

Chapter 8

Salivary Biomarkers in the Diagnosis and Monitoring of Metabolic and Endocrine Diseases



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Objectives

The present chapter revises the current knowledge about the use of salivary biomarkers for metabolic and endocrine disease diagnosis and monitoring in humans and different animal species. Currently, most information available comes from human medicine and studies performed with experimental animals such as mouse or rat, and less from other veterinary species. Furthermore, since obesity and its-related pathologies are currently recognised as the biggest worldwide public health crisis and socioeconomic problem in the twenty-first century, this chapter will focus most on this disease.

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8.1 Introduction

Endocrine and metabolic diseases are among the most common diseases that can affect humans and animals. In recent decades there has been an increase in the prevalence and incidence of many of them, especially those related to nutrition and metabolism. Metabolic disease is considered a disease or disorder that disrupt normal metabolism on a cellular level. Currently, the most often occurring metabolic disease is obesity and diseases associated with it, among which endocrine disease such as diabetes mellitus (DM). Endocrine diseases comprise disorders that result in altered production of endocrine hormone(s) (also referred as hormone imbalance) or development of lesions (such as nodules or tumors) in the endocrine system, which may or may not affect hormone levels. Close relationship between metabolic and endocrine disease exists since metabolic alterations can finally result in endocrine system disruption and vice versa. For instance, patients suffering from some endocrine diseases such as DM, hyperadrenocorticism or hypothyroidism present obesity.

Obesity is a multicausal disease that due to increased insulin resistance and chronic inflammatory status leads to increased risk of developing DM, cardiovascular diseases, cancer, and renal diseases among others. However, despite human obesity was recognised as a disease in 2008, and a number of studies were undertaken in order to increase knowledge about its pathogenesis, treatment options and prevention, its prevalence is keeping rising and it is considered to be one of the largest contributors to poor health in most countries (NCD Risk Factor Collaboration (NCD-RisC) 2016; Swinburn et al. 2019). Therefore, obesity was stated to be a worldwide public health crisis and socioeconomic problem in the twenty-first century (Hartman et al. 2016; Canfora et al. 2019). In a similar manner, obesity rates are increasing among veterinary species including dogs, cats, horses, resulting not only in worsened health and quality of life of these animals, but also decreased life span (Kealy et al. 2002). In cats and dogs, obesity was recognized to be a disease just in 2018 (<https://bit.ly/2vbTTYO>), endorsed by a total of 23 veterinary organisations.

In humans, obesity is usually diagnosed by calculating the body mass index (BMI) following the formula: $BMI = BW_{kg}/(\text{height}_m)^2$, where BW refers to body weight. People having BMI equal or above 30 are considered obese (Table 8.1). In animals, the obesity is considered as BW increase in 30% when compared with the ideal BW of the species. However, in many cases, it is very hard or even impossible to know the accurate ideal BW, thus body charts were developed in order to score body condition of an animal. The body condition scores (BCS) are based on the visual evaluation and palpation of the animals (Table 8.1). However, when the possible obesity-related risks have to be evaluated, the serum biochemistry is performed. Traditionally, triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol, glucose and insulin are determined in serum, since alterations in these analytes were related with the increased risk to develop cardiovascular diseases and diabetes mellitus among others. However, recently new biomarkers such as adipokines, oxidative stress markers, or acute phase proteins have emerged contributing to the pathogenesis of the disease.

Table 8.1 Weight groups according Body Mass Index (BMI) in humans and Body Condition Score (BCS) Charts in animals

Weight group	Humans	Animals		Description
	BMI	5 point BCS	9 point BCS	
Underweight	< 18.5	1–2	1–4	Ribs – easily palpated without or with poor fat coverage; Bone structures are prominent and easily identifiable; Muscle tone and mass – depressed; Subcutaneous fat – little or no; Abdomen – very collected. Hair – poor quality.
Normal-weight	18.5–25	3	5	Ribs – easily palpated with fat coverage; Bone prominences are palpable but not visible; Muscle tone and mass – normal; Subcutaneous fat – little; no accumulations; Abdomen – collected but not pronounced; Hair – good quality.
Overweight	25–30	4	6–7	Ribs – palpated with difficulty due to the accumulation of superimposed fat; Bone prominences cannot be identified; Muscle tone and mass – normal; Subcutaneous fat – in some areas evident accumulation (e.g., tale base); Abdomen – not collected; Hair – good or reduced quality.
Obese	> 30	5	8–9	Ribs – impossible to palpate due to superimposed fat; Bone prominences are hardly palpable but not visible; Muscle tone and mass – normal to depressed; Subcutaneous fat is evident and there are accumulations in the neck, base of the tail and abdominal region; Abdomen – not collected, rounded appearance; Hair – good or reduced quality.

