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Advances in the identification and development of biomarkers for health and welfare assessment: a One-Health perspective

Avances en la identificación y desarrollo de biomarcadores de salud y bienestar desde una perspectiva One Health

Dña. Lorena Franco Martínez

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FACULTAD DE VETERINARIA

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PhD thesis summary

"Advances in the identification and development of biomarkers for health and welfare assessment: a One-Health perspective"

Avances en la identificación y desarrollo de biomarcadores de salud y bienestar desde una perspectiva One Health

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Silvia Martínez Subiela

Asta Tvarijonaviciute

Nada en la vida debe ser temido, solamente comprendido.
Ahora es el momento de comprender más, para temer menos.

Marie Skłodowska-Curie

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Contents

Contents

Doctoral Thesis as compendium of publications

The present PhD thesis is presented as a compendium of 16 published works, in accordance with the authorization of the directors of the PhD thesis and the Academic Commission responsible for the Veterinary Sciences PhD program. This PhD thesis is composed by the following articles' references:

Book chapter

I. Franco-Martínez, L.; Rubio, C.P.; Contreras-Aguilar, M.D. Methodology Assays for the Salivary Biomarkers' Identification and Measurement. In *Saliva in Health and Disease*; Springer International Publishing: Cham, **2020**, 67–95.

Articles

1. Franco-Martínez, L.; Martínez-Subiela, S.; Cerón, J.J.; Tecles, F.; Eckersall, P.D.; Oravcova, K.; Tvarijonaviciute, A. Biomarkers of health and welfare: A One Health perspective from the laboratory side. *Res. Vet. Sci.* **2020**, *128*, 299–307.
2. Franco-Martínez, L.; Martínez-Subiela, S.; Escribano, D.; Schlosser, S.; Nöbauer, K.; Razzazi-Fazeli, E.; Romero, D.; Cerón, J.J.; Tvarijonaviciute, A. Alterations in haemolymph proteome of Mytilus galloprovincialis mussel after an induced injury. *Fish Shellfish Immunol.* **2018**, *75*, 41–47.
3. Franco-Martínez, L.; González-Hernández, J.M.; Horvatić, A.; Guillemin, N.; Cerón, J.J.; Martínez-Subiela, S.; Sentandreu, M.Á.; Brkljačić, M.; Mrljak, V.; Tvarijonaviciute, A.; et al. Differences on salivary proteome at rest and in response to an acute exercise in men and women: A pilot study. *J. Proteomics.* **2020**, *214*.

4. Franco-Martínez, L.; Tvarijonaviciute, A.; Martínez-Subiela, S.; Márquez, G.; Martínez Díaz, N.; Cugat, R.; Cerón, J.J.; Jiménez-Reyes, P. Changes in lactate, ferritin, and uric acid in saliva after repeated explosive effort sequences. *J. Sports Med. Phys. Fitness* **2019**, *59*, 902–909.

5. Franco-Martínez, L.; Villar, M.; Tvarijonaviciute, A.; Escribano, D.; Bernal, L.J.; Cerón, J.J.; Thomas, M. del C.; Mateos-Hernández, L.; Tecles, F.; de la Fuente, J.; et al. Serum proteome of dogs at subclinical and clinical onset of canine leishmaniosis. *Transbound. Emerg. Dis.* **2019**, *0*, 1–10.

6. Franco-Martínez, L.; Tvarijonaviciute, A.; Horvatić, A.; Guillemin, N.; Bernal, L.J.; Barić Rafaj, R.; Cerón, J.J.; del Carmen Thomas, M.; López, M.C.; Tecles, F.; et al. Changes in saliva of dogs with canine leishmaniosis: a proteomic approach. *Vet. Parasitol.* **2019**, *272*, 44–52.

7. Franco-Martínez, L.; Tvarijonaviciute, A.; Horvatić, A.; Guillemin, N.; Cerón, J.J.; Escribano, D.; Eckersall, D.; Kocatürk, M.; Yilmaz, Z.; Lamy, E.; et al. Changes in salivary analytes in canine parvovirus: A high-resolution quantitative proteomic study. *Comp. Immunol. Microbiol. Infect. Dis.* **2018**, *60*, 1–10.

8. Franco-Martínez, L.; Horvatić, A.; Gelemanović, A.; Samardžija, M.; Mrljak, V.; Contreras-Aguilar, M.D.; Martínez-Subiela, S.; And, R.D.; Tvarijonaviciute, A. Changes in the salivary proteome associated with canine pyometra. *Front. Vet. Sci.* **2020**.

9. Franco-Martínez, L.; Gelemanović, A.; Horvatić, A.; Contreras-Aguilar, M.D.; Dabrowski, R.; Mrljak, V.; Cerón, J.J.; Martínez Subiela, S.; Tvarijonaviciute, A. Changes in Serum and Salivary Proteins in Canine Mammary Tumors. *Animals* **2020**, *10*, 1–44.

10. Franco, L.; Romero, D.; García-Navarro, J.A.; Teles, M.; Tvarijonaviciute, A. Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of Mytilus galloprovincialis exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure. *Mar. Pollut. Bull.* **2016**, *102*, 30–35.

11. Franco-Martínez, L.; Romero, D.; García-Navarro, J.A.; Tecles, F.; Teles, M.; Tvarijonaviciute, A. Measurement of p-nitrophenyl acetate esterase activity (EA), total antioxidant capacity (TAC), total oxidant status (TOS) and acetylcholinesterase (AChE) in

gills and digestive gland of Mytilus galloprovincialis exposed to binary mixtures of Pb, Cd and Cu. *Environ. Sci. Pollut. Res.* **2016**, *23*, 25385–25392.

12. Franco-Martínez, L.; Romero, D.; Rubio, C.P.; Tecles, F.; Martínez-Subiela, S.; Teles, M.; Tvarijonaviciute, A. New potential biomarkers of oxidative stress in Mytilus galloprovincialis: Analytical validation and overlap performance. *Comp. Biochem. Physiol. Part - B Biochem. Mol. Biol.* **2018**, *221–222*, 44–49.

13. Oliveira, M.; Franco, L.; Balasch, J.C.; Fierro-Castro, C.; Tvarijonaviciute, A.; Soares, A.M.V.M.; Tort, L.; Teles, M. Tools to assess effects of human pharmaceuticals in fish: A case study with gemfibrozil. *Ecol. Indic.* **2018**, *95*, 1100–1107.

14. Franco-Martinez, L.; Tvarijonaviciute, A.; Martinez-Subiela, S.; Teles, M.; Tort, L. Chemiluminescent assay as an alternative to radioimmunoassay for the measurement of cortisol in plasma and skin mucus of Oncorhynchus mykiss. *Ecol. Indic.* **2019**, *98*, 634–640.

15. Franco-Martínez, L.; Tvarijonaviciute, A.; Mateo, S. V.; Cerón, J.J.; Romero, D.; Oliveira, M.; Teles, M.; Martínez-Subiela, S. Evaluation of C-reactive-like protein in *Mytilus galloprovincialis*. *Ecol. Indic.* **2019**, *106*, 105537.

In addition, six **appendices** were included with data from experiments related to the work carried out during the thesis. This data is planned to be submitted for possible publications in the near future.

I. *Teaching the One Health concept to undergraduate veterinary students: a pilot study.*

II. *Effects of filtration and alpha-amylase depletion on salivary biochemical analysis.*

III. *Adenosine deaminase (ADA) and its isoenzymes in saliva: analytical validation of an automated assay and applications in physical and psychological stress.*

IV. *The serum and saliva proteome of dogs with diabetes mellitus.*

V. *Changes in saliva and serum proteins in cows with mastitis: a proteomic approach.*

VI. *Development of a monoclonal antibody for the measurement of C-reactive protein in multispecies rapid and portable assays.*

Abbreviations

2-DE: two-dimensional electrophoresis

AChE: acetylcholinesterase

ADA: adenosine deaminase

AOPP: advanced oxidation protein products

APP: acute-phase protein

AST: aspartate aminotransferase

CK: creatinine kinase

COR: cortisol

CRP: c-reactive protein

CUPRAC: cupric reducing antioxidant capacity

CV: coefficient of variation

DNA: deoxyribonucleic acid

EA: esterase activity

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FRAP: ferric reducing ability of plasma

gGT: γ-glutamyl transferase

H_2O_2: hydrogen peroxide

Hp: haptoglobin

IDA: information-dependent acquisition

IEF: Isoelectric focussing

IgG: immunoglobulin G

IPG: immobilized pH gradient

LC-MS: liquid chromatography-mass spectrometry

LC-MS/MS: liquid Chromatography with tandem mass spectrometry

LLD: lower limit of detection

NSS: normal saline solution

OH: One Health

r: coefficient of correlation

RNA: ribonucleic acid

ROS: reactive oxygen species

RP-LC-MS/MS: reverse-phase liquid chromatography coupled with mass spectrometry

sAA: alpha-amylase

SD: standard deviation

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SOD: CuZn superoxide dismutase

TAC: total antioxidant capacity

TEAC: trolox equivalent antioxidant capacity

TOS: total oxidant status

TP: total protein

UA: uric acid

WB: western-Blot

WBC: white blood cell

Introduction

The One Health concept (OH) can be defined as the combined effort of multiple disciplines to attain optimal health for humans, animals, and the environment (Wong and Kogan, 2013), highlighting the increased value of inter- and trans-disciplinary collaborations in the last years (Häsler *et al.*, 2014). However, there is still a lack of integration between agencies responsible for animal, human and environmental health, despite the broad support that has been mobilized by many international organizations (Kahn, 2012). One example of the benefits of collaborative approaches is the COVID-19 pandemic, in which the services of human health have been overwhelmed and the support of veterinary laboratories has been of help for its diagnostic (OIE, 2020). These veterinary laboratories and their personnel are commonly familiarised with similar equipment and techniques, quality controls, biosecurity and bioprotection as used in human labs and can measure a high number of samples in routine.

A biomarker is defined by the World Health Organization as any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological (Yoshizawa *et al.*, 2013a). The wide use of biomarkers contributes to increasing the life expectancy and quality for the patients since they allow earlier decision making in terms of diagnosis, prevention or treatment monitoring, as well as improves the disease-prevention or treatment (Wagner, Verma and Srivastava, 2004). However, most studies focussed on biomarkers are applied in a tight range of species such as humans and domestic or laboratory animals, being their potential applications in the context of OH still scarcely developed.

An example of the possible applications of OH in the identification and measurement of biomarkers is the fact that, sometimes, an easily accessible commercial assay can be adapted and validated for its use in a different species that it was designed for, increasing the assay

availability and its possible applications. This may be especially relevant for physiological pathways highly conserved between different species such as stress (Marco-Ramell *et al.*, 2016), oxidative status (Apak, R., Özyürek, M., Güçlü, K., Çapanoğlu, 2016) or acute phase response (Schrödl *et al.*, 2016).

In relation to sample types, generally, serum or plasma obtained from blood are the most widely used for clinical analysis (Argüelles *et al.*, 2004). However, in the last years, the use of less-invasive samples such as saliva, hair, faeces, urine, or skin mucus in case of fish, offer various advantages compared to blood. They are faster and easier to obtain, do not need specific professional personnel for its collection (Lindsay and Costello, 2016), and most importantly, they are in most cases pain and stress-free. Thus, in parallel to the potential applications of OH from adapting assays from one species to others, some analyses that are designated for its use in one sample type can be adapted and validated for their use in non-invasive samples.

Last, there is a wide range of techniques available nowadays for biomarker identification and measurements, and the type and biochemical characteristics of each biomarker will determine the most suitable assay in each case (Cerón, Eckersall and Martínez-Subiela, 2005). For example, spectrophotometric or immunological assays are widely used for single biomarker measurement since they are easily integrated into laboratories and provides with rapid and economic turnouts in most cases; while other techniques such as “omics” enable the simultaneous identification of a wide range of biomarkers by the analysis of hundreds of analytes present in a tissue or fluid, being more expensive and time-consuming. Thus, in biomarker’s research, it is recommended the employment of different techniques depending on the objectives of the study and the characteristics of the biomarker of interest. In many cases, the combined use of different techniques would provide the best results. For example, it is possible to identify a panel of potential biomarkers by omics, perform the clinical and analytical validations by spectrophotometric or immunological assays, and finally develop portable immunoassays for its measurement in different contexts.

Objectives

The general objective of this PhD thesis was to increase the knowledge about the identification, measurement and validation of novel biomarkers of health and welfare that can be used in different contexts and species under the "One-Health" approach. Besides, the use of non-invasive samples such as saliva was prioritized whenever it was possible. Under this idea, different species were used: humans, dogs which are used frequently as a model for the study of human diseases, cows that are prone to develop mastitis being a disease of high importance from the One-Health perspective, and fish and mussels which are species far away from humans and dogs from a phylogenetic perspective but that due to its use for environmental monitoring are of high interest under the concept of "One-Health".

The specific objectives were:

1. To review the state of the art of the search and validation of novel biomarkers in different species and sample types and to evaluate the applications of the "One-Health" concept in a clinical pathology laboratory (Article 1, Book Chapter and Appendices I-II).
2. To perform proteomic and biochemical studies in different experimental and clinical situations for the discovery of novel biomarkers. The studies in humans and mussels would focus on situations where muscle damage can be produced. Whereas the studies in dogs and cows would be focused on different diseases that can have special interest from the OH concept due to their clinical or social importance or prevalence. In addition, a model of environmental pollution will be made in mussels and fish since these species are commonly used as sentinels of environmental health.

   The models to evaluate will be:

Model 1: muscular damage (Articles 2-4 and Appendix III).

Model 2: pathologies (Articles 5-9 and Appendix IV-V).

Model 3: environmental conditions such as hypoxia or pollution (Articles 10-14)

3. To identify a common biomarker by the application of the OH concept and to develop a fast assay for its measurement. To assess if C-reactive protein - a known major acute-phase protein in humans and dogs- is present in mussels and if a monoclonal antibody against CRP can be created that would have cross-reactivity with human, canine and mussel samples permitting the development of a new rapid and portable assay based on lateral-flow technique (Article 15 and Appendix VI).

Extended summary

Materials and methods

1.1. Biomedical Ethics

The current PhD thesis was approved by the Local Ethical Committee of the Veterinary Medicine School, Murcia University (CEEA 479/2018; ID: 2038/2018).

All experimental procedures were carried out according to the 3 R's principles of Animal Experimentation following Spanish legislation (Law 32/2007 and RD53/2013) that agrees with the International Guiding Principles for Biomedical Research Involving Animals (EU 2010/63). In all cases handling and sampling were performed by accredited researchers and/or veterinarians.

All procedures performed in studies involving human participants (Articles 2 and 4) followed the ethical standards of the institutional and/or national research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

For the articles using dogs, experimental procedures were conducted and approved by the Ethics committee of the University of Uludag, Turkey (Article 5), University of Murcia (protocol number: 276/2016) and the Regional Government of Murcia (identification code number: A13151002) (Articles 6-7), and University of Murcia and Ministry of agriculture, livestock, fishing and aquaculture, Region of Murcia (A13170503) (Articles 8-9). In those studies were client-owned dogs were employed (Articles 5-9 and Appendix IV), owner's consent were obtained. The study performed in cows (Appendix V) was approved by the Ethics committee of the Lithuanian University of Health Sciences, Lithuania.

1.2. Individuals and sampling procedure

Humans (Homo sapiens):

- For saliva collection, participants were asked not to exercise, eat, drink, smoke or brush their teeth for at least one hour before their participation in the study. Unstimulated saliva was collected for 1 minute by passive drool under supervision.
- Serum sampling was collected by blood collection via cephalic venipuncture and using tubes with a coagulation activator and a gel separator. Samples were centrifuged at 3000 or 3500 x g for 5 or 10 min.
- Capillary whole blood samples were drawn from the fingertip.

Dogs (Canis lupus familiaris) and cows (Bos taurus):

- Saliva specimens were collected by placing a small piece of sponge around the mouth. When the cotton swabs were thoroughly moist, they were placed in collection devices (Salivette saliva collection tube / V-Bottom, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany) and centrifuged (3000 x *g* for 10 min, 4 °C)(Contreras-Aguilar *et al.*, 2017).
- Blood sampling was obtained via jugular, cephalic or lateral saphenous venipuncture into tubes (Aquisel Valve-Stopper, Barcelona, Spain) without anticoagulant or with a coagulation activator and a gel separator. Samples were centrifuged at 3000 or 3500 x g for 5 or 10 min.

Mussels (Mytilus galloprovincialis):

- Hemolymph was extracted from the posterior adductor muscle by gentle aspiration with a 23G x 1" syringe, centrifuged (4000g, 5 min, 4°C) and the supernatant was collected in Eppendorf tubes.
- Gills and digestive glands were extracted from each mussel, weighed and frozen at −80 °C until analysis. On the day of analysis, all samples were thawed and mechanically homogenized with an automatic homogenizer (Precellys Evolution, Bertin Technologies, Saint-Quentin, Yvelines, France) in 1:4 (weight/volume, w/v) buffer. Homogenization was performed at 6500 rpm in three cycles of ten seconds, with a 10 s pause between cycles. After 30 min incubation at room temperature samples were centrifuged (3500 rpm, for 10 min, 4 °C). The supernatant was harvested in Eppendorf tubes and analysed.

Fish (Sparus aurata and *Oncorhynchus mykiss):*

- Skin mucus was collected following the methods described by Guardiola et al. (2016). Briefly, skin mucus was collected by carefully rasping the dorsolateral surface of the fish, using cell scrapers with enough care to avoid any skin damage and contamination with blood or excretions. Skin mucus samples were homogenized with 1 vol of Tris-buffered saline (TBS, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl), vigorously shaken and centrifuged (3,000 rpm, 10 min, 4 °C).
- Blood was collected from the caudal vein with heparinized syringes and used for plasma isolation (1,500 rpm, for 10 min, 4ºC).

In all cases unless otherwise stated, after collection, all samples were immediately stored at −80 °C until analysis.

1.3. Proteomic analysis

1.3.2. Gel proteomics

For proteomic analysis, the total protein content of each sample was quantified following Bradford determination (Bradford, 1976).

2-Dimensional electrophoresis (2-DE): IPG strips (GE Healthcare Life Sciences) were carried with equal protein amount of each sample and subjected to isoelectric focusing (IEF). The second dimension was performed by subjecting reduced and alkylated IPG strips to SDS-PAGE in homemade polyacrylamide gels. Analytical gels were stained with silver using a protocol compatible with MS analysis. A minimum of three gels were carried out for each condition. Gels were digitalized and 2-DE proteome maps were analysed by specific 2D-software (ImageMaster™ 2D platinum 7.0, GE Healthcare Life Sciences, Munich, Germany) to compare spot abundances between the different conditions. Finally, those spots which differed significantly between conditions were excised manually, digested with trypsin and analysed by Mass Tandem spectrometry.

