67	0.143	0.787	10	0.2	0.704	10	-0.943	**0.005**	10
Epididymal secretory protein E1	F1PAR9	0.829	**0.042**	10	-0.771	0.072	10	-0.429	0.397	10
Peptidyl-prolyl cis-trans isomerase	F1PLV2	0.486	0.329	10	-0.257	0.623	10	-0.943	**0.005**	10
Uncharacterized protein	E2RB37	0.6	0.208	10	-0.371	0.468	10	-0.886	**0.019**	10
Uncharacterized protein	F1PYR5	-0.5	0.667	10	1	**0.0**	10	-0.5	0.667	10
Glutathione S-transferase	E2R5B9	0.5	0.667	10	0.5	0.667	10	-1	**0.0**	10
Actin-related protein 2/3 complex subuni	E2QWU0	0.5	0.667	10	-1	**0.0**	10	0.5	0.667	10
Glutathione peroxidase	J9P028	0.5	0.667	9	-1	**0.0**	9	0.5	0.667	9

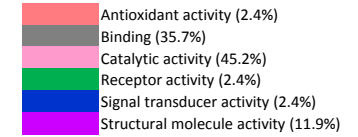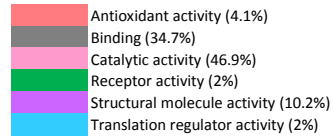| Protein	ID									
Rab GDP dissociation inhibitor	F1P8L7	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Valosin containing protein	E2RLQ9	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Uncharacterized protein	J9P730	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Uncharacterized protein	E2R886	0.5	0.667	9	0.5	0.667	9	-1	**0.0**	9
Uncharacterized protein	J9NTI8	-0.5	0.667	9	-0.5	0.667	9	1	**0.0**	9
Copper transport protein ATOX1	F1PBC8	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Uncharacterized protein	J9P9V0	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Hypoxanthine phosphoribosyltransferase	J9NVT2	-0.5	0.667	9	-0.5	0.667	9	1	**0.0**	9
Ras-related protein Rab-10	J9NUV0	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Leukotriene A(4) hydrolase	F6Y290	0.5	0.667	9	0.5	0.667	9	-1	**0.0**	9
Phospholipase B domain containing 1	F1P9N2	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Keratin, type I cytoskeletal 9	J9P0I1	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Actin, alpha, cardiac muscle 1	F2Z4N7	0.5	0.667	10	0.5	0.667	10	-1	**0.0**	10
Keratin 3	L7N094	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Superoxide dismutase [Cu-Zn]	F1Q462	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Uncharacterized protein	J9NUN7	-0.5	0.667	10	-0.5	0.667	10	1	**0.0**	10
Tyrosine 3-monooxygenase/tryptophan 5	J9NRH5	0.5	0.667	9	0.5	0.667	9	-1	**0.0**	9
Nucleoside diphosphate kinase A	Q50KA9	-0.5	0.667	10	-0.5	0.667	10	1	**0.0**	10