Endocrine disease diagnosis is traditionally performed by determination of the concentrations of the hormone of interest in serum and, frequently, dynamic function test, either *stimulating* or *suppressing* a particular hormonal axis and observing the appropriate hormonal response, are undertaken. However, general biochemistry analysis can permit to suspect ongoing endocrine disease, since alterations in lipids, acute phase proteins, or glucose among others occur in these diseases.

Nevertheless, in both metabolic and endocrine diseases, the use of saliva is getting attention mainly due to its non-invasive nature (see Chap. 2). Furthermore, saliva analysis is leaded not only to evaluate the possible utility of this biofluid as a

source of traditional biomarkers, but also for new biomarker identification with the aim to clarify and contribute to increased knowledge about pathophysiology of these diseases.

In the following sections we will review state-of-the-art of data obtained via sialochemistry, proteomics, metabolomics and microbiome analyses in metabolic and endocrine diseases.

8.2 Sialochemistry

8.2.1 Glucose

Humans It was hypothesized that glucose into saliva could enter due to leakage from blood across the basement membrane of salivary glands (Puttaswamy et al. 2017) and due to the capacity to cross the salivary gland epithelium in proportion to its concentration in blood (Abikshyeet et al. 2012). Microvascular alterations in the blood vessels due to DM could also contribute to enter glucose into saliva (Puttaswamy et al. 2017). Different authors have studied salivary glucose concentrations in healthy controls and patients with DM (Smriti et al. 2016; Carramolino-Cuéllar et al. 2017). And, although contradiction exists, saliva was suggested to be an useful biofluid for glucose monitoring, since moderate to positive correlation between salivary and serum glucose concentrations and between salivary glucose and serum glycated hemoglobin (HbA_{1c}) were observed (Abikshyeet et al. 2012; Panchbhai 2012; Gupta et al. 2017).

However, cautions should be taken when analyzing glucose metabolism-related biomarkers due to their stability in saliva samples. It was observed that salivary glucose is rapidly decomposed by oral bacteria and enzymes of saliva (Sandham and Kleinberg 1969a, b), thus samples should be refrigerated at all the moments and analyzed or stored at -80°C as soon as possible.

Animals One study in dogs indicate that glucose passes to saliva by a simply passive process from plasma (Langley et al. 1963). Intravenous glucose administration revealed delay in peak of around 25 min of salivary glucose with respect to serum, which could be responsible for the lack of correlation between serum and salivary levels (Muñoz-Prieto et al. 2019b). No studies have been reported evaluating salivary glucose concentrations in dogs with diabetes mellitus or other metabolic/endocrine diseases.

8.2.2 Fructosamine

Fructosamine is considered as an index of average blood glucose levels up to 2 weeks that is not affected by the diet. For this reason, this analyte is used as a blood glucose control index.

Humans Nakamoto et al. (2003) described positive correlation between salivary fructosamine glycated protein and serum HbA_{1c} and blood glucose and suggested its possible utility in diabetes diagnosis.

Animals A method for fructosamine determination in canine saliva was satisfactory validated showing adequate precision and accuracy and, as expected, no significant changes in its concentrations after intravenous glucose administration were observed (Muñoz-Prieto et al. 2019b). Further studies are now needed to evaluate the possible utility of salivary fructosamine in cases of diabetes. Nevertheless, it is important to mention, that fructosamine was described to decrease up to 65% even after being stored at -80°C , thus, samples should be analyzed in fresh (Muñoz-Prieto et al. 2019b). For this reason, some authors suggested determine glycated proteins, such as fructosamine glycated protein, in saliva since it was hypothesized that glycated proteins were more stable (Nakamoto et al. 2003).

8.2.3 Insulin

Humans Insulin-like immunoreactivity in saliva has been reported by different groups of authors (Marchetti et al. 1986; Fekete et al. 1993; Messenger et al. 2003).

There are two main hypotheses regarding the insulin presence in the saliva:

1. Insulin is actively synthesized and secreted by salivary gland. This hypothesis is based mainly on animal studies, in which insulin mRNA have been found in salivary glands and that salivary glands were sensitive to changes in glucose levels (Shubnikova et al. 1984; Kerr et al. 1995; Taouis et al. 1995). Furthermore, synthesis of this hormone was confirmed in humans and animal models through detection of radiolabeled product in salivary gland tissue incubated in vitro with [³H]leucine (Murakami et al. 1982).
2. Insulin enters saliva from blood by ultrafiltration. This hypothesis is supported by studies that detected relation (although delayed) between blood and saliva insulin levels together with the lower salivary insulin concentrations and the observation that salivary insulin was affected by *iv* glucose administration (Messenger et al. 2003).