Western Blotting assays were performed as described previously by Jonsson et al. (2006), employing nitrocellulose membranes and a Mini Trans-Blot device from Bio-Rad. Briefly, after the blotting run (25 V/1 h), the membranes were blocked with 5% roti (B6429 Sigma Aldrich, St. Louis, USA) overnight, incubated with primary antibody for two hours,

and secondary antibody for one hour. Blots probed for the protein of interest were developed with Pierce ECL2 kit (Pierce, Thermo Fisher Scientific, USA) and Typhoon 9410 (GE Healthcare, Wilmington, MA, USA) or ImageQuant LAS 500 (GE Healthcare) scanners. Calculations of band intensities and molecular weights (Mw) were made with the ImageJ software.

1.3.2. Gel-free proteomics

For the gel-free proteomic studies, two approaches were employed in the present PhD thesis.

a) Labelling of proteins by Tandem Mass Tags (TMT, Thermo Fisher Scientific) were employed in Articles 2, 5, 7-9 and Appendices IV-V. From each sample, 35 µg of acetone-precipitated proteins were subjected to reduction, alkylation, digestion and labelling using 6-plex Tandem Mass Tag reagents according to manufacturer instructions (Thermo Scientific) with some minor modifications, as described previously (Martínez-Subiela *et al.*, 2017).

b) Protein precipitation and digestion was used in the case of Article 6. Serum protein extracts (100 µg serum from each dog in every time point), were methanol/chloroform precipitated and resuspended in 30 µl of sample buffer. Proteins were gel concentrated, trypsin digested and the resulting peptides desalted as previously described (Villar *et al.*, 2015).

Samples were stored at –80 ºC until mass spectrometry analysis.

1.3.3. Mass spectrometry

For gel-based proteomics, the spots of interest were excised manually and the proteins were washed, silver-destained, reduced with dithiothreitol and alkylated with iodoacetamide. In-gel digestion was performed with trypsin (Trypsin Gold, Mass Spectrometry Grade, Pro- mega, Madison, WI) for 8 h at 37 °C. Afterwards, peptides were extracted with three portions of 30 µl of 5% trifluoro acidic acid in 50% aqueous acetonitrile supported by ultrasonication for 10 min per change. Extracted peptides were dried down in a vacuum concentrator (Eppendorf, Hamburg, Germany) and separated with Ekspert nano-LC 425 (Sciex).

In the case of gel-free proteomics, the LC–MS/MS analysis was performed on Dionex Ultimate 3000 RSLS nanoflow system (Dionex, Camberley, UK) and Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) as described elsewhere (Bilić *et al.*, 2018).

For Article 6, samples were analysed by reverse-phase liquid chromatography coupled with mass spectrometry (RP-LC-MS/MS) using an EkspertTM nanoLC 415 system coupled to a 6600 TripleTOF® mass spectrometer (AB SCIEX; Framingham, US) through Information-Dependent Acquisition (IDA) followed by SWATH (Sequential Windowed data-independent Acquisition of the Total High-resolution Mass Spectra).

1.4. Biochemical analysis

All determinations were performed in automated biochemistry analysers (Olympus AU600/AU400 Automatic Chemistry Analyzer, Olympus Europe GmbH, Germany) unless otherwise stated.

Table 1. Biochemical parameters measured in this PhD thesis and method employed.

Biomarker	**Method**
Acetylcholinesterase (AChE)	Ellman *et al.*, 1961
Advanced oxidation protein products (AOPP)	Witko-Sarsat *et al.*, 1996
Alpha-amylase (sAA)	Rohleder and Nater, 2009
Aspartate aminotransferase (AST)	OSR6209 aspartate aminotransferase (AST), Beckman Coulter, Ireland
C-reactive protein (CRP)	OSR 6147 Olympus Life and Material Science Europe GmbH
Cortisol (COR) (chemiluminescence)[a]	COR Cortisol, REF LKC01, Siemens Health Diagnostics, Deerfield, IL
Cortisol (COR) (radioimmunoassay)[b]	Rotllant *et al.*, 2001
Creatine kinase (CK)	Campos *et al.*, 2009
Esterase activity (EA)	Haagen and Brock, 1992; Tvarijonaviciute *et al.*, 2012
Ferric reducing ability of plasma (FRAP)	Benzie and Strain, 1996
Ferritin	F. Hoffmann-La Roche Ltd
Haptoglobin (Hp)	Kit haptoglobin Tridelta phase range, Tridelta Development

Lactate (whole blood)	Lactate Pro lT-1710 (Arkray, Kyoto, Japan) portable lactate analyser
Lactate (colourimetrical)	OSr6193, lactate, Beckman coulter, Ireland
Lactate (HPLC/MS)	Chuang *et al.*, 2009
Superoxide dismutase (SOD)	SD125, Randox Laboratories Limited, United Kingdom
Thiols	Jocelyn, 1987; Costa, Santos and Lima, 2006
Total antioxidant capacity (TAC/ trolox equivalent antioxidant capacity 2 [TEAC2])	Erel, 2004; Tvarijonaviciute *et al.*, 2012
Total protein content (TP)	Beckman Coulter OSR6132
Trolox equivalent antioxidant capacity (TEAC1)	Arnao, Cano and Acosta, 1998
Troponin	LKTI1, Siemens Healthcare, United Kingdom
Total oxidant status (TOS)	Erel, 2005; Barbosa *et al.*, 2014
Uric acid (UA)	Fossati, Prencipe and Berti, 1980

[a], reaction performed in Immulite® 1000 analyser; Immulite System; Siemens Health Diagnostics, Deerfield, USA; [b], assay was performed using Scintillation Counter Wallac 1409, PerkinElmer

1.5. CRP-like protein purification by affinity chromatography in mussels

The digestive gland homogenates from six mussels were pooled, filtrated with 1.2 µm filters (Minisart® Syringe Filters, Sartorius) and concentrated using Amicon 3K (Millipore) until a final volume of 4 mL. The affinity chromatography column was prepared by the method described elsewhere (Pontet, Engler and Jayle, 1978; Onishi, Shimizu and Kajikawa, 1994) with some modifications: 2.5 g of Epoxy- activated Sepharose 6B (GE Healthcare) were suspended in ultrapure water and washed on a sintered-glass filter for one hour with 200 mL of ultrapure water. Then, 0.24 g of ligand O-phosphoryletanolamine (GE Healthcare) were dissolved in 200 mM $NaHCO_3$ + 500 mM NaCl pH 11. Coupling was performed by gently mixing the suspension in a stoppered vessel for 16 hours at 30 °C. The gel was left in contact for 4 hours with 1M ethanolamine pH 8 buffer to block any remaining active group. Then, the gel was washed 3 times alternating 0.1 M acetate buffer + 500 mM NaCl pH 4 and 100 mM Tris-HCl buffer containing 500 mM NaCl, pH 8. The gel was packed in a glass column (XK16, GE Healthcare). Elution operations were performed at 1 ml/min flow rate, monitored at 280 nm with an automated liquid

chromatography system (ÄKTA pure, GE Healthcare) and collected by fractions of 1 ml. Eluted fractions from the absorbance peak were then pooled and concentrated using Amicon 3K (Millipore).

1.6. Analytical validation

The analytical performance of the biochemical methods was assessed by the evaluation of precision, accuracy, and sensitivity, according to previous protocols (Kjelgaard-Hansen and Jacobsen, 2011).

1.5.1 Intra- and inter-assay precision assessment.

For intra- and inter-assay coefficient of variation (CV) calculation, samples with high, medium, and low analyte values were measured five times in a single assay run or in five consecutive days, respectively. For the determination of inter-assay CV, the samples were stored at −80 °C in aliquots to avoid freeze and thawed cycles.

1.5.2. Accuracy assessment

To evaluate the ability of the assays to recover the amount of analyte added to baseline samples, linearity under dilution and/or spiking recovery studies were performed.

Linearity under dilution was performed by the use of samples serially diluted from 1:2 up to 1:64 ratio in ultrapure water, buffer, or normal saline solution (NSS). Regression analyses were performed by the comparison of the measured analyte concentrations versus the expected analyte concentration.

Spiking recovery was performed by the mixing of two samples with different analyte values at different ratios. Recovery (in per cent) was calculated for each pool comparing expected and observed values.

1.5.3. Sensitivity

The sensitivity of the assays was assessed by the determination of the lower limit of detection (LLD). LLD refers to the lowest concentration of analyte that can be distinguished from a specimen of zero value. It was calculated based on 10 replicate determinations of the zero standards (ultrapure water, buffer, or NSS) as the mean value plus two standard deviations.

1.5.4. Stability

The effects of storage were evaluated by analysing a minimum of five samples at the moment of their preparation and after 7, 15, 60 and 120 days of -80ºC storage. To avoid the possible effects of repetitive thawing and freezing, samples were frozen in aliquots and only the vials needed for each run were employed.

Experimental design, results and discussion of the different objectives

2.1. Objective 1.

The first objective of the present PhD thesis was to evaluate the application of the "One-Health" concept in the search of novel biomarkers in different species and sample types in a clinical pathology laboratory.

This objective was covered by two reviews of the literature corresponding to Article 1, Book Chapter 1, and Appendices I-II:

- Article 1: Biomarkers of health and welfare: A One Health perspective from the laboratory side.
- Book Chapter 1: Methodology Assays for the Salivary Biomarkers' Identification and Measurement.
- Appendix I: Teaching the One Health concept to undergraduate veterinary students: a pilot study.
- Appendix II: Effects of filtration and alpha-amylase depletion on salivary biochemical analysis.

Article 1: Biomarkers of health and welfare: A One Health perspective from the laboratory side.

This study aims to provide an introductory overview of the potential applications of a One Health perspective to the biomarker's discovery and measurement.

Experimental design

A review of the state of the art about One Health and its possible applications in clinical pathology laboratories was performed. The review includes information about the OH application for different techniques, sample type or species, as well as describing the main steps and consideration for a successful adaptation of methods.

This study was performed in collaboration with researchers from the University of Glasgow (United Kingdom).

Results and discussion

Based on the interconnection between humans, animals and the environment, the One Health concept emphasises the added value of the application of trans- and inter-disciplinary strategies for the assessment of health and environmental questions. In this sense, the application of OH approaches in the search and measurement of biomarkers can provide with improvements in terms of financial saving, ethics, quantity and quality of relevant information, larger audience, among other advantages.

However, the application of the OH approach into biomarkers' research in laboratories is still scarce. In this study, we focused in the implementation of OH in biomarkers of health and welfare by (1) providing some general ideas about the type of biomarkers that can be used; (2) indicating the different sample types that can be analysed; (3) describing the main methods that can be used for their measurement; (4) defining the basis of a proper analytical validation in the sample type that it is going to be used; and (5) providing key points for a correct selection of biomarkers such as species-related particularities.

Book Chapter 1: Methodology Assays for the Salivary Biomarkers' Identification and Measurement.

This book chapter aimed to provide an overview of the most employed techniques for the identification and measurement of biomarkers of health and welfare in saliva for both humans and animals.

Experimental design

A revision of the state of the art was performed to offer a schematic view of the most important methods to discover and measure salivary biomarkers in human and veterinary species. The methodologies were divided into spectrophotometric assays, immunoassays and "omics" only for academic purposes, and their main advantages and disadvantages for their use in saliva

samples were discussed. Additionally, the basis for a correct validation of these techniques to be used in saliva was described.

Results and discussion

Even if saliva is useful reflecting the body's health and well-being (Schulz, Cooper-White and Punyadeera, 2013), the widespread implementation of saliva as a diagnostic fluid is still scarce in comparison to blood because there are some barriers such as lower biomolecular concentration or high intra- and inter-individual variations (Schulz, Cooper-White and Punyadeera, 2013; Yoshizawa *et al.*, 2013b).

Spectrophotometric assays, that consist of the quantitative measurement of the interaction of light with material at a selected wavelength (Evenson, 2001) are widely employed for saliva analysis including but not limited to: (1) ultraviolet and visible (UV-Vis) absorption spectroscopy or colourimetry, which is used in saliva for the monitoring of chronic kidney disease (Bibi, Green and Nagler, 2008). (2) Atomic Spectrophotometry is employed in saliva of patients with oral squamous cell carcinoma (Shpitzer *et al.*, 2007), while (3) near-infrared spectrophotometry is employed for the measuring of response to stress in saliva (Khaustova *et al.*, 2009, 2010). In general, spectrophotometric techniques are economic, fast, robust and required minimum sample preparation demands, being among the most used methods for saliva analyses.

Immunoassays are based on the biorecognition between an antibody and an antigen, which makes them highly specifics methods (Ju, Lai and Yan, 2017). Immunoassays include labelling-free formats, in which an observable detection signal is directly produced by the immunoreaction, and labelling formats in which signal molecules to label antigens or antibodies that produce detectable analytical signals on the immunoreaction are needed (Ju, Lai and Yan, 2017). Immunoassays include Enzyme-Linked Immunoassays (EIAs), which are among the most employed techniques for clinical diagnosis, the wash-free alphaLisa, chemiluminescence immunoassays, fluoroimmunoassays, radioimmunoassays, liquid biopsy or multiplex immunoassays, among others. Thus, there are hundreds to thousands of immunoassays to be employed in saliva for the measurement of a wide range of analytes.

On the other hand, omics techniques englobe different approaches for the study of biological molecules including DNA (genomic and epigenomics), RNA (transcriptomics), proteins

(proteomics), metabolites (metabolomics), and others such as lipids and microbiome (lipidomics and microbiomics, respectively) (Figure 1). These techniques have high sensitivity providing quantitative results and enables to analysing simultaneously hundreds of analytes, allowing detecting small but accurate differences in these molecules. This allows to evaluate entire pathways and provides with a broad overview of the hierarchical linkage between genotype and phenotype (Guillemin *et al.*, 2016), enabling a holistic view of many conditions while increasing exponentially the number of potential biomarkers (Horvatić *et al.*, 2016). The term used for the study of saliva using omics techniques is 'salivaomics' (Yan *et al.*, 2008), and salivaomics has been employed in the early diagnosis, prognosis, treatment monitoring, recurrence prediction, and personalized therapeutic strategies of a wide variety of conditions (Wong, 2007; Yang *et al.*, 2016; Kaczor-Urbanowicz *et al.*, 2017).

Figure 1. The different and complementary components of salivaomics.

In any case, every technique needs to be validated for their use in saliva. For this, several different approaches may be used depending on the characteristics of the biomarker, potential application, and techniques used. The analytical validation ensures that the analyte of interest is measured precisely and accurately, and should consist at least in precision, accuracy, and

sensitivity studies. On the other hand, the clinical validation discerns if the assays can differ between controls and test populations, evaluating its sensitivity and specificity. Since omics are highly expensive and time-consuming, normally, the identified analytes of interest by omics are further validated using spectrophotometric or immunoassays.

In conclusion, saliva can provide with broad information about the health and well-being status if the correct technique is employed. Each type of assay has its own advantages and withdraws, and their use would depend on several factors such as the analyte or species of interest. We envision that, in parallel with the development of these methodologies, saliva would continue to gain increased attention for the diagnostic and monitoring of a wide range of conditions in both humans and animals.

Appendix I: Teaching the One Health concept to undergraduate veterinary students: a pilot study.

This study aimed to evaluate the usefulness of an active learning-based methodology for teaching One Health among veterinary students.

Experimental design

The awareness about OH was assessed in n=55 second-year students of Veterinary Medicine of the University of Murcia, Spain (academic year 2019/2020) before (questionnaire 1, Q1) and after (questionnaire 2, Q2) an OH module using online surveys.

The One Health course execution consisted of three phases: (1) a one-hour masterclass; (2) seminars based on the flipped classroom in which the students were divided into groups and had to prepare a 5-minute presentation or video about an OH related topic; (3) a plenary session in which ten presentations were exposed to all the students.

Results and discussion

Before the course, when the students were asked to rate their knowledge about OH from very low to very good, 81% rated ‘low’ or ‘very low’. In contrast, after the course, 97% of respondents considered that they had intermediate, good or very good knowledge of OH ($P<0.001$) (Figure 2).

In Q1, almost half of the respondents did not mention humans, animals or the environment in their description of OH before the course, while in Q2 > 75% mentioned these three components in addition to inter- and trans-disciplinary collaborations. The estimated level of importance of certain topics such as human health, animal health, environment, economy, zoonoses or legislation into the OH concept was asked to the students prior and after the course. After the course, the perceived importance for all topics was statistically increased ($p<0.05$). Thus, these results may point out that this short active-learning-based course permitted to gain at least basic knowledge about OH for most students.

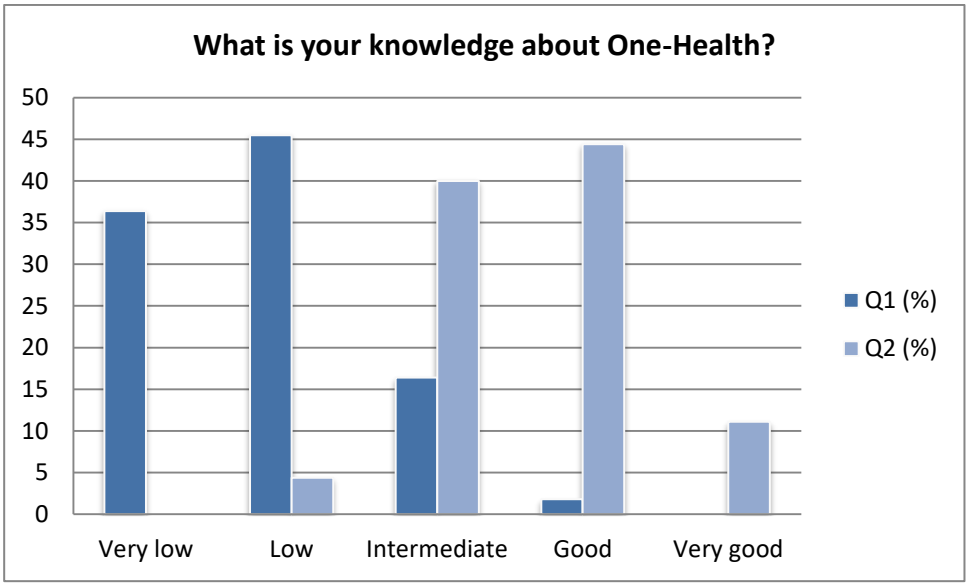


Figure 2. Perceived level of knowledge about OH in undergraduate veterinary students before (q1) and after (Q2) an OH module.