Rab GDP dissociation inhibitor	J9P1V4	-1	**0.0**	8	0.5	0.667	8	0.5	0.667	8
Tubulin beta 1 class VI	J9P716	0.5	0.667	10	0.5	0.667	10	-1	**0.0**	10
Thrombospondin 1	F1PBI6	-0.5	0.667	8	-0.5	0.667	8	1	**0.0**	8
Uncharacterized protein	E2RQ18	0.5	0.667	9	-1	**0.0**	9	0.5	0.667	9
Cystatin	E2RJE4	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Uncharacterized protein	J9P8J3	1	**0.0**	10	0.5	0.667	10	0.5	0.667	10
Oligodendrocyte transcription factor 2	J9P6I6	0.5	0.667	9	0.5	0.667	9	-1	**0.0**	9
Caspase activity and apoptosis inhibitor 1	J9P2V6	-1	**0.0**	10	0.5	0.667	10	0.5	0.667	10
Ankyrin repeat domain 13B	J9P1D5	0.5	0.667	10	-1	**0.0**	10	0.5	0.667	10
Aspartic peptidase retroviral like 1	F1PXE7	-1	**0.0**	9	0.5	0.667	9	0.5	0.667	9
Ankyrin repeat domain 52	F1PV95	0.5	0.667	9	-1	**0.0**	9	0.5	0.667	9
Tyrosine-protein phosphatase non-recept	F1PU81	0.5	0.667	9	-1	**0.0**	9	0.5	0.667	9
Tissue alpha-L-fucosidase	F1PAF0	0.5	0.667	10	-1	**0.0**	10	0.5	0.667	10
Platelet-activating factor acetylhydrolase	F1P9E5	0.5	0.667	9	-1	**0.0**	9	0.5	0.667	9
Dihydropyrimidine dehydrogenase [NAI	E2RSQ9	1	**0.0**	10	0.5	0.667	10	-1	**0.0**	10
Platelet activating factor acetylhydrolase	E2RN02	1	**0.0**	9	-0.5	0.667	9	-0.5	0.667	9
Potassium channel tetramerization doma	E2RJY0	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Phospholipase A2 inhibitor and LY6/PL	E2RGK7	-1	**0.0**	9	0.5	0.667	9	0.5	0.667	9
Uncharacterized protein	E2RBC6	0.5	0.667	8	0.5	0.667	8	-1	**0.0**	8
Carboxypeptidase D	E2R830	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
Uncharacterized protein	E2QUP2	-0.5	0.667	8	1	**0.0**	8	-0.5	0.667	8
Mitochondrial fission 1 protein	E2QTL3	0.5	0.667	10	0.5	0.667	10	-1	**0.0**	10
Lipocalin like 1	E2R6E0	0.5	0.667	8	-1	**0.0**	8	0.5	0.667	8
Uncharacterized protein	F1PUY9	1	**0.0**	10	-0.5	0.667	10	-0.5	0.667	10
G protein subunit alpha L	F1PPQ4	-0.5	0.667	10	1	**0.0**	10	-0.5	0.667	10
Uncharacterized protein	F6Y478	0.5	0.667	10	0.5	0.667	10	-1	**0.0**	10
Desmoglein-3	Q7YRU7	0.5	0.667	9	0.5	0.667	9	-1	**0.0**	9
S100 calcium binding protein P	F1P778	1	**0.0**	8	-0.5	0.667	8	-0.5	0.667	8
Coiled-coil domain containing 91	E2R446	0.5	0.667	9	-1	**0.0**	9	0.5	0.667	9
Vinculin	J9P4F3	0.5	0.667	9	-1	**0.0**	9	0.5	0.667	9