In subjects with normal glucose tolerance, both normal weight and obese, the concentrations of insulin in saliva increase after oral glucose tolerance test, although with a delay in the peak of approximately 30–45 min (Marchetti et al. 1986; Pasic and Pickup 1988; Fekete et al. 1993). In healthy school-age girls, salivary insulin levels were correlated with serum insulin and BMI, while this correlation was not detected in boys (Tvarijonavičiute et al. 2019). Nevertheless, insulin handling by salivary glands of patients with type I and II diabetes seem to differ (Marchetti et al. 1986; Pasic and Pickup 1988). Interestingly, strong positive correlation between salivary and serum insulin was observed when pooled data of patients with type I diabetes were studied, while the correlation did not exist when comparing salivary

and serum insulin concentrations of each individual separately (Pasic and Pickup 1988). For this reason, some authors indicate that the use of salivary insulin levels for diabetes control would not be reasonable (Pasic and Pickup 1988).

Animals Rat salivary glands and saliva were shown to present biologically active insulin-like immunoreactivity that can participate in metabolic regulations via amino acid uptake and lipogenesis (Taouis et al. 1995). Furthermore, as stated above, it was demonstrated the active secretion of insulin by salivary glands in rats and mice (Kerr et al. 1995). Kerr et al. (1995) observed that the behavior of salivary insulin in healthy normal mice differs from the mice with induced diabetes, being much lower in those with diabetes and suggesting that salivary glands can act as extrapancreatic source of insulin in cases of diabetes.

In dogs, intravenous glucose administration resulted in a delayed increase of insulin in saliva with respect to serum of about 10 min (Muñoz-Prieto et al. 2019b). The significant increase was noted in saliva 15 min after injection remaining high 45 min after. This delay, could be the reason of the lack of correlation between serum and saliva insulin concentrations. Unfortunately, no data exist about salivary insulin levels in dogs with metabolic diseases such as obesity or diabetes mellitus.

8.2.4 Lipids

Dysregulation of lipid metabolism is related to development of a number of pathologies including metabolic diseases obesity and diabetes mellitus among others (Gianfrancesco et al. 2018; Hou et al. 2019). In order to evaluate, diagnose or monitor lipid metabolism, in human medicine, triglycerides, cholesterol, low density lipoproteins (LDC) and high density lipoproteins (HDL) are determined in serum. Both HDL and LDL are predominantly involved in cholesterol metabolism. HDL is considered the “good” lipoprotein with protective role on vascular system, while increases LDL were associated with arteriosclerosis. It is important to notice, that the lipid and lipoprotein distribution in serum of humans and different animal species differ, for this reason, caution should be taken when selecting appropriate animal model for human dyslipidemia studies (Table 8.2) (Yin et al. 2012).

Humans Very few information about salivary lipids is available (Matczuk et al. 2017). From eight main groups of lipids, five were identified in saliva (Table 8.3). Salivary lipid profile in different major salivary glands is similar, while considerable

Table 8.2 Species having high density lipoprotein (HDL) or low density lipoprotein (LDL) as a main serum lipoprotein

Main lipoprotein	Species
HDL	Dog, cat, horse, ruminants, rat, mouse
LDL	Humans, Most of primates, rabbit, hamster, Guinea pig, pig, camel, rhinoceros

Table 8.3 Lipid groups present in saliva

Nr.	Group ^a	Described in Saliva
1	Fatty acyls	✓
2	Glycerolipids	✓
3	Glycerophospholipids	✓
4	Sphingolipids	✓
5	Sterol lipids	✓
6	Prenol lipids	
7	Saccharolipids	
8	Poliketides	

^aLipid classification according to International lipid Classification and nomenclature Committee and LIPID MAPS consortium

differences were observed in minor glands (Rabinowitz and Shannon 1975). Although, this is not of big importance when total saliva is used, it should be taken in consideration in patients with salivary gland pathologies. Just for instance, alterations in salivary lipid profile was reported in patients with cystic fibrosis, a disease that involves impaired secretion by the endocrine glands, including salivary glands (Matczuk et al. 2017). Nevertheless, no studies exist evaluating possible salivary lipid profile changes in presence of systemic metabolic diseases.

Animals In animals, only experimental studies in rats were reported describing salivary lipid profiles and their possible alterations in pathologies. For instance, experimental model consisting in diabetes induction in rats with streptozotocin resulted in increased lipids, and in particular stearic and linoleic acids that were normalized after successful treatment with insulin (Anderson and Garrett 1986; Morris et al. 1992; Mahay et al. 2004). However, no studies exist about salivary lipid profiles in naturally occurring pathologies in animals.

8.2.5 Adipokines

Adipokines are proteins mainly synthesized in the adipose tissue, although in the last years their synthesis by other tissues such as salivary gland was confirmed by different researchers (Katsiougianis et al. 2006). Adiponectin and leptin are two of the most studied adipokines in metabolic diseases due to their close relationship with insulin resistance and inflammation among others (Bastard et al. 2006). The presence of both adiponectin and leptin in saliva has been described by different groups of authors.