Last, the second questionnaire included two queries related to their opinion about the OH course. The first question asked if they did like the experience, being liked by two-thirds of the respondents. The second question was a free writing query in which they were asked for their opinion and suggestions for further courses. The students reported three main drawbacks for the course: (1) the groups consisted on 8-10 students, making them difficult to distribute the work and some students did not collaborate with the rest but they get the same qualification than their partners; (2) The time of exposition – which was set to five minutes- was considered too short; and (3) OH should be approached in further detail. Most of these inconveniences could be attributed to the short time for OH assigned in the veterinary curricula that conditioned the low number of groups, the exposition time and the incapacity of approach OH deeper. However,

besides these issues, the vast majority of the students provided with a positive opinion of the course and learned about OH.

In conclusion, the data recapitulated in the present study could have important implications for potential novel approaches for the developing of a One Health-based curricula. The focus on the recognition and learning of OH principles could improve the veterinary students capacity for performing inter and intra-collaborations, which are of high importance in their future profession.

Appendix II. Effects of filtration and alpha-amylase depletion on salivary biochemical analysis.

This study aimed to assess the possible effects of common saliva processing protocols on a battery of biomarkers.

Experimental design

A total of n=14 human saliva samples were employed in the present study. Each saliva sample was divided into three aliquots as follows: the first aliquot consisted of saliva samples that were only mixed and centrifuged (3500 *x g*, 10 min, 4ºC) (Pre-treatment group). The second aliquot was mixed, centrifuged (3500 *x g*, 10 min, 4ºC) and filtered being passed through a 0.45 µm commercial filter (Millipore, PAIS) (Filtered group); while the third aliquot had the same treatment as Filtered groups and was after passed through a home-made column with potato starch for the affinity depletion of alpha-amylase, as described by (Deutsch *et al.*, 2008) (Amylase depleted group). For each aliquot, a battery of biochemical biomarkers consisting on amylase, ALT, AST, GGT, ALP, lipase, CK, calcium, phosphorus, total protein, microalbumin, urea, creatinine, cholesterol, triglycerides and uric acid were measured in the same run to avoid storage or inter-assay related imprecisions.

Results and discussion

Changes in ALT, AST, GGT, lipase, CK, cholesterol and triglycerides were observed in

Filtration in comparison to Pre-treatment groups. After the depletion of alpha-amylase, differences of statistical relevance in amylase, ALT, AST, GGT, ALP, lipase, CK, calcium,

phosphorus, total proteins, urea, creatinine, cholesterol, triglycerides and uric acid were found when compared to pre-treatment values. In addition, filtered and alpha-amylase depleted samples revealed differences on amylase, ALT, AST, GGT, ALP, lipase, CK, calcium, phosphorus, total protein, urea, creatinine, triglycerides and uric acid. Although the changes in alpha-amylase and total protein were expected after depletion, the causes of the differences in the rest of biomarkers are unknown and should be further evaluated.

In conclusion, the routinely employed protocols for the preparation of saliva samples caused changes in most of the biochemical biomarkers measured. Thus, researchers should evaluate the possible influence of these protocols in their biomarkers of interest to avoid possible false results.

2.2. Objective 2

The Objective 2, consisting on proteomic and biochemical studies in different experimental and clinical situations for the discovery of novel biomarkers, was divided into three sections, each one corresponding to a different model.

Studies in humans and mussels were focused on situations where muscle damage can be produced and the studies in dogs and cows were focused in different diseases of high importance in the OH concept. In addition, a model of environmental evaluation was made in mussels and fish since these species are commonly used as sentinels of environmental health.

The use of the least invasive samples (saliva, haemolymph or skin mucus) was employed when possible.

2.2.1. Model 1: muscular damage

Model 1, corresponding to situations of muscular damage, was covered by Articles 2-4 and Appendix III.

- Article 2: Alterations in haemolymph proteome of *Mytilus galloprovincialis* mussel after an induced injury.
- Article 3: Differences on salivary proteome at rest and in response to an acute exercise in men and women: A pilot study.

- Article 4: Changes in lactate, ferritin, and uric acid in saliva after repeated explosive effort sequences.
- Appendix III: Adenosine deaminase (ADA) and its isoenzymes in saliva: analytical validation of an automated assay and applications in physical and psychological stress.

Article 2: Alterations in haemolymph proteome of Mytilus galloprovincialis mussel after an induced injury.

This experiment was carried out to identify biomarkers of muscular damage in a simple organism such as mussels that could be applied to other species.

Experimental design

In this study, *M. galloprovincialis* mussels were exposed to puncture of the adductor muscle for three consecutive days to promote muscular damage. After 48h of rest, haemolymph of these animals (Test group, n=16) and untreated group (Control group, n=16) was collected, pooled, and analysed using 2-dimensional electrophoresis proteomic approach was performed (Figure 3).

This study was performed in collaboration with researchers from the University of Veterinary Medicine of Vienna (Austria); and the Universitat Autònoma de Barcelona (Spain).

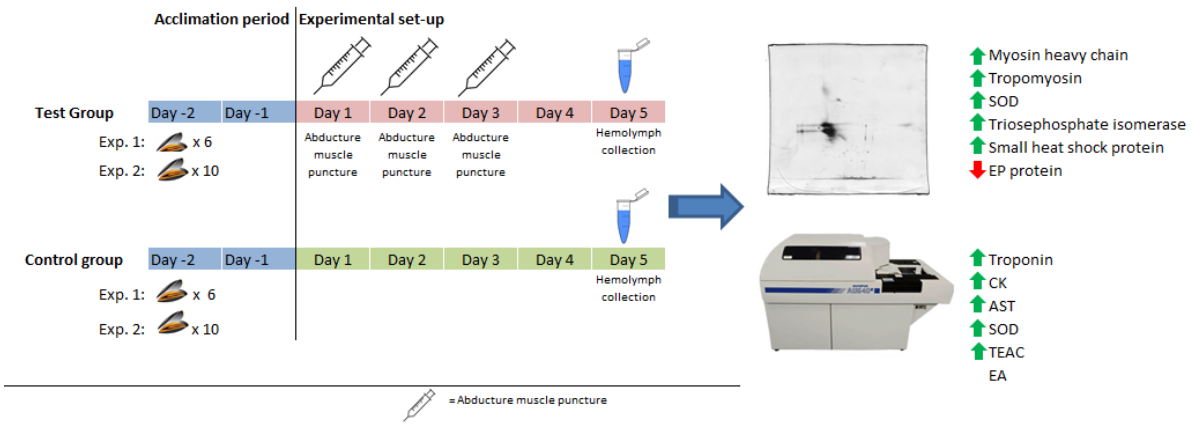


Figure 3. Experimental design and main results of Article 2.

Results and discussion

When the haemolymph proteomes of both groups were analysed and compared, five proteins identified as myosin heavy chain, tropomyosin, CuZn superoxide dismutase (SOD), triosephosphate isomerase and small heat shock protein were found to be in higher abundance in the Test group, while EP protein was in lower abundance. The results were verified by spectrophotometric assay in the case of SOD, and by western blotting in the case of tropomyosin.

Due that some of the proteins that were differently modulated between the two groups were related to muscular damage and oxidative stress, other commonly employed biomarkers for the analysis of these processes were also measured to evaluate their possible utility pointing tissue damage. Troponin, creatine kinase (CK), and aspartate aminotransferase (AST) were selected as biomarkers of muscle damage; and SOD, trolox equivalent antioxidant capacity (TEAC), and esterase activity (EA) were selected as biomarkers of oxidative stress. All the biomarkers showed higher values in the Test group in comparison to Controls, being these differences of significant ($p<0.05$) relevance in all cases except for EA.

In conclusion, there are changes in the haemolymph proteome in mussels with muscle damage, mainly in proteins related to muscular tissue and oxidative stress. Some of these and other related proteins can be easily measured and showed changes in the haemolymph of mussels employing automated assays designed for their use in other species, which can be an example of the applications of the One Health approach.

Article 3: Differences on salivary proteome at rest and in response to an acute exercise in men and women: A pilot study.

This study had two major goals. First, to evaluate possible changes in saliva proteome in response to exercise that could be employed as non-invasive biomarkers of muscular damage; and second, to compare the possible sex-related influence in saliva proteome at rest and in response to exercise.

Experimental design

Extended summary

Saliva samples from physically active sports sciences' students (five men and five women) were collected and analysed using Tandem Mass Tags following by LC-MS/MS proteomic approach at rest (T0) and 24h after a resistance session consisting in 6 sets of 10 repetitions maximum of full squats (T1).

This study was performed in collaboration with researchers from the Universidad Europea de Canarias (Spain); University of Zagreb (Croatia); Instituto de Agroquímica y Tecnología de Alimentos (CSIC, Paterna) (Spain); and King Juan Carlos University, Madrid (Spain).

Results and discussion

The analysis of the saliva proteomes allowed the identification of 274 proteins. Of those, a total of 87 proteins showed different abundance between the different groups (Figure 4).

Sixty-five proteins were found to be differentially modulated between men and women at rest. Some of these proteins were previously reported to be at different concentrations between men and women, such as in the case of lysozymes (Yeh *et al.*, 1997). Salivary alpha-amylase (sAA), which showed higher abundance at rest in women in the proteomic study, was further validated by a colourimetric assay showing higher levels in women in comparison to men at T0.

Six proteins identified as peroxiredoxin, cystatin-b, and four actins (actin, alpha skeletal muscle; actin, aortic smooth muscle; actin, gamma-enteric smooth muscle; and actin, alpha cardiac muscle 1) were modulated by the exercise. Actins, peroxiredoxin and cystatin-b had been previously related to exercise and had been proposed as biomarkers of sports performance (Wadley, Aldred and Coles, 2016; Tékus *et al.*, 2017; Stattin *et al.*, 2019) or muscular damage (Gornik and Lauc, 2008).

Sixteen proteins were modulated by the interaction of exercise and sex. Of those, catalase in blood had been described to be differentially modulated by exercise in a sex-dependant manner (Souglis *et al.*, 2018).

Taking overall, this study provided evidence of changes in saliva proteome due to (1) sex; (2) exercise; and (3) the interaction of sex and exercise. These differences associated with sex and/or exercise should be taken into consideration for athletes, coaches or researchers in sports

sciences. Additionally, the modulated proteins after the exercise could be considered as potential non-invasive biomarkers of sports performance or muscular damage and should be studied in further detail.

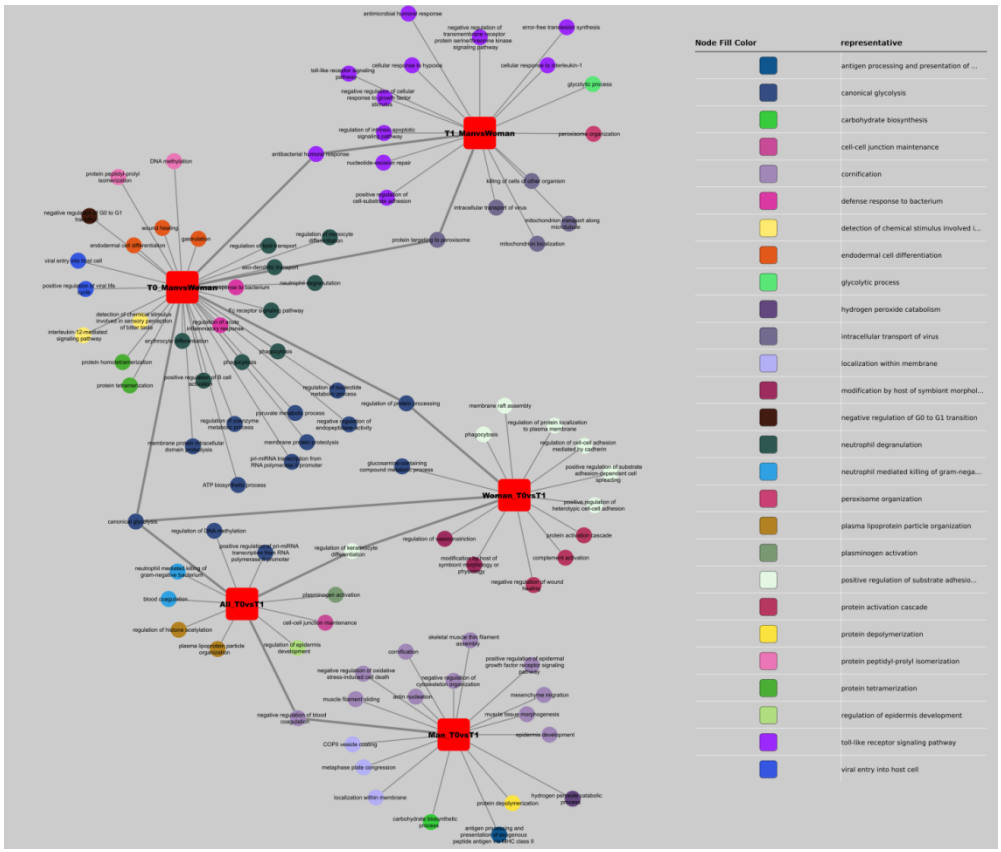


Figure 4. Graphical representation of proteins differentially expressed between the different comparisons: men versus woman before and after the exercise (T0_ ManvsWoman and T1_ManvsWoman, respectively), all individuals before and after exercise (All_T0vsT1), and men and woman before versus after the exercise (Man_T0vsT1 and Woman_T0vsT1, respectively).

Article 4: Changes in lactate, ferritin, and uric acid in saliva after repeated explosive effort sequences.

This study aimed to evaluate the possible changes in the saliva of a battery of already-used biomarkers in sports sciences. For this, lactate, ferritin and uric acid were measured in saliva of trained and untrained subjects during an exercise consisting of repeated explosive effort sequences. Additionally, the possible influence of how data is expressed was assessed.

Experimental design

Extended summary

Saliva samples from a total of 18 physically active men were employed. They were divided into two groups according to their previous experience in repeated explosive effort sequences (sprints) into: Untrained group consisting in n=11 sports sciences students, and Trained group consisting n=7 Spanish national high-level sprinters.

The sprints session consisted of a standardized 5 min warm-up protocol followed by 8 series of 60m sprint at the highest possible speed, separated by 4 min recovery between series. Saliva was sampled before the exercise (T1) and immediately after each session (T2 to T9). All sessions were performed at the same time of the day and under similar environmental conditions. The extent of fatigue was quantified by two methods: (1) by analysing the increment in time between the first and the eight sprints; and (2) by calculating the jump height difference between the first and eight sprints (change in countermovement jump, CMJ).

Lactate, ferritin and uric acid concentrations in saliva were validated and measured at each time point for each subject and blood lactate was measured after each saliva sampling. Finally, data of saliva analytes was presented without any correction and corrected by protein content and salivary flow rate in order to evaluate its possible influence in results interpretation.

This study was performed in collaboration with researchers from the Catholic University of San Antonio, Murcia (Spain); and Quirón Hospital, Barcelona (Spain).

Results and discussion

First, the validity of the exercise model was probed by the decreases in speed loss and CMJ, and by the increase of blood lactate in all-time points when compared to T1, which is commonly employed as a reference standard for tissue hypoxia during exercise (McLellan, Cheung and Jacobs, 1991; Urhausen *et al.*, 1993; Santos *et al.*, 2006; Ohkuwa *et al.*, 2007).

The three analytes measured in saliva showed adequate linearity and low imprecision and, for lactate in saliva the results were further validated by HPLC/MS. Lactate in saliva increased during the exercise session in both groups when compared to T1 concentrations (Figure 5) and T1 lactate was 5-fold lower and showed lower increased during sprints in Trained than Untrained group. Lactate in saliva and blood was positively correlated only in the Untrained group, which could be explained by the higher parasympathetic tone in untrained subjects that modifies the

kinetics of lactate in saliva but no in blood, as proposed elsewhere (Tékus *et al.*, 2012). A difference in the time of filtration of lactate from blood to salivary glands between trained and untrained subjects (Santos *et al.*, 2006) could also explain our findings. The correction of lactate in saliva by protein content provided with higher correlation with blood lactate and showed increases of statistical relevance at earlier time points.

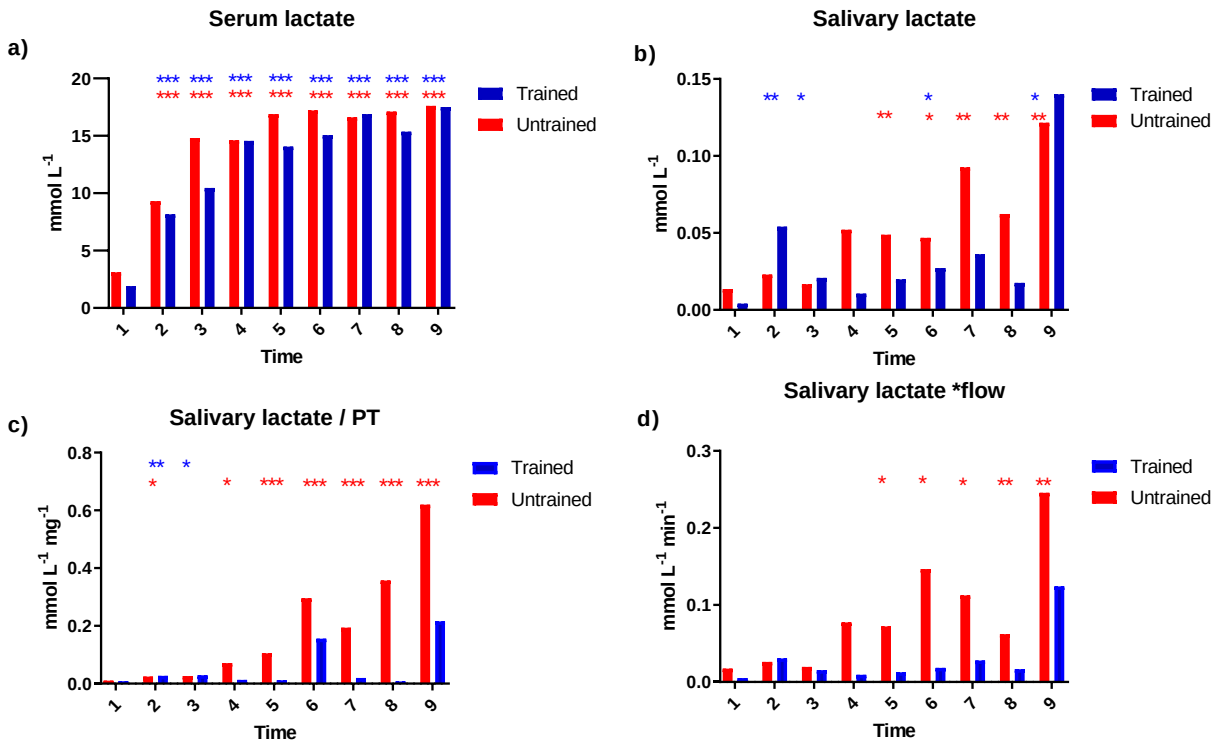


Figure 5 Median concentration of lactate before (T1) and after consecutive 60m explosive effort sequences (T3 to T9). a) Blood lactate, b) Salivary lactate (absolute concentration), c) Salivary lactate concentration after correcting by salivary protein

No changes of statistical relevance were observed for ferritin nor uric acid in our model with or without correcting data by protein content or flow rate. These results were unexpected since ferritin in serum and saliva are positively correlated (Canatan and Akdeniz, 2012) and serum ferritin increases after endurance training (Govus *et al.*, 2014); similarly uric acid was reported to increase after 30-minute exercise sessions (González *et al.*, 2008; Deminice *et al.*, 2010). We

hypothesize that the lack of changes in ferritin and uric acid in saliva observed could be associated with the low length of the hypoxic exposure in our study that may be insufficient to promote changes in these analytes.