PANTHER GO-Slim Molecular Function
Total # Genes: 42   Total # function hits: 32

- Antioxidant activity (6.3%)
- Binding (40.6%)
- Catalytic activity (43.8%)
- Receptor activity (3.1%)
- Structural molecule activity (6.3%)
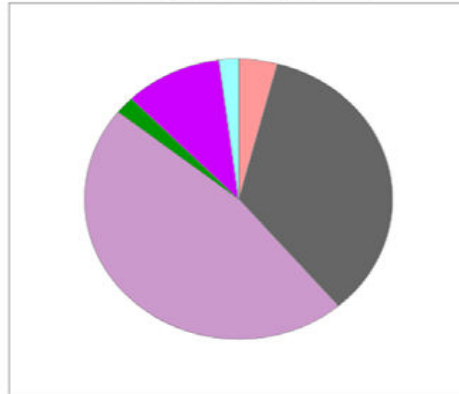
PANTHER GO-Slim Molecular Function
Total # Genes: 50   Total # function hits: 42

- Antioxidant activity (2.4%)
- Binding (35.7%)
- Catalytic activity (45.2%)
- Receptor activity (2.4%)
- Signal transducer activity (2.4%)
- Structural molecule activity (11.9%)
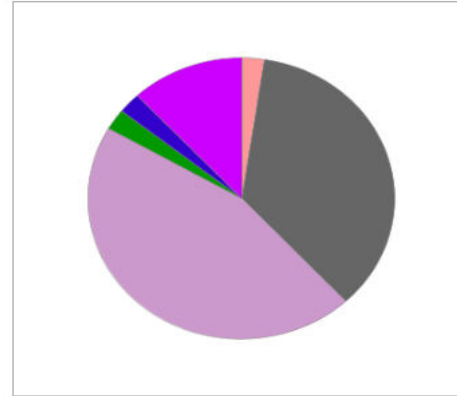
PANTHER GO-Slim Molecular Function
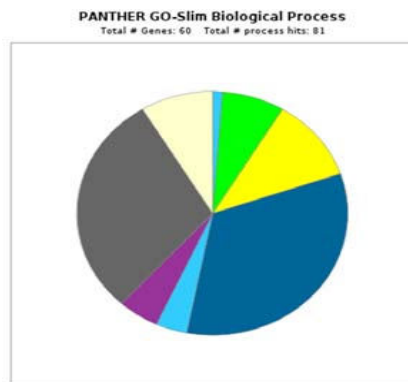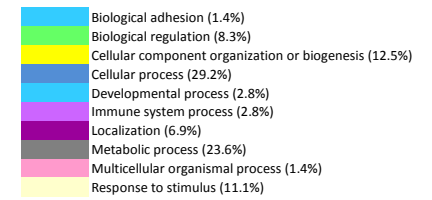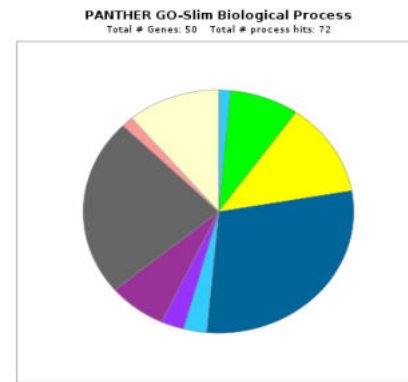Total # Genes: 60   Total # function hits: 49

- Antioxidant activity (4.1%)
- Binding (34.7%)
- Catalytic activity (46.9%)
- Receptor activity (2%)
- Structural molecule activity (10.2%)
- Translation regulator activity (2%)

Supplementary Figure 3 - Protein Analysis Through Gene List Analysis (PANTHER) for molecular functions of the salivary proteins correlated with triglycerides (up - left), cholesterol (up - right) and glucosa (down).
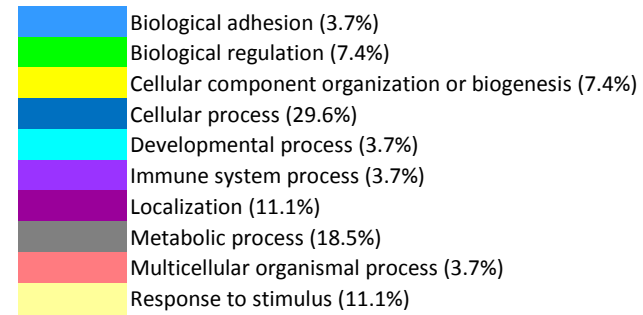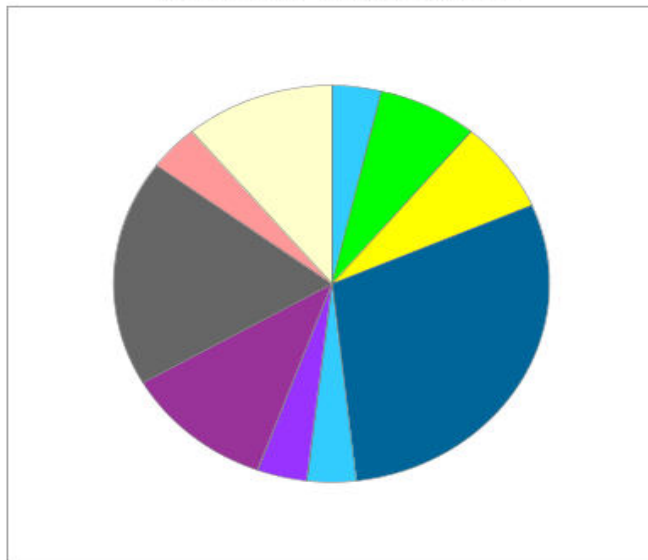
PANTHER GO-Slim Biological Process
Total # Genes: 42   Total # process hits: 45

- Biological adhesion (2.2%)
- Biological regulation (6.7%)
- Cellular component organization or biogenesis (11.1%)
- Cellular process (33.3%)
- Localization (6.7%)
- Metabolic process (33.3%)
- Response to stimulus (6.7%)