Humans Salivary adiponectin concentrations correlate positively with serum and, thus, saliva was indicated as appropriate biofluid for this adipokine measurement in different clinical situations such as insulin resistance and obesity (Desai and Mathews 2014; Nigro et al. 2015; Teke et al. 2019) and could serve as convenient

adjunct method in predicting cardio-metabolic risks in the population (Attlee et al. 2019). Salivary leptin concentrations were shown to be higher in overweight individuals and patients with diabetes mellitus as compared with normal weight individuals (Jayachandran et al. 2017; Tvarijonavičiute et al. 2017). However, no association between salivary adiponectin or leptin was detected with metabolic syndrome (Thanakun et al. 2014). Furthermore, cautions must be taken in presence of the gingivitis, since an increase in salivary adiponectin was described (Meriç et al. 2018).

Animals Salivary adiponectin was described in dogs and its correlation with serum was reported (Tvarijonavičiute et al. 2014). Furthermore, salivary adiponectin concentrations were lower, although not statistically significantly, in obese dogs in comparison to normal weight or overweight dogs (Muñoz-Prieto et al. 2019a). However, as occur in humans, salivary adiponectin was shown to be increased in dogs with gingivitis naturally occurring and due to teeth cleaning procedures (Tvarijonavičiute et al. 2014), thus oral health should be evaluated in the dogs when saliva for adiponectin determination is collected.

In pigs, salivary leptin was related with body weight, food ingestion and inflammation (Schmidt et al. 2016).

8.2.6 *Inflammatory Biomarkers*

Some metabolic diseases, such as obesity or diabetes mellitus, are related with the presence of low grade inflammation. For this reason, acute phase proteins, especially C-reactive protein (CRP) and interleukins are usually used to evaluate pro-inflammatory status of patients with metabolic and endocrine diseases (Bastard et al. 2006).

Humans Utility of CRP and interleukins in saliva were proved to be useful biomarkers in studies of obesity, metabolic syndrome or diabetes mellitus being levels of pro-inflammatory biomarkers elevated in these metabolic pathologies (Naidoo et al. 2012; Dezayee and Al-Nimer 2016; Hartman et al. 2016; Balaji et al. 2017; Janem et al. 2017; Tvarijonavičiute et al. 2019).

Animals The association of salivary S100, a protein participating in the regulation of the immune homeostasis and inflammation (Bao et al. 2012), with metabolic diseases was suggested, since its increase in submandibular glands of rats, after 2 months of induced-hyperglycemia, was observed and linked to the inflammatory response and impaired metabolic and energy production processes (Alves et al. 2013). Furthermore, S100 was higher in saliva of dogs with Obesity Related Metabolic Disease (ORMD) as compared with overweight obese dogs without ORMD (Lucena et al. 2019).

8.2.7 *Antioxidants*

Humans Presence of pro-oxidant status has been reported in different metabolic diseases. However, studies on salivary oxidative biomarker behavior in metabolic diseases are scarce, although some reported results evidence alterations in levels of these biomarkers including malondialdehyde, uric acid, superoxide dismutase, total oxidant status (TOS) and total antioxidant status (TAS) in saliva of patients with diabetes mellitus, gestational DM, obesity, hepato-metabolic comorbidities (Hartman et al. 2016; Madi et al. 2016; Troisi et al. 2019b; Zygula et al. 2019). In addition, oxidative stress was shown to be a strong inducer of alkaline phosphatase in various tissues (Torino et al. 2016). And its salivary concentrations were higher in patients with chronic periodontitis with type-2 diabetes mellitus than chronic periodontitis without diabetes mellitus and healthy patients (Sridharan et al. 2017; De et al. 2018). The adequate metabolic control and periodontal treatment result in normalisation of levels of oxidative stress biomarkers in saliva (Aral et al. 2017).

Animals Despite the limited number of studies about salivary antioxidants and metabolic diseases in animals, in dogs, the presence of oxidative stress was suggested in dogs with ORMD, since alterations in salivary glutathione S-transferase, superoxide dismutase and Hsp70 were observed in presence of ORMD (Lucena et al. 2019). Furthermore, a 3-month experimental-weight loss resulted in increased salivary levels of copper chaperone ATOX1 and alkaline phosphatase in purebred Beagles (unpublished data), leading to the hypothesis of a reduction in oxidative stress, resulting in elevation of antioxidant defense markers, as it was reported in serum in humans (Bawahab et al. 2017).

Rats with insulin resistance presented higher values of superoxide dismutase, catalase, peroxidase and total antioxidant status in the parotid glands in comparison with the control rats (Zalewska et al. 2014).

8.2.8 *Stress Markers*

Humans Among stress-related biomarkers, the majority of the studies highlight the disturbance of salivary cortisol, cortisone and salivary alpha amylase levels in metabolic and endocrine diseases. These biomarkers were related with BMI, fasting glucose concentrations and insulin sensitivity among others (Incollingo Rodriguez et al. 2015; Aldossari et al. 2019; Liu et al. 2019).