In the present study, high inter-individual variability was observed. This can be attributed to different degrees of fatigue or different kinetics for saliva biomarkers that should be analysed in further studies with a larger population.

The model of repeated explosive effort sequences employed in the present study provides with several conclusions. (1) The measurement of lactate in saliva could potentially be employed as a non-invasive alternative to the commonly-employed blood lactate for the evaluation of fatigue. (2) The correction of lactate in saliva by protein content showed a higher correlation with blood lactate and allows detecting fatigue earlier. This points out the benefits of evaluating the ways of reporting data for salivary biomarkers. (3) The high inter-individual variation and the existence of different saliva dynamics between trained and untrained individuals should be further studied.

Appendix III: Adenosine deaminase (ADA) and its isoenzymes in saliva: analytical validation of an automated assay and applications in physical and psychological stress.

This study aimed to validate an automated assay for the measurement of total adenosine deaminase (tADA) and its isoenzymes (ADA1 and ADA2) in human saliva using a high-throughput automated method and to evaluate if ADA and its isoenzymes could change in different situations of physical and psychological stress.

Experimental design

Saliva samples from a total of thirty-three participants were employed in the study. tADA was measured by using a commercially available assay, and ADA1 and ADA2 isoenzymes were assessed by the addition of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) to the samples, an ADA1 inhibitor (Muraoka *et al.*, 1990). The results after EHNA inhibition corresponded to ADA2, while ADA1 was calculated subtracting ADA2 from tADA. The two methods were adapted for

their use in human saliva, including the calculation of the most appropriate EHNA concentration, an analytical validation, and the possible influence in the way of reporting data was evaluated.

After method development, ADA and its isoenzymes were measured in saliva and compared to other biomarkers in different situations: (1) resistance training leading to failure, as described in Article 2, in which ADA and IgA were measured in saliva and serum, and white blood cells were assessed in the blood; (2) repeated explosive effort sequences, as described in Article 4; in which ADA was measured in saliva and lactate was assessed in blood; and (3) psychological stress by trier social stress test, based on previous reports (Nater *et al.*, 2006; Campbell and Ehlert, 2012), being ADA and alpha-amylase measured in saliva.

Results and discussion

The automated assay employed in the study was easy to perform and set-up, economic, rapid, requires low sample volumes and the addition of EHNA directly to the commercial reagent up to 0.12mM concentration allowed the measurement of ADA2 and the estimation of ADA1 isoforms in human saliva and serum. The assays showed an adequate analytical performance according to with the limits for good method functionality (U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), 2001; Cossio *et al.*, 2012). Our results suggested that ADA activity is higher in human serum than in saliva, and the predominant isoforms were ADA2 for serum and ADA1 in the case of saliva.

The situation of training leading to failure did not cause the alteration of any biomarker, including IgA which has been reported to be modulated in a wide variety of intense exercises such as marathon (Nieman *et al.*, 2002) or 50km ski race (Tomasi *et al.*, 1982). However, the correlations observed between tADA and ADA2 with salivary IgA ($r=0.622$ and $r= 0.738$, respectively) and blood leukocytes ($r=-0.596$ and $r=-0.726$, respectively) ($p<0.05$ for all cases) could indicate that ADA and its isoenzymes in saliva may be considered as potential biomarkers for the evaluation of the immune system.

For the assessment of ADA in a situation of repeated explosive effort sequences, two populations with different tolerance to fatigue were included, since different kinetics between these populations were reported previously (Franco-Martínez *et al.*, 2019). In accordance decreases of statistically relevance in tADA after 3, 4, 5, 6 and 8 sprints and a negative correlation between

tADA in saliva and blood lactate ($r=-0.381$, $p<0.001$) were observed only in the untrained group. Thus, our results highlighted the differences in dynamics between trained and untrained subjects, and tADA could point out the effects of exercise on the immune system in untrained subjects.

When ADA was assessed in a situation of psychological stress such as Trier Social Test, no correlations with salivary alpha-amylase nor differences between pre- and post-measurements were observed, although a tendency to decreased tADA and ADA1 after 15 minutes, was present ($p>0.05$). Thus, ADA in saliva could not be a suitable biomarker for the assessment of short-term psychological stress, at least under the circumstances of the present study.

In conclusion, tADA and its isoenzymes ADA1 and ADA2 can be measured accurately and precisely in saliva and serum samples in a rapid, economic, and reproducible way. ADA and its isoenzymes in saliva were correlated to other already-used biomarkers in the two situations of physical stress, making them feasible alternatives in these contexts that deserve further study.

2.2.2. Model 2: pathologies

The Model 2, corresponding to situations of pathology, was covered by Articles 5-9 and Appendices IV-V.

- *Article 5: Serum proteome of dogs at subclinical and clinical onset of canine leishmaniosis.*
- *Article 6: Changes in saliva of dogs with canine leishmaniosis: a proteomic approach.*
- *Article 7: Changes in salivary analytes in canine parvovirus: A high-resolution quantitative proteomic study.*
- *Article 8: Changes in the salivary proteome associated with canine pyometra.*
- *Article 9: Changes in serum and salivary proteins in canine mammary tumours.*
- *Appendix IV: The serum and saliva proteome of dogs with diabetes mellitus.*
- *Appendix V: Changes in saliva and serum proteins in cows with mastitis: a proteomic approach.*

Articles 5-9 and Appendices IV-V: proteomic studies for the identification of biomarkers of disease in dogs and cows.

For the cover of the Model 2, different pathologies were studied from a high- -throughput quantitative proteomics approach. In all cases except for Appendix V, the pathologies were important and common canine diseases. Dogs were selected since they were proposed as animal models for human medicine and, similarly by applying a One Health concept, advances in human medicine and research such as proteomic studies in saliva can be applied to dogs. On the other hand, mastitis in cows was studied because is the most frequent infectious problem in dairy cow and it has huge importance from an OH approach in terms of societal impact, animal health and welfare, food security and safety, responsible use of antibiotics and economy, among other fields.

Experimental design

In these studies, serum or/and salivary proteomes were analysed by the use of gel-free proteomics techniques with the aims of identifying potential biomarkers for each disease. Proteomics was performed using Tandem Mass Tags approach unless otherwise stated (Article 5).

For bioinformatics analysis, the genes encoding the differentially abundant proteins between DM and HC groups were used to determine the GO terms over-represented in DM using Protein Analysis Through Evolutionary Relationships (PANTHER) classification tool (http://www.pantherdb.org/) or Cytoscape (v3.6.1) plug-in ClueGO (v2.5.0) (Bindea *et al.*, 2009).

Article 5: Serum proteome in leishmaniosis. In this study, serum samples from the same dogs (n=4) were analysed using a high-throughput label-based quantitative LC-MS/MS approach in three different moments of the development of an experimental-induced canine leishmaniosis through intravenous injection with 1 x 10^6 stationary-phase infective *L. infantum* promastigotes: pre-infection (T0), increased in ferritin and CRP serum levels with absence of external clinical signs (T1), and when at least two clinical signs that can appear in canine leishmaniosis were present (T2).

This study was performed in collaboration with researchers from the Consejo Superior de Investigaciones Científicas (CSIC, Castilla–La Mancha) (Spain), Consejo Superior de

Investigaciones Científicas (CSIC, Granada) (Spain), Université Paris-Est (France), and Oklahoma State University (United States).

Article 6: Saliva proteome in leishmaniosis. Saliva samples from the same dogs (n=4) were analysed by high-resolution quantitative proteomics approach before (T0) and after an experimental infection with *Leishmania infantum* and the development of clinical signs (T1). The animals consisted of four beagle dogs from the University of Murcia that were subjected to experimental infection through intravenous injection with 1 x 10^6 stationary-phase infective *L. infantum* promastigotes. Dogs were clinically and analytically evaluated monthly and T1 was sampled when they showed external symptoms of the disease (Figure 6).

This study was performed in collaboration with researchers from the University of Zagreb (Croatia), and Consejo Superior de Investigaciones Científicas (CSIC, Granada) Spain.

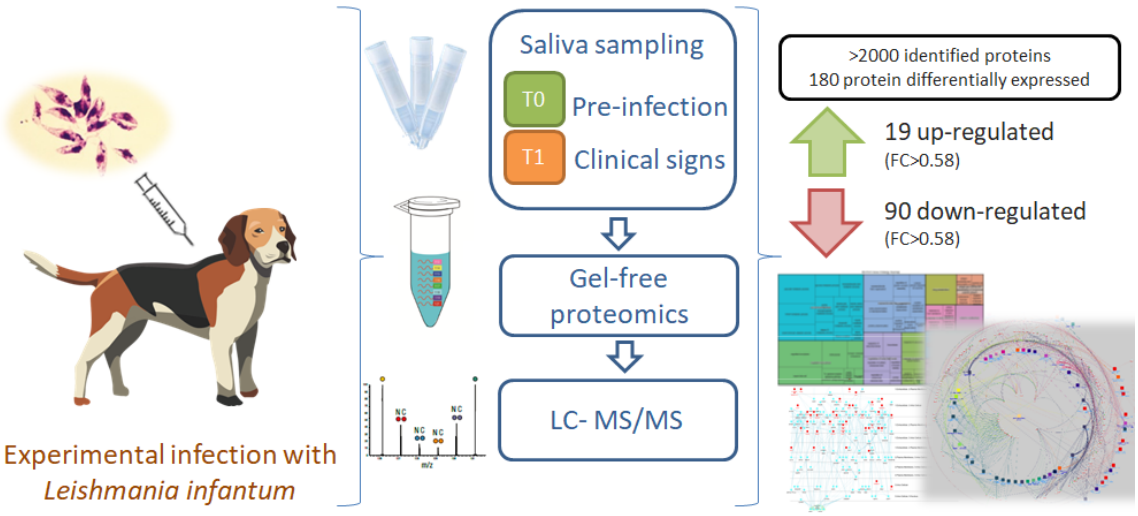


Figure 6. Experimental design and main results of Article 6.

Article 7: Saliva proteome in parvovirosis. Saliva samples from healthy dogs (Healthy group, n=5) and dogs diagnosed with severe canine parvovirosis that survived (Survival group, n=3) or perish because of the disease (Non-surviving group, n=3) were employed. All animals were client-owned dogs that were presented to the Veterinary Teaching Hospital of the Uludag University

(Turkey). Parvovirosis infection was diagnosed by the existence of compatible clinical including acute bloody diarrhoea, vomiting, anorexia and dehydration; haematological signs including leukopenia, lymphopenia and neutropenia; and positive test results of the commercially available faecal diagnostic test (Antigen Rapid PVE kit, Animal Genetics, Inc., Suwon, Korea). Healthy dogs did not show any clinical or analytical alteration and were negative to the commercially available faecal diagnostic test. All dogs with parvovirosis were treated as described elsewhere (Yilmaz and Senturk, 2007).

The data obtained in the proteomic study were used for Gene Ontology (GO) analysis by conversion of canine genes encoding proteins differentially expressed to their human orthologs and determining to GO terms over-represented using Cytoscape (v3.6.1).

This study was performed in collaboration with researchers from the University of Zagreb, (Croatia); Uludag University (Turkey); Universitat Autònoma de Barcelona (Spain); University of Glasgow (United Kingdom); and University of Évora (Portugal).

Article 8: Saliva proteome in pyometra. Saliva samples from client-owned bitches of different breeds presented to the Department and Clinic of Animal Reproduction, University of Life Sciences in Lublin, Poland were analysed by Tandem Mass Tags-based proteomic approach. All animals were in diestrus and, according to their health status, they were classified as healthy controls (H group, n=5) or dogs with open-cervix pyometra (P group, n=5). Pyometra diagnosis was based on clinical examination (with all cases showing pyrexia, apathy, polydipsia-polyuria, and anorexia), haematology (with increased WBC count and band neutrophil counts), biochemistry (with increased globulins and CRP), and abdominal ultrasound findings consistent with pyometra, as described previously (Tecles *et al.*, 2018). Besides, haptoglobin in saliva was further validated by a time-resolved fluorometry-based immunoassay in an independent sample set consisting of n=7 healthy and n=8 bitches with pyometra.

This study was performed in collaboration with researchers from the Mediterranean Institute for Life Sciences (MedILS) (Croatia), University of Zagreb (Croatia), and University of Life Sciences in Lublin (Poland).

Article 9: Serum and saliva proteomes in mammary tumours. Paired serum and saliva samples of n=5 healthy (HC) and n=5 bitches with mammary tumours (CMT) were analysed using Tandem Mass Tags proteomic approach. All bitches were spayed less than one year ago to minimize hormonal influence and, for CMT, TNM classification was grades 1 and 2 in all cases (i.e. tumour diameter ≤3 cm, and absence of regional lymph node or distant metastases) (Owen, 1980). An independent sample set (n=25) were used to validate serum albumin as a candidate biomarker of canine mammary tumours.

This study was performed in collaboration with researchers from the Mediterranean Institute for Life Sciences (MedILS) (Croatia), University of Zagreb (Croatia), and University of Life Sciences in Lublin (Poland).

Appendix IV: Serum and saliva proteomes in diabetes mellitus. Paired serum and saliva samples of dogs with untreated diabetes mellitus (DM, n=5) and healthy dogs (HC, n=5) were analysed using Tandem Mass Tag-based proteomic approach. Also, serum samples from healthy (n=13) and dogs with DM (n=13) were used for the validation of haptoglobin in serum using a commercial kit in an independent sample set. Dogs with diabetes mellitus were diagnosed based on clinical signs including polyuria, polydipsia and polyphagia and laboratory findings including glucosuria and hyperglycaemia (glucose >200 mg/dL) as previously described (Durocher *et al.*, 2008).

This study was performed in collaboration with researchers from the Mediterranean Institute for Life Sciences (MedILS) (Croatia), and the University of Zagreb (Croatia).

Appendix V: Serum and saliva proteomes in cows with mastitis. Paired serum and saliva samples from cows with mastitis (M, n=18) and healthy controls (HC, n=18) from a dairy farm in Lithuania were analysed using Tandem Mass Tag (Thermo Fisher Scientific). HC did not show any clinical signs nor abnormalities in milk analysis, with somatic cell count <150 000 cells/mL in all cases, while all animals from M group showed clinical signs compatible with mastitis and presented clots and pathogens (*Streptococcus uberis* and/or *Streptococcus agalactiae*).

This study was performed in collaboration with researchers from Lithuanian University of Health Sciences (Lithuania), Mediterranean Institute for Life Sciences (MedILS) (Croatia), and University of Zagreb (Croatia).

Results and discussion

Article 5: Serum proteome in leishmaniosis. A total of 169 proteins were identified, of which 74 were differentially represented between the three time points. Additionally, haptoglobin was further validated by a colourimetric assay, verifying the higher concentrations observed in positive to Leishmania dogs with clinical signs in comparison to asymptomatic ones.

When compared T0 and T1, only complement C8 alpha chain was differentially modulated, showing a decrease a T1. Since some studies have related the complement cascade activation to anti-Leishmania defence (Solbach and Laskay, 1999; de Amorim *et al.*, 2011), the decrease of complement C8 at T1 could be interpreted as a less-effective response of the dog and the beginning of the onset of the disease.

When T1 and T2 serum proteomes were compared, 30 proteins were found to be differentially modulated. Of those, only adiponectin and transferrin were decreased at T2. Adiponectin is related to inflammation and immune response and is deregulated in several inflammatory conditions such as acute pancreatitis (Paek *et al.*, 2014) or diabetes mellitus (Kim *et al.*, 2015). On the other side, transferrin is a negative acute-phase protein in dogs (Cerón, Eckersall and Martínez-Subiela, 2005) and it has been found in lower concentrations in dogs with canine leishmaniosis in comparison to healthy ones (Burillo *et al.*, 1994). Among the proteins up-regulated in T2 in comparison to T1, some proteins such as complement C8 and a variety of immunoglobulin's are directly related to immune defence mechanisms.

The comparison between T0 and T2 revealed 13 up-regulated proteins at T2. These proteins included immunoglobulins and other proteins related to defence such as sphingomyelin phosphodiesterase acid-like 3A, which has been proposed as a potential novel anti-inflammatory protein presented in macrophages (Traini *et al.*, 2014).

Lastly, the pathways that are related to the differentially expressed proteins were identified by bioinformatics. At the symptomatic stage, the proteins were mainly related to blood coagulation,

gonadotropin-releasing hormone receptor pathway, pentose phosphate and plasminogen activating cascade pathways.

In conclusion, this study revealed several biological pathways such as blood coagulation or gonadotropin-release hormone receptor that were modulated in canine leishmaniosis. Additionally, 74 proteins that could be employed as potential serum biomarkers of early diagnosis or disease progression in canine leishmaniosis were identified, and haptoglobin was further verified in an independent sample set as a potential biomarker for the follow-up of canine leishmaniosis.