PANTHER GO-Slim Biological Process
Total # Genes: 50   Total # process hits: 72

- Biological adhesion (1.4%)
- Biological regulation (8.3%)
- Cellular component organization or biogenesis (12.5%)
- Cellular process (29.2%)
- Developmental process (2.8%)
- Immune system process (2.8%)
- Localization (6.9%)
- Metabolic process (23.6%)
- Multicellular organismal process (1.4%)
- Response to stimulus (11.1%)

PANTHER GO-Slim Biological Process
Total # Genes: 60   Total # process hits: 81

- Biological adhesion (1.2%)
- Biological regulation (7.4%)
- Cellular component organization or biogenesis (11.1%)
- Cellular process (33.3%)
- Developmental process (3.7%)
- Localization (4.7%)
- Metabolic process (29.6%)
- Response to stimulus (8.6%)

Supplementary Figure 4 - Protein Analysis Through Gene List Analysis (PANTHER) for biological processes of the salivary proteins correlated with triglycerides (up - left), cholesterol (up - right) and glucosa (down).

PANTHER GO-Slim Biological Process
Total # Genes: 21   Total # process hits: 27

- Biological adhesion (3.7%)
- Biological regulation (7.4%)
- Cellular component organization or biogenesis (7.4%)
- Cellular process (29.6%)
- Developmental process (3.7%)
- Immune system process (3.7%)
- Localization (11.1%)
- Metabolic process (18.5%)
- Multicellular organismal process (3.7%)
- Response to stimulus (11.1%)

Supplementary Figure 5- Protein Analysis Through Gene List Analysis (PANTHER, PCS) for biological processes applied to the proteins that were differently expressed in their abundance between PRE and POST BW loss groups through univariate and multivariate analysis.

PANTHER GO-Slim Molecular Function
Total # Genes: 21   Total # function hits: 11

Binding (63.6%)
Catalytic activity (27.3%)
Structural molecule activity (9.1%)
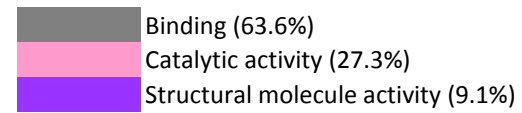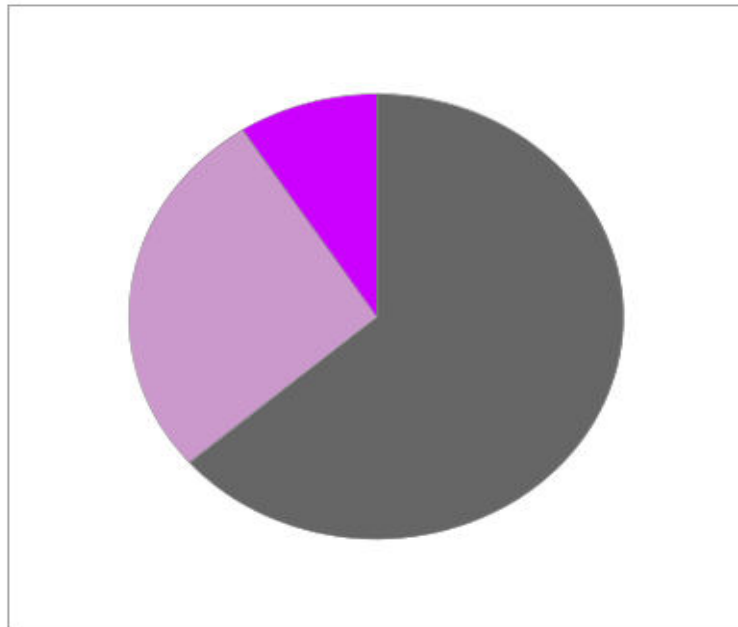
Supplementary Figure 6- Protein Analysis Through Gene List Analysis (PANTHER, PCS) for molecular functions applied to the proteins that were differently expressed in their abundance between PRE and POST BW loss groups through univariate and multivariate analysis.

UNIVERSIDADE DE ÉVORA
INSTITUTO DE INVESTIGAÇÃO
E FORMAÇÃO AVANÇADA