In endocrine diseases, although varying results have been reported, salivary cortisol and cortisone have been suggested to reflect inappropriate production of cortisol in the organism – both excess (e.g., Cushing’s syndrome with a sensitivity of 92–100% and a specificity of 93–100%) and insufficiency (e.g., Addison’s disease) and to monitor in a non-invasive manner the response to treatment (Gilbert and Lim

2008; Blair et al. 2017). It is important to notice, that saliva collection for cortisol determination when its inappropriate production is suspected should be performed at midnight to eliminate bias due to circadian rhythm (Viardot et al. 2005; Gilbert and Lim 2008).

For the reasons described above and because of their high stability in saliva (i.e. cortisol is stable in saliva up to 6 weeks at room temperature when preserved with citric acid 10 g/L) salivary stress-related biomarkers were reported as promising biomarkers in studies of metabolic and endocrine diseases (Chen et al. 1992; Hartman et al. 2016; Blair et al. 2017).

Animals In dogs, salivary cortisol usefulness to diagnose hiperadrenocortisims was studied (Wenger-Riggenbach et al. 2010). The authors observed that although salivary cortisol in dogs with diagnosed hiperadrenocortisims was higher, overlap between healthy (n = 21) and diseased dogs (n = 6) were detected (2 healthy dogs showed elevated results and 2 dogs with hiperadrenocortisims showed low results) (Wenger-Riggenbach et al. 2010). Nevertheless, low number of animals was used, thus future studies are required in order to clarify the clinical utility of salivary cortisol determination in dogs. Nevertheless, it is important to highlight that when salivary cortisol is up to be determined, sex and neuter status, age, regular living environment, time in environment before testing, testing environment, owner presence during testing, and collection media should be taken into consideration (Cobb et al. 2016). In addition, methods for sample collection, storage and analysis should be acknowledged when comparing results from different studies (Cobb et al. 2016; Damián et al. 2018).

Furthermore, some of the stress-related biomarkers, such as salivary alpha amylase, were observed while using proteomic approach to be altered in metabolic/endocrine diseases in animals (for more information see section *Proteomics*).

8.2.9 Thyroid Hormones

Humans There is a controversy about the possible use of thyroid hormone measurements in saliva for the diagnosis of hypothyroidism. A report that used a RIA, indicated a good correlation between saliva and serum concentrations of thyroxine (T4) ($r = 0.74$) and a good agreement between saliva T4 values and the functional state of the thyroid (Putz et al. 2009), being this high correlation later confirmed (Gotovtseva and Korot'ko 2002). Meanwhile, Al-Hindawi et al. (2017) did not observe significant differences in free T4 and TSH in saliva between patients with hypothyroidism and healthy individuals, although the values of saliva were parallels to their values in serum. It is of interest to note that in this later study ELISA kits that usually are less sensitive than RIA were used. Furthermore, samples were stored at -20°C during a not defined time, fact that could have influenced concentrations of the hormones. The sensitivity of the assays has been indicated as one of the main

limitations for the measurement of T4 in saliva since concentrations of T4 in this fluid is much lower than in serum (1/100) (Vining et al. 1983).

Animals Till the date, thyroid hormones were not evaluated in saliva of animals in relation to metabolic and/or endocrine diseases.

8.2.10 Trace Elements

Humans Trace elements, such as magnesium, zinc, and calcium levels in saliva were suggested to be useful biomarkers for differentiating patients with type 2 diabetes mellitus from non-diabetics being higher as in controls (Marín Martínez et al. 2018). Furthermore, the salivary magnesium could serve as a marker of high cardiovascular risk since its levels were associated with abdominal obesity in men (Marín Martínez et al. 2018).

8.3 Proteomics

Proteomics means the analysis of the total protein set expressed by a cell or an organism. Different approaches can be used for proteomic analysis, namely expression proteomics, functional proteomics and structural proteomics. In this part of the chapter, we will focus mainly in expression proteomics studies that have been performed in the context of metabolic diseases. Expression proteomics is mainly used to generate a large qualitative data set with the expression levels of the proteins present in the sample. This provides a global analysis of protein composition, post-translational modifications, and the dynamic nature of protein expression.

Salivary proteomics has been shown particularly valuable, since it allows to have a source of information about proteins from a fluid that is collected under a non-invasive way. This opened new doors for disease biomarker discovery, which can be particularly interesting also in the case of endocrine and metabolic diseases. Among these last, diabetes was the one receiving greater attention in terms of saliva proteome. Although fewer, some studies did also report changes in salivary proteome induced by obesity. A review about what is known in humans and animals will be subsequently presented.

Humans Studies in adults and children showed existing differences in salivary proteome of obese individuals in comparison with normal-weight controls. Just for instance, Rangé et al. (2012) observed higher levels of albumin, α - and β - haemoglobin chains, as well as α -defensins 1,2 and 3 in obese individuals, comparatively to normal weight ones. These authors emphasized the interest of saliva as a biological fluid to monitor inflammatory status in obesity, which is considered a low-grade inflammatory condition. Furthermore, differences between obese and non-obese

women were observed at the level of proteins such as α -amylase, zinc- α 2 glycoprotein and cystatins, among others (Lamy et al. 2015).