Article 6: Saliva proteome in leishmaniosis. A total of 2218 proteins were identified and 90 unique proteins showed different abundance between T0 and T1. Of those, 66 showed a fold change higher than ± 2-fold. Among the proteins most down-regulated in canine leishmaniosis were thymosin beta-10 and beta-4, haemoglobin, carbonic anhydrase 2 and apolipoprotein A-1. These proteins participate in wound healing stimulation, or antioxidant and antimicrobial defence, among others functions. Opposing these, the proteins most up-regulated in the saliva of dogs after the development of canine leishmaniosis included immunoglobulins, olfactomedin-4 and ceruloplasmin-like protein, which has defence and antioxidant functions.

Some of the proteins that were observed to change in this study such as immunoglobulins, thymosin beta-4, vitamin D binding protein or apolipoprotein A were previously proposed as biomarkers of leishmaniosis (Escribano *et al.*, 2016; Cantos-Barreda *et al.*, 2017; Jaegger *et al.*, 2017).

Additionally, the cellular pathways that were over-represented by the modulated proteins were evaluated. They were mainly related to antioxidant activity, binding, and catalytic activity, being these pathways the most affected in other canine diseases such as parvovirosis (Article 6). The network created by bioinformatic analysis revealed that carbohydrate metabolism may play an important role in the development of the disease, which was also described in a previous study that analysed serum in dogs with leishmaniosis (Saunders *et al.*, 2010).

In conclusion, this study provides evidence of changes in saliva composition occurring after the development of canine leishmaniosis. The proteins identified in this study could be considered as

possible non-invasive biomarkers of diagnosis of canine leishmaniosis and should be further studied to verify their real utility.

Article 7: Saliva proteome in parvovirosis. The proteomic study quantified a total of 1516 peptides, of which 190 proteins were shown to be differentially modulated between the three studied groups. The comparison between Healthy and Survival groups revealed 90 proteins that were differentially modulated. The comparison between Healthy and Non-surviving groups showed 90 proteins with different abundance; while the comparison between the two groups with canine parvovirosis revealed 10 proteins that were modulated differently.

In general, the bioinformatics analysis of the proteins that were differentially modulated in saliva between the three groups pointed out alterations in coagulation and inflammation systems, which are two closely-related processes that are typically affected during canine parvovirosis (Esmon *et al.*, 1999; Coorens *et al.*, 2017). When diseased (Survival + Non-surviving groups) were compared to Healthy, more of 85% of the differentially expressed proteins were related to catalytic activity and binding pathways (Figure 7). Some of the proteins that participated in binding or catalytic activity such as apolipoprotein A-1, or neutrophil esterase had been previously related to parvoviral enteritis or other diseases in dogs and humans (Scott *et al.*, 2002; Escribano *et al.*, 2016), being proposed as feasible biomarkers.

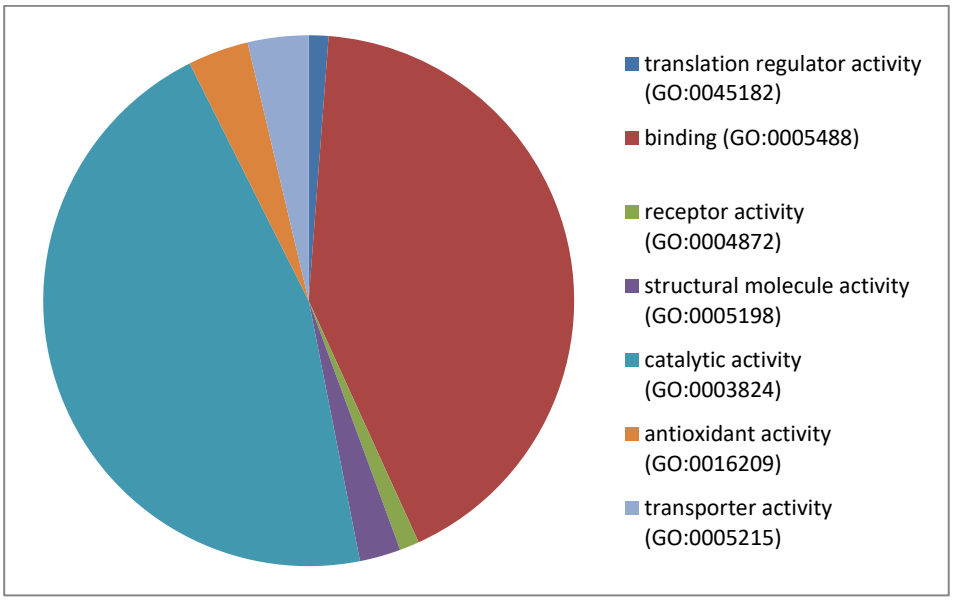


Figure 7: Functional characterization of genes differentially expressed in dogs with parvoviral enteritis.

In conclusion, this study provided for the first time an overview of the saliva proteome in dogs with canine parvovirosis with different outcomes. This information allowed the description of the biological roles of the proteins affected during the disease, which could be considered as potential non-invasive biomarkers for the diagnosis or prognosis of the disease.

Article 8: Saliva proteome in pyometra. A total of 707 proteins were quantified in saliva, 16 of these were found to be differentially modulated between H and P groups.

Vimentin, S100A12 and haptoglobin were among the most up-regulated proteins in pyometra. In agreement with our finding, vimentin has been associated with sepsis (Strela *et al.*, 2020); while S100A12 gene expression was described to be upregulated in the uterus of bitches with pyometra (Bukowska *et al.*, 2014), and S100A12 in serum and plasma has been proposed as a diagnostic and prognostic biomarker of sepsis in humans (Tosson *et al.*, 2018; Dubois *et al.*, 2019). Haptoglobin is a moderate acute phase protein in dogs, been proposed as a serum biomarker of the postoperative period in bitches with pyometra (Dabrowski *et al.*, 2009). In this study, the proteomic results for haptoglobin were further verified by a time-resolved fluorometry based immunoassay validated for its use in canine saliva, showing an increase in haptoglobin in the saliva of bitches with pyometra in comparison to healthy ones.

The bioinformatics analysis according to Protein Analysis Through Evolutionary Relationships (PANTHER) classification tool revealed that the differentially modulated proteins were mainly associated to binding and catalytic activities molecular functions, and were mainly related to pro-inflammatory mediators, acute phase proteins and sepsis.

In conclusion, this study revealed changes in the salivary proteome of bitches with pyometra, reflecting different physiopathological changes occurring in this disease. Some of the proteins described in this study were previously related to sepsis or pyometra but, to the best of our knowledge, they were proposed as potential saliva biomarkers for canine pyometra here for the first time. Thus, the proteins identified as differentially expressed in the salivary proteome in bitches with pyometra could be a source of potential non-invasive biomarkers for this disease and should be confirmed in future studies.

Article 9: Serum and saliva proteomes in mammary tumours. The proteomic analyses quantified a total of 379 proteins in serum and 730 proteins in saliva. Of these, 35 and 49 were observed to be differentially modulated between HC and CMT in serum and saliva, respectively. While some proteins were previously related to canine mammary tumours or breast cancer, for others their relationship has been described in this study for the first time.

In serum, fibrinogen A alpha chain was the most upregulated protein. This protein may have a protective role against tumour growth and metastasis (Wang *et al.*, 2020), and has been proposed as a biomarker for certain types of breast cancer (Cheng *et al.*, 2005). On the other side, interleukin 13 receptor subunit alpha 2 precursor and immunoglobulin gamma heavy chains A and D were the proteins most downregulated. These proteins play key roles in the immune response against cancer, being interleukin 13 proposed as a predictor of outcome in breast cancer (Kwon, Choi and Bae, 2018), and needing further studies to clarify the downregulation observed in immunoglobulins and its relation to CMT. In addition, acute phase proteins were modulated in serum in CMT, including higher haptoglobin and lower albumin. Haptoglobin increases in serum were reported previously in CMT (Planellas *et al.*, 2009; Tecles *et al.*, 2009); while the downregulation of albumin was further verified using a commercially available kit in 10 healthy and CMT dogs.

In saliva, several members of the calcium-binding S-100A family (S100A2, S100A4, and S100A6) were among the most upregulated proteins in CMT. These proteins were described to be associated to different cancer progression and worse prognosis in humans (Komatsu *et al.*, 2000; Platt-Higgins *et al.*, 2000; Rudland *et al.*, 2000; Pedersen *et al.*, 2002; Wang *et al.*, 2010; Bai *et al.*, 2018; Maděrka *et al.*, 2019), although others related S100A6 expression with better prognosis in breast cancer (Zhang *et al.*, 2017). However, no reports were found about S100A family members and canine mammary tumours. Calmodulin like proteins, that were upregulated in saliva in CMT, were overexpressed in serum in patients with breast cancer (Hait and Lazo, 1986) and have been proposed as a therapeutic strategy against cancer (Fancy *et al.*, 2018). Kallikrein-1-like protein was the only downregulated protein in saliva in CMT. This protein has been related to cancer previously, although its roles are still controversial with studies associating kallikrein with better

(Wolf *et al.*, 2001) or worse income (Avgeris, Mavridis and Scorilas, 2012), and its expression in breast tissues is hormonally regulated (Yousef *et al.*, 1999).

Taking overall, the serum and saliva proteomes in bitches with CMT are different from healthy dogs. Bioinformatics analyses revealed that most of the modulated proteins have binding or catalytic activity molecular functions. Some of the differentially expressed proteins found in the present study such as haptoglobin or S100A4 have been related to CMT or human breast cancer previously, which confirm the validity of our study as a model for the evaluation of CMT or breast cancer; while others including kallikrein 1 and interleukin 13 receptor have been described in CMT here for the first time and their role as potential novel biomarkers for CMT should be further evaluated. Overall, this data reflects the changes that occur in the proteome of serum and saliva in dogs with CMT and could be a potential source of new biomarkers for this disease.

Appendix IV: Serum and saliva proteomes in diabetes mellitus. The proteomic analysis quantified 389 and 767 proteins in serum and saliva, respectively, being 16 proteins in serum and 26 in saliva differentially represented between the two groups.

In serum, most of the modulated proteins had a catalytic activity or binding molecular functions. Some of the differently expressed proteins were apolipoprotein D, haptoglobin and transferrin, which may be explained by the inflammation caused by DM (Fernández-Real, López-Bermejo and Ricart, 2002). In the case of haptoglobin, the proteomic results were verified by a commercial kit, showing higher haptoglobin in dogs with DM in comparison to healthy ones. These proteins have been previously related to DM (Awadallah *et al.*, 1978; McMillan, 1989; Sadrzadeh and Bozorgmehr, 2004; Kruger *et al.*, 2010; Golizeh *et al.*, 2017); however, they were described in canine DM in this study for the first time.

In concordance to serum, most proteins differently modulated in saliva in DM were associated with catalytic activity and binding molecular functions. Proteins with catalytic activity such as heat shock cognate 71 kDa protein (Karthik *et al.*, 2012; Safaei *et al.*, 2017), triosephosphate isomerase (Chen *et al.*, 2013) and enolase 2 (Sreekumar *et al.*, 2002) were previously related to DM in other species. Similarly, proteins identified in this study with binding molecular functions were related to DM or its associated complications in the literature. For example, S100A family proteins were reported to be upregulated in the saliva of diabetic human adults (Caseiro *et al.*,

2013) and children (Cabras *et al.*, 2010), and were proposed as a predictive factor for diabetes-related microvascular complications (Caseiro *et al.*, 2013; Lim *et al.*, 2019), while alterations in calmodulins promoted the development of severe DM in mice (Epstein, Overbeek and Means, 1989).

In conclusion, there are changes in serum and saliva proteome in dogs with diabetes mellitus that provide with information about the affected pathways and could be a source of potential biomarkers of the disease. Serum proteome bioinformatics analysis revealed changes in the oxidative status, defence and inflammation, and some proteins such as transferrin and haptoglobin were not described previously in canine DM. Saliva bioinformatics analysis revealed changes in cellular and metabolic processes related to the pathogenesis of DM and insulin resistance, and while some of the modulated proteins in saliva such as HSPA8, NME2 or S100A9 have been previously related to DM complications such as hypertension or micro-vascular alterations before, others such as S100A2 and S100A11 were described in DM in this study for the first time.

Appendix V: Serum and saliva proteomes in cows with mastitis. A total of 1299 and 2192 proteins were identified from serum and saliva samples, respectively. Of those, in serum, 29 were differentially modulated between HC and M, being 19 up-regulated. Serum amyloid A (SAA), serpin A3, immunoglobulin lambda and kappa, alpha1-antichymotrypsin, vitamin K-dependent protein Z, complement factor and prothrombin were among the most up-regulated, while plasma retinol-binding protein, serum albumin showed the most downregulation.

In saliva, 63 proteins were observed to change significantly between the two groups, being 45 of them upregulated. Lung and nasal epithelium carcinoma-associated protein, Keratin, Lysozyme, Beta-lactoglobulin, Beta-casein, S100 calcium-binding protein were the most up-regulated proteins; opposing Profilin 1, Von Ebner gland protein, Leukocyte elastase inhibitor and Actin that showed the most marked downregulation. Some of these proteins have important roles related to inflammation or defence.

In conclusion, this is the first report in which paired saliva and serum samples of cows with mastitis were analysed using TMT and compared to a healthy control group. This approach allowed not only the identification of potential biomarkers of the disease in both serum and

saliva – which may provide important benefits from the animal, human & environmental points of view -, but also may pose an initial step for further knowledge of the physiopathology of mastitis that can allow earlier diagnosis and better treatments.

2.2.3. Model 3: environmental contamination

Model 3, corresponding to the evaluation of the environment, was covered by Articles 10-14.

- *Article 10: Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of Mytilus galloprovincialis exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure.*
- *Article 11: Measurement of p-nitrophenyl acetate esterase activity (EA), total antioxidant capacity (TAC), total oxidant status (TOS) and acetylcholinesterase (AChE) in gills and digestive gland of Mytilus galloprovincialis exposed to binary mixtures of Pb, Cd and Cu.*
- *Article 12: New potential biomarkers of oxidative stress in Mytilus galloprovincialis: Analytical validation and overlap performance.*
- *Article 13: Tools to assess effects of human pharmaceuticals in fish: A case study with gemfibrozil.*
- *Article 14: Chemiluminescent assay as an alternative to radioimmunoassay for the measurement of cortisol in plasma and skin mucus of Oncorhynchus mykiss.*

Articles 10-14: innovations in the assessment of the environmental state.

For the cover of the Model 3, the environmental state was analysed indirectly by the changes observed in two common bioindicators of marine ecosystems: mussels (*Mytilus galloprovincialis*) and fish (*Oncorhynchus mykiss* and *Sparus aurata*). The One Health approach was applied by the employment of biomarkers employed in other species, the use of less-invasive samples, and by the promotion of alternative technologies to the commonly used in these species.

Experimental design

In these studies, different biomarkers were measured in fish and mussels after their exposure to different environmental conditions including heavy metal and drugs pollution, or hypoxia. When possible, the use of alternative samples such as haemolymph or skin mucus was preferred. In all cases, the biomarkers were assessed using automated measurements, which pose important advantages in comparison to manual techniques such as simplicity, higher throughput with more than 800 processed samples per hour, lower use of sample and reagent volumes, or higher precision and accuracy by decreasing errors due to human manipulation, which are of high importance when a high number of samples are employed such as in the case of environmental biomonitoring programs.

Article 10: Gills of mussels exposed to single and mixed heavy metals. In this study, EA, TAC and TOS were validated and determined in gills of mussels exposed to single metal and ternary combination of lead (Pb, 1000 μgL^{-1}), cadmium (Cd, 100 μgL^{-1}) and copper (Cu, 100 μgL^{-1}). The mussels were acclimated for two weeks under controlled conditions of water pH, osmolarity, temperature, continuous aeration and natural photoperiod, and were daily fed with microalgae *Isochrysis galbana*, clon t-ISO. After this, the toxicants were daily distributed during 7 days within three tanks with n=12 mussels for each group (Pb, Cd, Cu and Pb+Cd+Cu groups); being three tanks with n=12 animals each unexposed, composing control group (Figure 8). During the experiments, the animals were under the same conditions than during the acclimation period.

Before the measurement of these biomarkers, the methods were first optimised by evaluating two different buffers, as well as the possible effects of ultracentrifugation of samples (600 g for 15min, 13,000 g for 20min and 40,000 g for 90 min at 4 °C). After optimisation, EA, TAC and TOS were analytically validated in gills by assessing precision, accuracy, limit of detection and 4-months stability. The day of the analysis, all measures were performed in the same run in order to avoid possible inter-assay imprecision.

This study was performed in collaboration with researchers from the Universidad Autónoma de Barcelona (Spain).

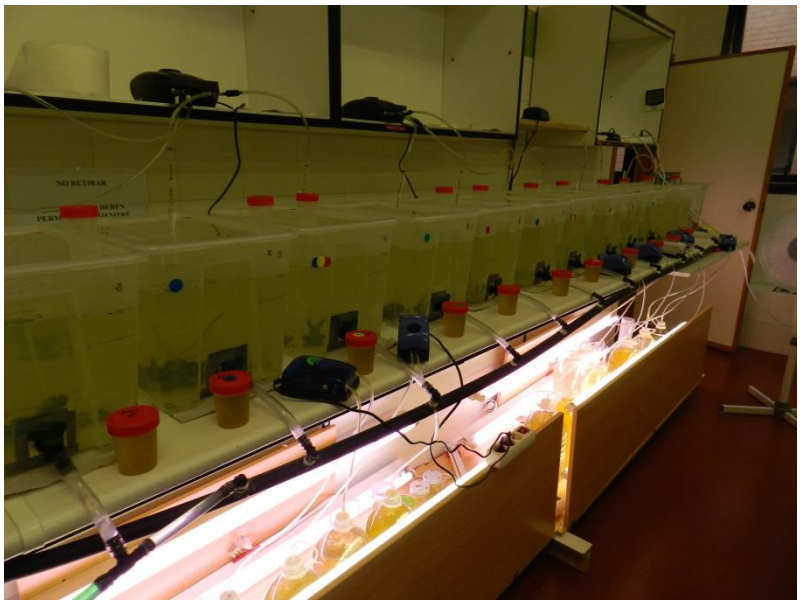


Figure 8. *Mytilus galloprovincialis* were exposure in tanks during seven days under controlled environmental conditions of pH, temperature, feeding, osmolarity and heavy metal concentration.

Article 11: Gills and digestive gland of mussels exposed to binary mixtures of heavy metals. In the present study, EA, TAC, TOS and AChE biomarkers were analysed in gills and digestive gland of (*M. galloprovincialis*) exposed to binary combinations of Pb, Cd and Cu. Mussels were acclimated for 14 days under controlled conditions of water pH, osmolarity, temperature, continuous aeration, natural photoperiod and fed daily with fed with microalgae *Isochrysis galbana*, clon t-ISO. Then they were divided into 4 groups (n=36 each), being 3 groups exposed for 7 days to a binary combination of (Pb, 1000 µgL−1), cadmium (Cd, 100 µgL−1) and copper (Cu, 100 µgL−1): (Pb+Cu, Cd+Pb and Cd+Cu groups) and one group were unexposed and employed as a control group. After the exposure period, EA, TAC, TOS and AChE were analysed in gills and digestive gland from six randomly selected animals of each group.

All the biomarkers were validated for its use in mussels' digestive gland, and AChE was also validated for its use in gills.

This study was performed in collaboration with researchers from the Universidad Autónoma de Barcelona (Spain).

Article 12: Novel biomarkers of oxidative stress in mussels. This study aimed to develop and validate novel automated spectrophotometric assays for the measurement of oxidative stress in gills, digestive gland and haemolymph of mussels in a hypoxic experimental set-up. Additionally, the need for correction according to the total protein content of each sample was evaluated. For this, first, n=15 mussels were employed for the validation of FRAP, CUPRAC, TEAC1, thiols and AOPP in gills, digestive gland and haemolymph. Then, after a two-day acclimation under controlled conditions of water pH, osmolarity, temperature, continuous aeration and natural photoperiod, mussels were randomly divided into control and hypoxic group (n=10 each). Controls were kept under the same conditions than during the acclimation period, while the hypoxic stress was elicited by the removal of the inter-valvar water and the exposition of the mussels to air. After 96h, FRAP, CUPRAC, TEAC1, thiols, AOPP, TEAC2, EA, TOS and AChE were measured in gills, digestive gland and haemolymph. Two animals from the hypoxic group died during the 96h period and therefore were excluded from the study.

This study was performed in collaboration with researchers from the Universidad Autónoma de Barcelona (Spain) and the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Portugal).

Article 13: Effects of human pharmaceuticals in fish. In this study, *Sparus aurata* fish were exposed via water to gemfibrozil (a human pharmaceutical) for 96h with different aims: first, to validate TOS, TAC and EA in fish gills; second, to study the relation between these oxidative stress biomarkers in gills and mRNA levels of stress and immune function genes (*gpx1, gr, cat, sod2, gst3, prdx6, hsp70, grpt75, bax, casp3, ll1β, tnfα, il6, il10* and *cox2*) in gills and head kidney; last, to evaluate the effects of human pharmaceutical on marine fish species.

Thus, *Sparus aurata* juveniles were acclimated to laboratory conditions for 3 weeks under controlled salinity, temperature, photoperiod and fed daily with commercial fish diet. After this period, animals were randomly divided into duplicated groups (n=7) and exposed to 0 (control group), 1.5, 15, 150, 1500 and 15000 µg/L gemfibrozil for 96h.

This study was performed in collaboration with researchers from the Universidad Autónoma de Barcelona (Spain) and the University of Aveiro (Portugal).

Article 14: Alternative to radioimmunoassays for plasma cortisol measurement in fish. In this article, an automated chemiluminescence assay (CIA) was analytically validated and evaluated as an alternative to the traditionally employed radioimmunoassay (RIA) for the measurement of cortisol in plasma and skin mucus of fish. Also, the assay capacity to differentiate between stressed and non-stressed fish were evaluated.

For this, paired plasma and skin mucus of sexually immature rainbow trout fish (*Oncorhynchus mykiss*) at different time points after 3 min of air exposure were assessed both by CIA and RIA. They were acclimated for 2 weeks under controlled laboratory conditions of temperature, aeration, oxygenation, water pH and photoperiod, and were daily fed with a commercial diet. After this, one group (non-stressed group, n=9) were maintained under the same condition than during acclimation period; while the rest of animals were quickly captured with a net and maintained in an air-exposed net for 3 minutes and then released back to the tanks for 1, 6 and 24 hours (Stressed-fish groups (T0, T6 and T24), n=9). Nine fish for each group (controls and T1, T6 and T24) were sacrificed by anaesthetic overdose with MS222 and skin mucus and blood was sampled.

This study was performed in collaboration with researchers from the Universidad Autónoma de Barcelona (Spain) and the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR, Portugal).

Results and discussion

Article 10: Gills of mussels exposed to single and mixed heavy metals. First, it was observed that the widely employed potassium-based buffer containing chemical elements such as EDTA, DTT or leupeptin (Fernández *et al.*, 2010, 2012; González-Fernández *et al.*, 2015) interfered with TOS measurements, and some elements were described to altered EA (Tvarijonaviciute *et al.*, 2012). Thus, for the present study, a potassium buffer without additives was employed. The ultracentrifugation of samples resulted in better assay repeatability due to increased homogeneity and was therefore used for the evaluation of EA, TOS and TAC.

The three assays for the measurement of oxidative biomarkers in mussel's gills were precise and provided with repeatedly accurate results. The stability studies revealed that the three parameters were stable for at least four months at -80ºC.

When the behaviours of the three biomarkers were assessed, EA did not show any difference of statistical relevance. Attending to other antioxidant biomarkers such as glutathione-dependent antioxidant, glutathione or protein carbonyl content that showed modulations after longer time-periods (Kaloyianni *et al.*, 2009; Fernández *et al.*, 2010; Canesi, 2015), it could be hypothesised that longer exposure times could be needed to observe changes in EA. TOS showed statistical lower concentrations only in the mussels exposed to cadmium and to the ternary mixture, which were the most toxic treatments. This might be explained by the increased formation of ROS and subsequent oxidative damage caused by heavy metals (Levine *et al.*, 2000; Livingstone, 2001; Dovzhenko *et al.*, 2005; Di Salvatore *et al.*, 2013). TAC tends to be higher in all groups except for Cu in comparison to controls, although it was not statistically significant, which may suggest a better regulation of copper in comparison to other metals, as proposed elsewhere (Zhou *et al.*, 2008).

Taking overall, the increase in antioxidant response (TAC) in Pb, Cd and ternary groups in conjunction with the decrease in oxidants (TOS) in Cd and ternary suggests an effective antioxidant response against ROS in these groups.

Article 11: Gills and digestive gland of mussels exposed to binary mixtures of heavy metals. All the parameters showed adequate analytical validation with ranges within the limits proposed by the U.S Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (2001). When results from gills and digestive gland were compared, the latter showed significantly higher values in all cases (Figure 9), and EA, TAC and TOS results were positively correlated between both tissues.

The highest EA was observed in Pb+Cu group for gills and digestive gland, being significantly higher than controls. For digestive gland, EA in Pb+Cd was also higher than in controls. These finding could be in line with the increased in other antioxidant biomarkers observed in mussels from heavy metal-polluted coasts (Fernández *et al.*, 2010). No differences were observed in TAC

in gills or digestive glands between the different groups, which were in concordance with previous studies that did not observe changes in TAC in mussels' haemolymph or gills exposed to heavy metals or organic pollutants for 6-7 days (Kaloyianni *et al.*, 2009; Franco *et al.*, 2016). On the other hand, the same study revealed TAC modulation when the exposure time was increased to 12 days (Kaloyianni *et al.*, 2009), which points out that the shortage of time of exposure of this study may be insufficient to cause changes in TAC. TOS was only significantly modulated in the gills of Pb+Cd group, showing a decrease in comparison to controls. Similar results were observed in our previous study (Franco *et al.*, 2016), in which TOS in gills were lower in mussels exposed to Cd or Cu+Pb+Cd, being the most toxic treatments in both studies. AChE was higher in the digestive gland of Cd+Cu group when compared to controls and to Pb+Cu (the less toxic mixture). There is a controversy in the literature about the behaviour of AChE in animals exposed to pollution, showing increases, no changes or decreases depending on the tissue, animal species, pollutant and other conditions (Sáenz *et al.*, 2010; Vidal-Liñán and Bellas, 2013; Vidal-Liñán *et al.*, 2014; Wan *et al.*, 2015) and, therefore, further studies are needed to confirm the utility of AChE in gills and digestive gland of mussels for the assessment of heavy metals pollution.

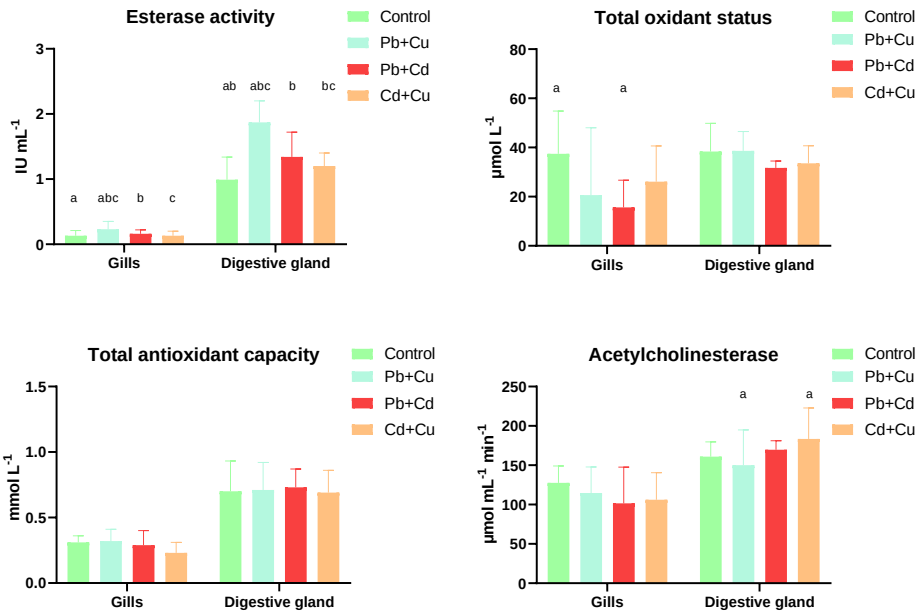


Figure 9. EA, TOS, TAC and AChE in gills and digestive gland of mussels exposed to binary mixtures of Pb, Cd and Cu. For each tissue, the same letters indicate statistical significance between groups ($p<0.05$).

In conclusion, the present study proves the validity of automated methods for the measurement of EA, TAC, TOS and AChE in different mussels' tissues, showing higher levels of these biomarkers in the digestive gland in comparison to gills. However, gills may be the tissue of election for oxidative stress evaluation since EA and TOS were significantly modulated in this tissue while, in contrast, the measurement of AChE activity in digestive gland could be more sensible for detection combined heavy metal toxicity in mussels.

Article 12: Novel biomarkers of oxidative stress in mussels. The analytical validation showed adequate intra- and inter-assay coefficients of variation in all cases except for TEAC1 and FRAP in haemolymph, which reached >75% inter-assay CV. Linearity studies showed linear regression equations with correlation coefficients close to 1 for all biomarkers and tissues, while spiking recovery percentages were among the acceptable 80-120% ranges in all cases except for TEAC1 in the haemolymph. The lower limits of detection were much lower than the range of values found in gills and digestive gland; while it was higher in the cases of haemolymph for TOS and AOPP in the control group. Therefore, the analytical validation was adequate in most cases, although certain precautions should be taken for future studies, such as to measure all the samples in the same batch to minimise the effects related to inter-assay imprecision. Significant differences were observed in protein content between control and hypoxic groups in gills and haemolymph, and thus, the need for correcting the results by protein content in these specimens was proposed.

All the biomarkers evaluated in this study showed significant changes between control and hypoxic groups: higher values in the hypoxic group were found in TEAC1 (gills), FRAP (digestive gland and haemolymph), AOPP (haemolymph) and AChE (gills); while TEAC1 (haemolymph), TEAC2 (haemolymph), CUPRAC (gills), thiols (gills), EA (haemolymph), and TOS (digestive gland) values were lower. For the biomarkers that composed the total antioxidant capacity (TEAC1, TEAC2, FRAP and CUPRAC), these observed differences may be caused by the different compounds measured by each assay which reflect different biochemical processes (Christensen *et al.*, 2015) and emphasises the importance of the use of a battery of biomarkers to obtain a wider overview of the antioxidant status (TAC) in biological samples. In the cases of other antioxidants (EA and thiols), they were decreased in haemolymph and gills, respectively, which

may indicate an increased consumption after their interactions with ROS (Dickinson and Forman, 2002). In relation with the two biomarkers that evaluates oxidants, AOPP increased in haemolymph of hypoxia group, while TOS decreased in digestive gland. The increase in AOPP may be due to the increase in protein oxidation caused, as reported in fish exposed to xenobiotics (Petrache *et al.*, 2012; Stanca *et al.*, 2013; Hermenean *et al.*, 2015), while the decrease in TOS in digestive gland could be a consequence of the changes in antioxidant capacity of the system. Lastly, AChE increased in gills of hypoxic animals, which were in concordance with previous reports that described increases in gills' AChE in animals exposed to municipal-effluents (Wan *et al.*, 2015).

Taken all together, the proposed novel biomarkers of oxidative stress have proved their potential usefulness in biomonitoring studies, since they are precise, accurate, economic, automated and showed an altered behaviour in a situation of oxidative stress. Moreover, since aquatic organisms have been proposed as model systems for the investigation of basic processes of cellular damage and protection against free radicals (Lushchak, 2011), these biomarkers in mussels and other species could be applied for other contexts such as tissue injury, diseases or ageing.

Article 13: Effects of human pharmaceuticals in fish. The analytical validation of EA, TOS and TAC in gills of fish showed adequate analytical results according to the U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), (2001), except inter-assay CV which almost raised 11 and 15% for EA and TOS, respectively. Thus, the measurement of all samples in a single batch, if possible, would reduce imprecision.

TOS was higher in the gills of animals exposed to the two highest concentration of gemfibrozil, which may point out and imbalance between the antioxidant and oxidant systems due to the increased production of ROS (Mimeault, Trudeau and Moon, 2006). On the contrary, EA and TAC did not show any change of statistical relevance. This may suggest the incapacity of delayed response to the increased oxidative response or, in the case of EA, that this enzyme could be not involved in the protection of the oxidative stress induced by gemfibrozil.

The transcriptomic profile revealed an upregulation of *gpx1* and *sod2* in gills (for the group exposed to the highest concentration) and head kidney (second highest) in comparison to controls. The gene expression of *hsp70* was only down-regulated in the head kidney (for all concentrations), suggesting a higher sensitivity of head kidney to gemfibrozil exposure. In contrast, the gene transcript expression of il10 gene was only downregulated in gills for all concentrations, which in mammals have been related to an impaired inflammatory progression (Saraiva and O'Garra, 2010). Thus, the transcriptomic study suggested that the exposure to gemfibrozil for 96h has a weak ability to alter the gene expression related to oxidative status after 96h and that more robust proinflammatory activities are present in the head kidney in comparison to gills.

Taking overall, the assays for the measurement of EA, TOS and TAC in fish's gills are precise, accurate, robust, cost-effective and fully automated, making them suitable for the evaluation of oxidative stress in environmental biomonitoring programs. TAC appeared to reflect the overall pattern of the antioxidant transcripts, which were not altered except for gpx1 and sod2. The effects of gemfibrozil at environmentally relevant doses were only detected in mRNA expression of some transcript genes such as il10 and hsp70; while although TOS was the most sensitive biomarker pointing out an increased oxidative pressure, it was only altered at concentrations that are not environmentally relevant.

Article 14: Alternative to radioimmunoassays for plasma cortisol measurement in fish. The validation of cortisol measurements by CIA in plasma and skin mucus of fish showed adequate precision and accuracy, with CVs lower than 15% and linear regression equations with correlation coefficients close to 1 in all cases. The lower limits of detection were much lower than the range of values found in plasma samples. For skin mucus, 10 samples were below the lower limit of detection; however, 9 of them corresponded to T0 and T24, when cortisol is expected to be at low concentrations due to the short and long time between the stress and sampling, respectively. When CIA and RIA results were compared, CIA reported higher cortisol values than RIA in all skin mucus and 28 out of 36 plasma samples, and Bland-Altman showing a wide 95% CI and the presence of proportional error. These differences could be attributed by the potential cross-reactivity of CIA in fish samples with other steroids, or by the potential interactions of

other substances in CIA. The cortisol correlation between plasma and skin mucus was significant with both methods, and similar to others reported previously (Bertotto *et al.*, 2010). The different diffusion of cortisol among different biological matrices makes these matrices suitable for cortisol evaluation in different situations: for example, those in which the diffusion is slower can be employed to avoid potential increases in hormone levels caused by sampling stress (Bertotto *et al.*, 2010).

When the effects of air-exposure were assessed at different time points, CIA revealed higher cortisol concentrations in plasma and skin mucus at T1 and T6 in comparison to T0. In contrast, RIA in plasma did not identify any differences of statistical relevance between T0 and T6. Although cautions due to the low sample size should be taken, this may suggest than CIA is more effective in detecting air-exposure-related stress after 6h than RIA. None of the techniques identified differences in cortisol in plasma or skin mucus at T24, which were in concordance with another study that reported cortisol decreases 24h after the stress (Guardiola, Cuesta and Esteban, 2016). When ROC analysis was performed using T0 as controls and T1 and T6 as test groups, it showed that values of CIA-measured cortisol above 7.515 and 0.9317 µg/dL (for plasma and skin mucus, respectively) were indicative of the effect of air exposure-induced stress in fish.

In conclusion, the measurements of cortisol by CIA in plasma and skin mucus samples of fish are precise, accurate and able to detect stress. This makes CIA a suitable alternative to RIA for the measurement of cortisol in fish providing with substantial progress from the technologic, ecologic, and economic viewpoints. Besides, the use of human assays for the evaluation of samples from other species could increase the economical savings and provide with wider availability of test, being key factors in biomonitoring, research and welfare programs.

2.3. Objective 3.

The third objective of the present PhD thesis was to identify a common biomarker by the application of the OH concept and to develop a fast assay for its measurement. The C-reactive protein is a known major acute-phase protein in humans and dogs; however, its possible presence in other organisms such as mussels was not investigated. Thus, studies were first

performed to identify if C-reactive protein was present in mussels and if it could be used as inflammation biomarkers in these species. Then, monoclonal anti-CRP antibodies were developed to create portable assay for the measurement of C-reactive protein in the three species based on lateral-flow technique.

Objective 3 was covered by 2 experiments corresponding to Article 15 and Appendix VI.

- *Article 15: Evaluation of C-reactive-like protein in Mytilus galloprovincialis.*
- *Appendix VI: Development of a novel Point of Care assay for the measurement of C-reactive protein in different species.*

Article 15: Evaluation of C-reactive-like protein in Mytilus galloprovincialis.