Salivary proteome of obese patients was also studied evaluating the effect that body weight loss could have. Bariatric surgery appeared to have effect at the level of salivary proteins like salivary amylase, cystatins and carbonic anhydrase VI (Lamy et al. 2015), proteins referred as potentially involved in oral food perception (Rodrigues et al. 2017). A different study, where weight loss was obtained after weeks of continuous physical activity and caloric restriction, showed also changes in salivary proteome, namely amylase, carbonic anhydrase VI and cystatins, but in opposite direction as changes detected in the study about the effect of bariatric surgery, suggesting that the weight-loss procedures can differently affect physiological changes, reflected in saliva (Simões and Lamy, *not published*).

Also in children, salivary proteome of overweight individuals present differences from normal-weight ones (Rodrigues et al. 2019). When these differences were accessed by in-gel based methods, namely two-dimensional electrophoresis (2-DE), protein spots identified as zinc- α 2 glycoprotein, α -amylase and S-type cystatins were observed increased in obese children (Rodrigues et al. 2019).

Concerning salivary α -amylase, different studies report different associations with obesity: some authors refer decreased copy number of the gene that codifies for this protein (Mejía-Benítez et al. 2015; Pinho et al. 2018) and decreased enzymatic activity (Lasisi et al. 2019); however, some other authors observed increased expression levels of spots identified as containing this salivary enzyme (Lamy et al. 2015). At the same time that these different results may seem contradictory, if we look to the proteoforms of the protein that are increased, is possible to see that are mainly forms with molecular masses lower than the one from the native form of the protein (Lamy et al. 2015). It is possible that these are non-active forms, probably resultant from higher proteolysis in the mouth of obese individuals. But this needs further elucidation. Finally, a recent study points that this relationship between obesity and salivary amylase is not simple and that starch intake can modify it (Rukh et al. 2017). Nevertheless, some authors failed to observe a relationship between salivary proteome and BMI (Mosca et al. 2019).

Several studies about saliva proteome in metabolic diseases were performed in the context of diabetes. By using a gel-free based approach, namely multidimensional liquid chromatography/tandem mass spectrometry (2D-LC-MS/MS), whole saliva from type-2 diabetic individuals was compared with non-diabetic ones, with the identification of 65 proteins with an increased greater than twofold, in diabetic, comparatively to control (Rao et al. 2009). The majority of these proteins are involved in metabolism or immune responses. The usefulness of saliva proteome assessment in diabetes were reinforced by the existence of changes in it according to glycemic control, both in adults (Bencharit et al. 2013) and children (Pappa et al. 2018).

Animals Comparatively to humans, there are less studies about salivary proteomics in animals. In the case of metabolic diseases, it is mainly concerning diabetes, using

rodent models and, only more recently, studies in dogs concerned with metabolic dysfunctions and obesity were published.

Among the different salivary proteins observed to be related with obesity and metabolic diseases, in animals, salivary α -amylase secretion has been suggested of interest to predict susceptibility for weight gain (Rodrigues et al. 2015). In the mentioned study, rats with susceptibility to obesity presented higher α -amylase levels prior to weight gain experimentally-induced with high-fat diet. But this relationship between this salivary protein and obesity needs to be further elucidated, since some studies observed decreased levels of this salivary protein after diet-induced obesity (Lasisi et al. 2019). A potential role of leptin in salivary α -amylase secretion has been recently suggested (Lamy et al. 2018), leading to the hypothesis that the changes in this salivary protein, associated with obesity, can be due to leptin action. In fact, decreased levels of expression levels and enzymatic activity of salivary α -amylase were observed when hyperleptinemia was induced in an animal model (Lamy et al. 2018), leading to the hypothesis that obese, by having higher circulating leptin levels could also have lower α -amylase levels in their saliva.

Recently, through a proteomic approach, the presence of salivary amylase in dog saliva samples has been reported (de Sousa-Pereira et al. 2015), being this presence supported by the measurement of its enzymatic activity in saliva from these animals (Contreras-Aguilar et al. 2017). Nevertheless, until the moment, a potential association between its levels and dog obesity was not reported.

Potential changes in salivary proteome of dogs submitted to weight loss were recently studied. An increase of BPIFA1 salivary levels was found in purebred Beagles dogs after a 3-month experimentally-weight loss (unpublished data), which goes in line with studies of obesity and insulin resistance in humans (Guo et al. 2017). This protein has been positively correlated with insulin action and associated with immune system and inflammatory pathways (Gubern et al. 2006). As such, the aforementioned study (unpublished data) goes in line with a possible improvement in insulin sensitivity after weight loss. Besides this, other salivary proteins, whose abundance was changed after weight loss, were proteins related with immune system/inflammation, oxidative stress and glucose metabolism. These were the cases of copper chaperone ATOX1 and alkaline phosphatase, for example, whose increased levels lead to the hypothesis of a reduction in oxidative stress, after weight loss (unpublished data).