In this study, the possible existence of C-reactive protein in mussels was investigated. CRP-like protein was identified and purified from *M. galloprovincialis* digestive gland homogenates. In addition, an automated method designed for its use in humans was analytically validated and was used to evaluate if CRP-like protein may be employed as an inflammation biomarker in mussels in two experimental set-ups.

Experimental design

CRP-like protein was purified using affinity chromatography from the digestive gland of n=6 mussels. Then, SDS-PAGE and western blotting were performed for characterization and verification of CRP-like protein using a commercial goat anti-human CRP antibody (Olympus® CRP OSR 6147 Olympus Life and Material Science Europe GmbH) as primary antibody and HRP conjugated anti-goat-IgG (A5420-1 mL, Sigma Aldrich) as secondary antibody.

An hypoxia experimental set-up was executed, in which samples from a previous study were employed (Franco-Martínez *et al.*, 2018). Briefly, after a two days acclimation, a group of animals (hypoxic group, n=10) were removed of their inter-valvar water and kept air-exposed, while others were kept under the same conditions than during acclimation period (control group, n=10). Two animals from the hypoxic group died during the experiment and excluded from the study, and the rest were sacrificed after 96h.

For the exposure to nanoplastics experimental set-up, samples from a previous study were employed (Brandts *et al.*, 2018). In brief, after 10 days of acclimation period, mussels were randomly distributed and exposed for 96 h to one of the following experimental conditions: control (0), 0.005, 0.05 and 0.5 mg/L polystyrene nanoplastics (PSNP) (n=12 for each group). For this experimental set-up, after the dissection of the digestive glands, they were immediately frozen in liquid nitrogen and stored -80ºC until further analysis.

This study was performed in collaboration with researchers from the Universidad Autónoma de Barcelona (Spain) and the University of Aveiro (Portugal).

Results and discussion

CRP-purification from digestive gland homogenates, which was performed by affinity chromatography based on CRP calcium-dependent binding affinity for phosphorylcholine as described previously (Volanakis and Kaplan, 1971; Kolberg, Høiby and Jantzen, 1997) showed a peak at 60mAU. The SDS-PAGE analysis of the purified CRP-like protein showed a multimeric pattern similar to the described for porcine CRP (Martinez-Subiela *et al.*, 2007), and the western blotted confirm the recognition of the primary antibody of a triple band patter of around 60, 33 and 29kDa. These bands could be composed by CRP monomers (29 and 33kDa) and dimers (60 kDa), being compatible to the presence of CRP described in other species such as fish or clamps (Olafsen, 1995; MacCarthy *et al.*, 2008).

The automated assay employed for the measurement of CRP-like protein from mussels' digestive gland homogenates showed and adequate analytical validation according to the limits for good method functionality (U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER), 2001). However, the inter-assay was above the acceptance range and, therefore when possible, all samples should be measured in the same batch to avoid imprecision.

When the behaviour of CRP-like protein was evaluated in two experimental set-ups, it was statistically higher in hypoxic animals and mussels exposed to 0.05 and 0.5 mg/L PSNP in comparison to their respective controls. In concordance to our results, increases of CRP were reported in humans with hypoxia (Masuda *et al.*, 2008; SHANG *et al.*, 2014). This increase in CRP under hypoxia may suggest that hypoxia causes inflammation, since these same animals also

suffered from oxidative stress (Franco-Martínez *et al.*, 2018) which has been related to inflammation elsewhere (SHANG *et al.*, 2014; Yilmaz Avci *et al.*, 2017), although CRP-like proteins may be increased directly as a response to the increase of ROS. In the same line, the increase of CRP-like protein in mussels exposed to nanoplastics could be a consequence of an imbalance in oxidative status caused by PSNP (Brandts *et al.*, 2018). Besides, a higher increase in CRP-like protein was observed in the group exposed to the highest PSNP concentration, which may suggest the potential of this protein not only as a biomarker of exposure but also of severity. This modulation of CRP depending on the severity of the disturbance was also reported in fish (Sinha *et al.*, 2001) and mammals previously (Gabay and Kushner, 1999; Martínez-Subiela *et al.*, 2002).

In conclusion, in this study, mussels' CRP-like protein was detected, characterized and measured for the first time. It was purified by affinity chromatography and verified by western blotting, measured by a validated economic automated high-throughput designed for its use in human samples, and the CRP-like potential for environmental biomonitoring purposes was evaluated by the exposure of mussels to two of the most pertinent threats to marine ecosystems: hypoxia and pollutants (Bijma *et al.*, 2013).

Appendix 6: Development of a monoclonal antibody for the measurement of C-reactive protein in multispecies rapid and portable assays.

This study entails the first step towards the development of a point-of-care (POC) assay for the measurement of CRP in different species. Although polyclonal antibodies may recognize different epitopes for CRP and are easier to develop, monoclonal antibodies are highly specific and more stable for continuous production.

Experimental design

The production of monoclonal antibodies anti CRP was performed following previous protocols consisting of mice immunization, cell fusion and cell selection (Yokoyama, 1999). C-reactive protein was purified from serum of dogs with inflammation using a commercial p-aminophenyl phosphorylcholine agarose (Reference 20307, Thermo Scientific), being the purification validated by SDS page and ELISA using a human immunoturbidimetric test for CRP measurement (CRP OSR 6147 Olympus Life and Material Science Europe GmbH) previously validated for its use in canine

serum (Martinez-Subiela and Cerón, 2005). Cell selection was performed by several cycles of cell separation and subsequent identification of cell lines that created antibodies that recognized purified canine CRP in ELISA.

This study was performed in collaboration with researchers from the University of Turin (Italy).

Results and discussion

The elution profiles of canine CRP from serum obtained by affinity columns showed a double band pattern of about 29 and 24 KDa, in agreement to previous reports (Parra, Cabezas-Herrera J and Cerón José Joaquín, 2005). Also, the canine CRP purificate was recognized by the commercial antibody previously validated for the measurement of CPR in dogs.

After immunization, cell fusion, and three cycles of cell selection (Figure 10), 17 cell clones were selected for further research. All cell lines showed adequate viability, and produce an adequate quantity of antibodies that recognized canine CRP by ELISA. For practical reasons, all cell lines were frozen in nitrogen liquid and three lines (each one of distant cell families) were maintained for further evaluation of their antibodies.

In conclusion, in the present study, a battery of monoclonal antibodies against canine CRP was created and validated by ELISA. Of those, three antibodies were selected for further evaluation, which may include the evaluation of affinity against CRP of other species including humans, their affinity in other specimens such as saliva, and their aptitude for the development of point-of-care assays.

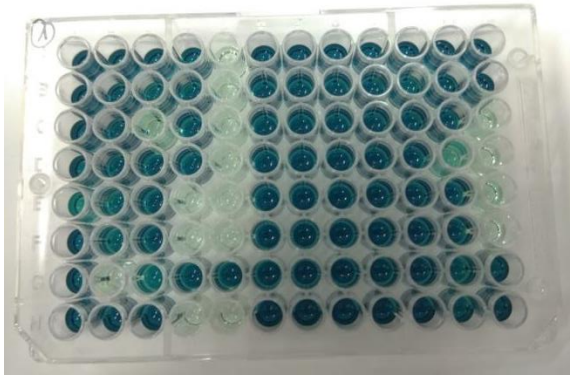


Figure 10. Representative results of ELISA screening evaluating the affinity of the produced monoclonal antibodies. Canine CRP was purified and fixed to the plaque. Each well from columns 1-11 and wells 12G-12H represents one candidate monoclonal antibodies, column 12 was composed by positive (A, B) and negative (C, D, E, F) controls.

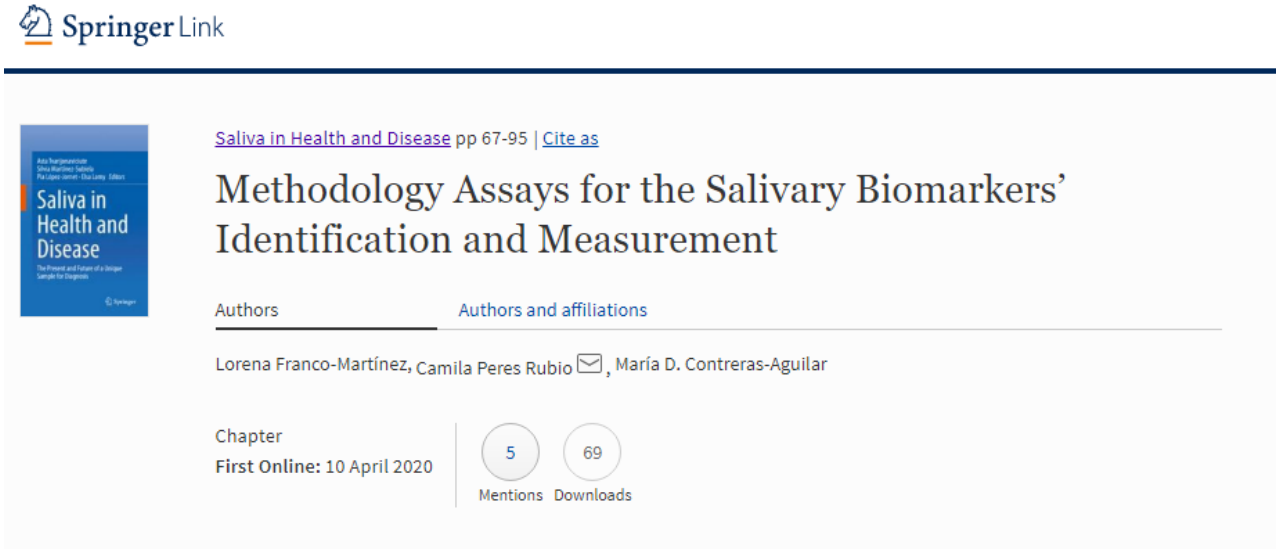
Items published

Objective 1

To review the state of the art of the search and validation of novel biomarkers in different species and sample types and to evaluate the applications of the "One-Health" concept in a clinical pathology laboratory

Items published

Book chapter 1



Editorial: Springer

Abstract: This contribution aims to offer a schematic view of the most important methods to discover and measure salivary biomarkers in human and veterinary species. In addition, examples of the application of these different techniques are given, and the importance of correct validation studies was highlighted.

URL: https://link.springer.com/chapter/10.1007/978-3-030-37681-9_4

Article 1

Research in Veterinary Science 128 (2020) 299–307

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Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Biomarkers of health and welfare: A One Health perspective from the laboratory side

Lorena Franco-Martínez[a], Silvia Martínez-Subiela[a], José Joaquín Cerón[a], Fernando Tecles[a], Peter David Eckersall[b], Katarina Oravcova[b], Asta Tvarijonaviciute[a,*]

Journal: Research in Veterinary Science

Abstract: A biomarker is any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The One World, One Health concept established that human and animal health and the environmental state are highly interconnected, sharing common aspects that can be applied globally in these three components. In this paper, we review how the concept of One Health can be applied to biomarkers of health and welfare, with a special focus on five points that can be applied to any biomarker when it is expected to be used to evaluate the human, animal or environmental health. Three of these points are: (1) the different biomarkers that can be used, (2) the different sample types where the biomarkers can be analysed, and (3) the main methods that can be used for their measurement. In addition, we will evaluate two key points needed for adequate use of a biomarker in any situation: (4) a proper analytical validation in the sample that it is going to be used, and (5) a correct selection of the biomarker. It is expected that this knowledge will help to have a broader idea about the use of biomarkers of health and welfare and also will contribute to a better and more accurate use of these biomarkers having in mind their One Health perspective.

URL: https://www.sciencedirect.com/science/article/pii/S003452881930565X?via%3Dihub

Objective 2

To perform proteomic and biochemical studies in different experimental and clinical situations for the discovery of novel biomarkers.

Model 1: muscular damage

Model 2: pathologies

Model 3: environmental conditions such as hypoxia or pollution

Article 2

Fish and Shellfish Immunology 75 (2018) 41–47

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Alterations in haemolymph proteome of *Mytilus galloprovincialis* mussel after an induced injury

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Journal: Fish and Shellfish Immunology

Abstract: A proteomic and biochemical approach was performed to assess the effects of an induced muscle injury on the haemolymph of bivalve molluscs. For this purpose, *Mytilus galloprovincialis* were exposed to puncture of adductor muscle for three consecutive days, and their haemolymph proteome was then compared to healthy animals using 2-dimensional electrophoresis (2-DE) to identify proteins that differed significantly in abundance. Those proteins were then subjected to tandem mass spectrometry and 6 proteins, namely myosin, tropomyosin, CuZn superoxide dismutase (SOD), triosephosphate isomerase, EP protein and small heat shock protein were identified. SOD and tropomyosin changes were verified by spectrophotometric measurements and western blotting, respectively. As some of the proteins identified are related to muscular damage and oxidative stress, other biomarkers associated with these processes that can be evaluated by automatic biochemical assays were measured including troponin, creatine kinase (CK), and aspartate aminotransferase (AST) for muscle damage, and SOD, trolox equivalent antioxidant capacity (TEAC) and esterase activity (EA) for oxidative stress. Significantly higher concentrations of troponin, CK, AST, and TEAC were observed in mussels after puncture, being also possible biomarkers of non-specific induced damage.

URL: https://www.sciencedirect.com/science/article/pii/S105046481830038X?via%3Dihub

Article 3

Journal of Proteomics 214 (2020) 103629

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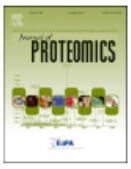
Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot



Differences on salivary proteome at rest and in response to an acute exercise in men and women: A pilot study

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Journal: Journal of Proteomics

Abstract: The aim of the present study was to evaluate the differences in salivary proteome at rest and in response to an acute exercise in men and women. For this, unstimulated whole saliva samples in rest and after a bout resistance exercise leading to failure (ELF) of both men and women (n = 5 for each sex) were subjected to isobaric Tandem Mass Tags (TMT) labelling followed by LC-MS/MS. A total of 274 proteins were identified and met the inclusion criteria. 16 proteins were modulated for the interaction sex*exercise, 6 were modulated because of the exercise, and 65 were differentially expressed between men and women at rest. In conclusion, these results indicate sex-related differences in the salivary proteome at rest and after an acute exercise, pointing out possible candidate biomarkers for sports performance and allowing further knowledge of the physiological processes occurring during ELF.

Significance: The present study describes for the first time the changes that occur in salivary proteome detected by TMT-based proteomics in response to an acute exercise and the differences in these changes depending on sex. Of the 274 protein identified, the 87 differentially expressed proteins and their related pathways were discussed, focusing on the sex- and exercise-related differences in the salivary proteome.

URL: https://www.sciencedirect.com/science/article/pii/S1874391919304014?via%3Dihub

Article 4

J Sports Med Phys Fitness. 2019 Jun;59(6):902-909. doi: 10.23736/S0022-4707.18.08792-3. Epub 2018 Jul 18.

Changes in Lactate, Ferritin, and Uric Acid in Saliva After Repeated Explosive Effort Sequences

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Journal: Journal of Sport Medicine and Physical Fitness

Abstract: BACKGROUND The aim of the present study was to evaluate the changes in a panel of biomarkers including lactate, ferritin and uric acid in saliva of untrained and trained subjects after repeated explosive effort sequences, and to analyse the differences in interpretation of these biomarkers depending on the way the data is expressed (without any correction or corrected by protein content or flow). METHODS Eighteen volunteers (11 untrained and 7 trained) performed 8 consecutive explosive effort sequences (60 m sprints). Blood and saliva samples were collected before and after each sequence. Salivary data was analysed as absolute concentration and after correcting by their protein content and flow. RESULTS Lactate in saliva showed increases with acute exercise, being these increases of higher magnitude in non-trained subjects. In addition, when corrected by total protein, lactate in saliva correlates with blood lactate in non-trained subjects. Ferritin and uric acid in saliva did not show changes after the effort sequences. CONCLUSIONS From the biomarkers evaluated, the measurement of lactate in saliva corrected by total protein could be useful for the assessment of fatigue induced during repeated explosive effort sequences and could be potentially used as a non-invasive alternative to blood lactate. This report also points out that way of reporting the saliva analytes could greatly influence the interpretation of the results and that lactate in saliva has a different dynamic in trained and non-trained individuals that should be further explored.

URL: http://www.ncbi.nlm.nih.gov/pubmed/30024129

Article 5

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ORIGINAL ARTICLE

Transboundary and Emerging Diseases WILEY

Serum proteome of dogs at subclinical and clinical onset of canine leishmaniosis

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Journal: Transboundary and Emerging Diseases

Abstract: The objective of this study was to identify changes in serum proteome in dogs that may occur after an experimental infection at subclinical and clinical stages of canine leishmaniosis (CanL). For this purpose, canine pre- and post-infection with Leishmania infantum serum proteomes in the same dogs were analysed by a high-throughput label-based quantitative LC-MS/MS proteomic approach. A total of 169 proteins were identified, and 74 of them including complement C8 alpha chain, adiponectin, transferrin, sphingomyelin phosphodiesterase acid-like 3A and immunoglobulins showed different modulation between the different stages of CanL. These proteins could be considered as potential serum biomarkers of early diagnostic or disease progression in CanL. Additionally, biological pathways modulated during CanL such as blood coagulation or gonadotropin-releasing hormone receptor were revealed, which could help to understand the pathological mechanisms of the disease.

URL: https://onlinelibrary.wiley.com/doi/abs/10.1111/tbed.13354

Article 6

Veterinary Parasitology 272 (2019) 44–52

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Research paper

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

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Journal: Veterinary Parasitology

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

URL: https://www.sciencedirect.com/science/article/pii/S0304401719301530?dgcid=coauthor

Article 7

Comparative Immunology, Microbiology and Infectious Diseases 60 (2018) 1–10

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Changes in salivary analytes in canine parvovirus: A high-resolution quantitative proteomic study

Lorena Franco-Martínez[a], Asta Tvarijonaviciute[a], Anita Horvatić[b], Nicolas Guillemin[b], José Joaquín Cerón[a], Damián Escribano[a,c], David Eckersall[b,d], Meriç Kocatürk[e], Zeki Yilmaz[e], Elsa Lamy[f], Silvia Martínez-Subiela[a,*], Vladimir Mrljak[b]

Journal: Comparative Immunology, Microbiology and Infectious Diseases

Abstract: The present study evaluated the changes in salivary proteome in parvoviral enteritis (PVE) in dogs through a high-throughput quantitative proteomic analysis. Saliva samples from healthy dogs and dogs with severe parvovirosis that survived or perished due to the disease were analysed and compared by Tandem Mass Tags (TMT) analysis. Proteomic analysis quantified 1516 peptides, and 287 (corresponding to 190 proteins) showed significantly different abundances between studied groups. Ten proteins were observed to change significantly between dogs that survived or perished due to PVE. Bioinformatics' analysis revealed that saliva reflects the involvement of different pathways in PVE such as catalytic activity and binding, and indicates antimicrobial humoral response as a pathway with a major role in the development of the disease. These results indicate that saliva proteins reflect physiopathological changes that occur in PVE and could be a potential source of biomarkers for this disease.