Also in the case of experimentally-weight loss in Beagle dogs, the salivary levels of angiopoietin like 5 protein was increased and strongly positively correlated with the percentage of weight loss (data not shown). This protein is involved in glucose metabolism and the observed relationship with body weight loss suggests a positive effect of this process in glucose regulation. These results are in accordance with a reported negative association between the levels of serum angiopoietin like 3 and 4 proteins and body weight, diabetes status, and parameters of glucose control across a wide range of BMI (Cinkajzlová et al. 2018). To the best of the authors' knowledge there were no reports of an association between angiopoietin like 5 and obesity.

The relationship between salivary proteome and metabolic diseases was recently demonstrated in a study performed with Obesity Related Metabolic Disease (ORMD) dogs (Lucena et al. 2019). Among the different proteins observed to be different in ORMD dogs, comparatively to control ones, salivary levels of Glucose-6-phosphate dehydrogenase were found to be increased. The levels of the enzyme glucose-6-phosphate dehydrogenase were already reported to be increased in diabetes (Hamzah et al. 2018). This enzyme is involved in the pentose phosphate pathway and its activity is related with the production of NADPH. It was suggested that, at an initial phase of diabetes, increases levels of this enzyme prevent the oxidative stress known to be associate to the development of diabetes and metabolic syndrome. Furthermore, other authors reported a decreased levels of glucose-6-phosphate dehydrogenase in rats with chronic hyperglycemia, resulting in increased oxidative stress (Xu et al. 2005). Other salivary proteins, referred as antioxidant and/or inflammatory biomarkers were also observed to be changed in dog ORMD, by this proteomic approach, and were referred in previous sections of this chapter.

The salivary proteins kallikreins were referred as associated with diabetes, in rodent model. A significant decrease of Kallikrein proteins after 2 months of induced-hyperglycemia in rats was observed, reinforcing results of the effect of chronic hyperglycemia on the proteome of submandibular glands (Alves et al. 2013). Kallikrein proteins are extracellular matrix protein constituents, which had already been reported having salivary levels decreased in type 2 diabetes human subjects (Rao et al. 2009).

8.4 Metabolomics

Being part of the “omic” sciences, metabolomics is the global assessment and validation of endogenous small-molecule metabolites within a biologic system. Salivary metabolomics gained interest in the last few years, becoming of interest to monitor biological status and for monitoring diseases.

Humans Salivary metabolomics is an emerging area, with a limited number of studies, at the moment. However, recently this approach has been used to study metabolic syndrome and fatty liver in obese children (Troisi et al. 2019a) and diabetes and diabetes-related periodontal disease in adults (Barnes et al. 2014). Distinct salivary metabolic signatures for pediatric obesity and its related fatty liver and metabolic syndrome were observed as defined mainly by energy, amino and organic acid metabolism, as well as in intestinal bacteria metabolism (Troisi et al. 2019a). These metabolic processes were associated with the diet, fatty acid synthase pathways, microbiota and intestinal mucins (Troisi et al. 2019a). In the same line, 69 out of 475 detected metabolites (14%) in saliva were over or under-expressed in saliva of patients with diabetes as compared to healthy controls (Barnes et al. 2014). Oxidative stress and anti-oxidative capacity through increased purin degradation signature and decreased redox balance and lipid metabolism through altered ω -3/

ω -6 fatty acid profiles were reported to be the main processes associated with diabetes and periodontal disease (Barnes et al. 2014). These data suggest about potentials that have salivary metabolomics studies to identify early occurring metabolic alterations in a non-invasive way, although future research is necessary to confirm these results and to further study the complex biological pathways, their interactions and their possible changes in metabolic and endocrine diseases.

Animals To date, metabolomics was not performed in saliva of animals to evaluate possible alterations in metabolic diseases. Future studies are needed to fill this knowledge gap.

8.5 Microbiome

Microbiome refers to the entire habitat, including microorganisms (bacteria, archaea, lower and upper eukaryotes, and viruses), their genomes (ie, genes), and environmental conditions (Marchesi and Ravel 2015). However some authors limit the definition of microbiome to the collection of genes and genomes of members of a microbiota (Marchesi and Ravel 2015) and in some literature these terms are often used interchangeably (Ursell et al. 2012).

Interest in the study of microbiome has increased in recent years in humans (Maguire and Maguire 2017; Thomas et al. 2017; Mohajeri et al. 2018) and animals (Deng and Swanson 2015; Trinh et al. 2018), since the evidence is growing that associated microorganisms make essential contributions to health and well-being, and changes in their amounts/proportions are associated with a number of different diseases. In the case of the oral microbiota, it has a significant impact on both the oral and systemic health (Dewhirst et al. 2010; Wade 2013; Verma et al. 2018) and is also a potential diagnostic indicator of several oral and systemic diseases (Gao et al. 2018).

Inter-individual variations were significantly larger than intra-individual variations for most of the dominant genera in the oral microbiota (Monteiro-Da-Silva et al. 2014; Barroso et al. 2015; Sato et al. 2015). Multiple factors and their interactions modulate oral microbiome. These factors are intrinsic or extrinsic to the individual (host) and include:

- (i) the genetic composition of the host (ex: ethnicity, gender, circadian rhythm);
- (ii) oral environment (ex: saliva composition; (Marsh et al. 2016; Lyng Pedersen and Belstrøm 2019));
- (iii) lifestyle, behaviour and diet;
- (iv) socioeconomic status (Cornejo Ulloa et al. 2019);
- (v) climatic conditions (Li et al. 2014) and geographical location (Shaw et al. 2017) although some authors report that the influence of geographical location on the oral microbiome is not significant (Nasidze et al. 2009).

It should also be mentioned, in order to understand the complexity and the dynamics of oral microbiota, composed of hundreds of taxa interacting across multiple spatial scales, that the different structures and tissues of the oral cavity present distinct microbial populations and there are scientific evidences that most oral microbes are site specialists (Aas et al. 2005; Mark Welch et al. 2016; Welch et al. 2019). Distinct microbial communities appear to be observed between dental, tongue, and salivary samples, with high levels of similarity observed between the tongue and salivary communities (Hall et al. 2017). Furthermore, bacterial communities vary along an ecological gradient from the front to the back of the mouth (Proctor et al. 2018).

Humans Scientific evidences exist that oral/salivary microbiome have a link with the presence of obesity and other metabolic diseases. Si et al. (2017) compared the oral microbiome from subgingival plaque with the gut microbiome and their results support the notion that metabolic disease can influence the non-gut human microbiome. In the same manner, other studies also observed differences in oral microbiota between overweight and normal-weight persons (Goodson et al. 2009; Wu et al. 2018; Mervish et al. 2019; Raju et al. 2019). Furthermore, the overweight was related to the greater diversity of microbial species, although lower total amounts (Mervish et al. 2019). Microbiota diversity and composition were significantly associated with body size and gender in school-age children (Raju et al. 2019). Overall, these studies emphasize the utility of local oral bacteria as potential biomarkers for systemic metabolic disease.

Some studies have confirmed that the oral diseases and diabetes mellitus are closely related (Negrato et al. 2013). According to Ebersole et al. (2008) the increased severity of periodontal disease associated with type 2 diabetes may reflect an alteration in the pathogenic potential of periodontal bacteria and/or a modification of the characteristics of the host's inflammatory response. Furthermore, oral microbiota is an important factor in the development of diabetes, affecting oral bone development and increasing the risk and severity of tooth loss (Xiao et al. 2017), what in periodontitis can compromise the chewing process and the sensory function of periodontal tissues (Borges et al. 2012). Saeb et al. (2019) observed a reduction of the biological and phylogenetic diversity in the diabetes and prediabetes oral microbiota in comparison with that in the normoglycemic oral microbiota and this was associated with an increase in the pathogenic content of the hyperglycemic microbiota.

The results of Tam and colleagues suggested that obesity alters composition and diversity of the oral microbiota in patients with type 2 diabetes mellitus, but the impact of glycemic control on oral microbiota, however, remains to be elucidated (Tam et al. 2018). Detailed information on the different taxa and species can be find in literature (Gao et al. 2018; Lu et al. 2019).

Animals To date there are only a few reports that assessed the characterization of dogs (Dewhirst et al. 2012; Sivakami et al. 2015) and cats (Sturgeon et al. 2014; Dewhirst et al. 2015; Adler et al. 2016) oral microbiomes using modern sequencing technology. Equine subgingival plaque microbiota shares many similarities with the

human, canine and feline oral microbiomes (Gao et al. 2016). Nevertheless, contrary to what happens in humans, the evaluation of the relationship between the oral microbiome and metabolic diseases is not yet documented.

There is a growing number of families having pets, mainly dogs and cats. Since many of these animals are treated as family members and are in direct contact with the people, it is expected that the exchange of microorganisms between humans and animals occur, through various routes, including saliva (Nishiyama et al. 2007; Song et al. 2013; Misic et al. 2015). However, dogs and their owners presented appreciable differences in oral microbiome, being their oral microbiotas not correlated with residing in the same household (Oh et al. 2015). Further studies are needed to study the oral microbiome relation with metabolic and endocrine disease in animals and the possible connection with changes in animal-owner microbiota.

8.6 Conclusion

Strong scientific evidences exist suggesting saliva to constitute a promising tool to evaluate metabolic diseases and endocrinopathies-related alterations.

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