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Article 8

ORIGINAL RESEARCH ARTICLE
Front. Vet. Sci., 11 June 2020 | https://doi.org/10.3389/fvets.2020.00277

Changes in the Salivary Proteome Associated With Canine Pyometra

Lorena Franco-Martínez[1], Anita Horvatić[2], Andrea Gelemanović[3], Marko Samardžija[2], Vladimir Mrljak[2], María Dolores Contreras-Aguilar[1], Silvia Martínez-Subiela[1], Roman Dąbrowski[4*] and Asta Tvarijonaviciute[1]

Journal: Frontiers in Veterinary Science

Abstract: The present study evaluated for the first time changes in the saliva proteome in bitches with pyometra through a high-throughput quantitative proteomic analysis. The aims were to explore whether saliva composition could reflect the physiopathological changes occurring in canine pyometra and to identify potential biomarkers of the disease. Saliva samples from six healthy (H) and six bitches with pyometra (P) were analyzed using tandem mass tags–based approach. Additionally, 15 samples were used for the validation of changes in haptoglobin (Hp) concentration in saliva of dogs with pyometra. Proteomic analysis quantified 707 proteins in saliva. Comparison of the two groups revealed 16 unique proteins significantly modulated in saliva, with S100A calcium-binding protein 12 (S100A12), vimentin, and Hp the most up-regulated in canine pyometra. According to PANTHER (Protein Analysis Through Evolutionary Relationships) classification tool, these proteins are mainly related to proinflammatory mediators, acute-phase proteins, and sepsis. In conclusion, it can be stated that there are changes in various proteins in saliva in canine pyometra reflecting different physiopathological changes occurring in this disease. These proteins could be a source of potential non-invasive biomarkers for this disease that should be confirmed in future studies.

URL: https://www.frontiersin.org/articles/10.3389/fvets.2020.00277/full

Article 9

animals

MDPI

Article

Changes in Serum and Salivary Proteins in Canine Mammary Tumors

Lorena Franco-Martínez [1], Andrea Gelemanović [2], Anita Horvatić [3], María Dolores Contreras-Aguilar [1], Roman Dąbrowski [4], Vladimir Mrljak [3], José Joaquín Cerón [1], Silvia Martínez-Subiela [1,*] and Asta Tvarijonaviciute [1]

Journal: Animals

Abstract: The aim of this study was to evaluate changes in serum and saliva proteomes in canine mammary tumors (CMT) using a high-throughput quantitative proteomic analysis in order to potentially discover possible biomarkers of this disease. Proteomes of paired serum and saliva samples from healthy controls (HC group, n = 5) and bitches with CMT (CMT group, n = 5) were analysed using a Tandem Mass Tags-based approach. Twenty-five dogs were used to validate serum albumin as a candidate biomarker in an independent sample set. The proteomic analysis quantified 379 and 730 proteins in serum and saliva, respectively. Of those, 35 proteins in serum and 49 in saliva were differentially represented. The verification of albumin in serum was in concordance with the proteomic data, showing lower levels in CMT when compared to the HC group. Some of the modulated proteins found in the present study such as haptoglobin or S100A4 have been related to CMT or human breast cancer previously, while others such as kallikrein-1 and immunoglobulin gamma-heavy chains A and D are described here for the first time. Our results indicate that saliva and serum proteomes can reflect physiopathological changes that occur in CMT in dogs and can be a potential source of biomarkers of the disease.

URL: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7222850/

Article 10

Marine Pollution Bulletin 102 (2016) 30–35

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of *Mytilus galloprovincialis* exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure

Journal: Marine Pollution Bulletin

Abstract: The aims of the present study were to optimize and validate methods for esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) determination in mussel' gills, and to establish the relationships between these biomarkers and Pb, Cd and Cu pollution, in single form and ternary mixture. Two different buffers for sample homogenization, the need of ultracentrifugation, and analytical validation were evaluated. Coefficients of variation, when buffer without additives and ultracentrifugation were used, were <15%, and recovery were 97%-109% in all cases. The EA response tends to decrease with treatments, TOS decreased significantly in Cd and ternary groups, while TAC tended to increase in treatments with Pb, Cd and ternary groups. In conclusion, the methods for EA, TOS and TAC measurements in gills of mussel were precise and accurate and could be interesting resources in biomonitoring programmes.

URL: http://dx.doi.org/10.1016/j.marpolbul.2015.12.010

Article 11

Environ Sci Pollut Res
DOI 10.1007/s11356-016-7677-y

RESEARCH ARTICLE

Measurement of p-nitrophenyl acetate esterase activity (EA), total antioxidant capacity (TAC), total oxidant status (TOS) and acetylcholinesterase (AChE) in gills and digestive gland of *Mytilus galloprovincialis* exposed to binary mixtures of Pb, Cd and Cu

Lorena Franco-Martinez[1] · Diego Romero[1] · José A. García-Navarro[1] · Fernando Tecles[2] · Mariana Teles[3] · Asta Tvarijonaviciute[4]

Journal: Environmental Science and Pollution Research

Abstract: The aims of the present work were (1) to evaluate oxidative stress biomarkers and AChE in two tissues of wild mussel (*Mytilus galloprovincialis*) of high biochemical activity and accumulation capacity (gills and digestive gland) and (2) to study the behaviour of these biomarkers in presence of heavy metals. For this, EA, TOS, TAC and AChE were measured in tissues of mussels exposed to binary combination of Pb, Cd and Cu. Mussels (n = 36) were exposed to one of the binary mixtures of Pb (1000 mu g L-1), Cd (100 mu g L-1) and Cu (100 mu g L-1) for 7 days, under controlled conditions. Gills and digestive gland were extracted and frozen at -80 A degrees C until analysis. The automatic methods employed for the measurement of EA, TAC, TOS and AChE in *M. galloprovincialis* revealed higher levels of these biomarkers in digestive gland than gills. Study results suggest that gills would be the tissue of election for study oxidative stress markers, whereas digestive tissue should be selected for AChE measurements in case of evaluation of combined metal toxicity in mussels.

URL: http://link.springer.com/10.1007/s11356-016-7677-y

Article 12

Comparative Biochemistry and Physiology, Part B 221–222 (2018) 44–49

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

journal homepage: www.elsevier.com/locate/cbpb

New potential biomarkers of oxidative stress in *Mytilus galloprovincialis*: Analytical validation and overlap performance

Lorena Franco-Martínez[a], Diego Romero[b], Camila Peres Rubio[a], Fernando Tecles[a], Silvia Martínez-Subiela[a], Mariana Teles[c,d], Asta Tvarijonaviciute[a,*]

Journal: Comparative Biochemistry and Physiology, Part B

Abstract: The aim of the present report was to develop and validate new automated spectrophotometric assays for measurement of total antioxidant capacity (TAC), thiols, and advanced oxidation protein products (AOPP) in mussel gills, digestive gland and hemolymph samples, and to evaluate their possible utility in biomonitoring programs. Total antioxidant capacity (TAC) was measured by different methods: trolox equivalent antioxidant capacity (TEAC1 and TEAC2), cupric reducing antioxidant capacity (CUPRAC), and ferric reducing ability of plasma (FRAP). The assays were precise, accurate and provided low limits of detection. When oxidative stress was promoted by inducing hypoxia and the behaviour of these biomarkers between hypoxic and controls mussels were compared, statistically significant differences were observed in all biomarkers and tissues evaluated. The results of the present study demonstrated that these biomarkers, not previously studied in mussels, show a potential use as biomarkers of oxidative stress in this species since they were validated and showed changes under a state of oxidative stress.

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Article 13

Ecological Indicators 95 (2018) 1100–1107

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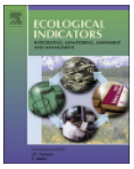
Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind



Tools to assess effects of human pharmaceuticals in fish: A case study with gemfibrozil

M. Oliveira[a,*], L. Franco[b], J.C. Balasch[c], C. Fierro-Castro[c], A. Tvarijonaviciute[d], A.M.V.M. Soares[a], L. Tort[c], M. Teles[c,*]

Journal: Ecological Indicators

Abstract: There is an increasing need to assess animal welfare and subtle alterations that may indicate the presence of environmental stressors. Among the most widely used endpoints are the assessment of the activities of specific enzymes and the alterations of the expression levels of target genes. Enzymes associated with oxidative stress are frequently assessed but the results frequently render enzyme-dependent altered levels that make analyses difficult. In this study, *Sparus aurata* (gilthead sea bream) juveniles, were exposed, via water, to gemfibrozil (GEM) for a 96 h period to i) validate methods to measure total oxidative status (TOS), total antioxidant capacity (TAC), and esterase activity (EA) in gills; ii) study the relation between assessed gills' oxidative stress responses and the mRNA levels of genes related to stress and immune function (in the gills and head kidney); iii) contribute to the knowledge of the effects of this human pharmaceutical on marine fish species. Overall, the obtained data confirmed the methodological precision and accuracy of TOS, TAC and EA measurements in the gills. GEM exposure did not alter TAC in gills but increased TOS. GEM modulated mRNA levels of genes, such as glutathione peroxidase 1 (gpx1) and interleukin 1β (il1 β) in the gills and head kidney of *S. aurata*.

URL: https://www.sciencedirect.com/science/article/pii/S1470160X17308415?via%3Dihub

Article 14

Ecological Indicators 98 (2019) 634–640

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Original Articles

Chemiluminescent assay as an alternative to radioimmunoassay for the measurement of cortisol in plasma and skin mucus of *Oncorhynchus mykiss*

L. Franco-Martinez[a], A. Tvarijonaviciute[a], S. Martinez-Subiela[a], M. Teles[b,*,1], L. Tort[c,*,1]

Journal: Ecological Indicators

Abstract: The aims of the present study were to validate an automated chemiluminescence assay (CIA) for cortisol determination in plasma and skin mucus of fish, to compare the results produced with those obtained by radioimmunoassay (RIA), and to evaluate the assay capacity to differentiate between stressed and non-stressed fish. Cortisol hormone was measured using both CIA and RIA in plasma and skin mucus of 36 rainbow trout (*Oncorhynchus mykiss*) at different time points after 3 min of air exposure. For CIA, analytical validation consisting of intra- and inter-assay coefficient of variation (CV), limit of detection, and linearity studies were performed. In addition, correlation and agreement between the CIA and RIA were evaluated. In all cases, intra- and inter-assay CV for CIA measurements were lower than 10 and 11%, respectively. Cortisol results with CIA were statistically significantly higher than those obtained with RIA in both tissues ($p < 0.001$). Strong positive correlation was observed between the two methods ($r = 0.999$, $p < 0.001$ and $r = 0.993$, $p = 0.03$ for plasma and skin mucus, respectively). The agreement between the two techniques was examined by Bland-Altman plots, which identified wide confidence intervals and outliers for cortisol (plasma n = 3, skin mucus n = 2) results. Both assays were able to differentiate between pre- and post-stressed fish in both biological matrices. In conclusion, CIA assay is precise and accurate for measuring cortisol in plasma and skin mucus of fish and is able to discriminate among stressed and control animals, showing a strong correlation with RIA, making it a reliable method for stress assessment in fish.

URL: https://www.sciencedirect.com/science/article/pii/S1470160X18309099?via%3Dihub

Objective 3

To identify a common biomarker by the application of the OH concept and to develop a fast assay for its measurement: the case of C-reactive protein.

Article 15

Ecological Indicators 106 (2019) 105537

Contents lists available at ScienceDirect

Ecological Indicators

journal homepage: www.elsevier.com/locate/ecolind

Evaluation of C-reactive-like protein in *Mytilus galloprovincialis*

Lorena Franco-Martínez[a], Asta Tvarijonaviciute[a,*], Sandra V. Mateo[a], José J. Cerón[a], Diego Romero[b], Miguel Oliveira[c], Mariana Teles[d], Silvia Martínez-Subiela[a]

Journal: Ecological Indicators

Abstract: C-reactive protein (CRP) is an acute phase protein of the innate immune system with an important role as biomarker in many biological processes in mammals. However, there is a lack of information regarding CRP in molluscs and there is no data about the presence of CRP-like protein in mussels. In the present work, CPR-like protein was identified and purified from the digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. In addition, it was demonstrated that CRP-like protein can be measured accurately and precisely by a high-throughput automated method. Finally, higher levels of CRP-like protein in mussels exposed to hypoxia and nanoplastics were detected when compared to controls. The results obtained indicate that CRP-like protein could be a suitable novel biomarker of inflammatory response in mussels for biomonitoring programs being economic, easy and rapid to determine.

URL: https://linkinghub.elsevier.com/retrieve/pii/S1470160X19305229

Conclusions

From the discussion of the results obtained during the present PhD thesis, six conclusions can be derived:

1. The One Health approach can be applied to laboratory conditions by the use of the same biomarker in different species, sample type or diseases.
2. Non-invasive samples such as saliva and skin mucus are valuable biofluids that showed changes in their composition after muscular damage, diseases or hypoxia.
3. Muscular damage can be identified using a panel of potential salivary biomarkers in humans and haemolymph of mussels, including lactate, actin, tropomyosin and cystatin-B.
4. Both saliva and serum are suitable biofluids for the measurement of biomarkers that can provide valuable clinical information in different diseases such as parvoviral enteritis, leishmaniosis, pyometra, mammary tumours, diabetes mellitus and mastitis. In the present PhD thesis, more than 6700 proteins were identified by proteomic analyses with more than 447 being modulated in these conditions, providing with a large battery of candidate biomarkers for its further evaluation.
5. Biomarkers used in human and veterinary medicine for the evaluation of oxidative status, inflammation and stress can be applied in fish and mussels for the biomonitoring of physiological effects associated with the environmental conditions. These biomarkers can have potential in environmental biomonitoring programs related to pollution and hypoxia.
6. C-reactive protein was identified and described in mussels for the first time in the present PhD thesis. In addition, anti-C-reactive protein monoclonal antibodies have been developed during this PhD thesis, which could serve as an initial step for the development of rapid and portable assays for the diagnosis of inflammation in different species.

Resumen general

El concepto “Una Salud” (One Health, OH) puede definirse como la combinación de esfuerzos por parte de múltiples disciplinas para alcanzar un estado óptimo de salud humana, animal y medioambiental (Wong and Kogan, 2013). Este concepto ha puesto de manifiesto el valor añadido de las colaboraciones intra e interdisciplinares durante los últimos años (Häsler *et al.*, 2014); sin embargo, aún no hay una total integración entre las diferentes agencias responsables de la salud humana, animal y el medio ambiente, a pesar del amplio apoyo que está recibiendo este concepto por parte de diversas organizaciones internacionales (Kahn, 2012). Un ejemplo de los posibles beneficios de un enfoque OH es la pandemia de COVID-19, durante la cual los servicios de salud humana se han visto desbordados y algunos laboratorios de salud animal han servido de apoyo para el diagnóstico de la enfermedad (OIE, 2020). Estos laboratorios de salud animal y su personal, además de estar acostumbrados a trabajar en condiciones similares a la de los laboratorios de salud humana, incluyendo equipos, técnicas, controles de calidad, bioseguridad y bioprotección, también suelen trabajar con un gran número de muestras de manera rutinaria.

Según la Organización Mundial de la Salud, un biomarcador puede definirse como cualquier medida que refleje una interacción entre un sistema biológico y un peligro potencial que puede ser de naturaleza química, física o biológica (Yoshizawa *et al.*, 2013a). El uso adecuado de los biomarcadores contribuye a aumentar la esperanza y calidad de vida de los pacientes al permitir una más temprana toma de decisiones en torno al diagnóstico, pronóstico y respuesta al tratamiento, así como mejorar la prevención y tratamiento de enfermedades (Wagner, Verma and Srivastava, 2004). Sin embargo, la mayoría de los estudios se realizan en un pequeño rango de especies como los humanos o los animales domésticos o de laboratorio, siendo por el momento sus potenciales aplicaciones dentro del contexto OH escasamente desarrolladas.

Un ejemplo de las posibles aplicaciones del concepto OH en la identificación y medición de biomarcadores es el hecho que, en ocasiones, un ensayo comercial puede ser adaptado y validado para su uso en especies diferentes a las que fue diseñado, lo que incrementa sus aplicaciones potenciales. Esto puede ser especialmente importante en aquellas rutas fisiológicas que están altamente conservadas entre las diferentes especies, como el estrés (Marco-Ramell *et al.*, 2016), el estrés oxidativo (Apak, R., Özyürek, M., Güçlü, K., Çapanoğlu, 2016) o las proteínas de fase aguda (Schrödl *et al.*, 2016).

Generalmente, las muestras biológicas más empleadas para la realización de análisis clínicos son el suero y el plasma sanguíneo (Argüelles *et al.*, 2004). Sin embargo, en los últimos años, el uso de muestras menos invasivas como la saliva, el pelo, las heces, la orina, o el moco cutáneo en el caso de los peces, ofrece una serie de ventajas en comparación con la sangre. Estas muestras son más rápidas y fáciles de obtener, no requieren de material específico ni de profesionales para su recolección (Lindsay and Costello, 2016) y, lo más importante, son en la mayoría de casos libres de estrés y dolor. Por ello, en paralelo a las posibles aplicaciones de aplicar el concepto OH para adaptar un ensayo de una especie a otra, algunos ensayos pueden adaptarse y validarse para su uso en muestras no invasivas.

Actualmente existe un amplio número de técnicas disponibles para la identificación y medición de biomarcadores, estando la elección de la más adecuada condicionada por el tipo de biomarcador y sus características bioquímicas (Cerón, Eckersall and Martínez-Subiela, 2005). Por ejemplo, los ensayos espectrofotométricos o los inmunoensayos se emplean comúnmente para la medición de biomarcadores ya que se integran fácilmente en los laboratorios y proveen de mediciones rápidas y económicas en la mayoría de casos.; por contra, otras técnicas como las "ómicas" aunque permiten la rápida identificación de una amplia gama de biomarcadores al analizar simultáneamente cientos o miles de analitos presentes en un tejido o fluido son técnicas más complicadas, largas y caras de realizar. La elección de la técnica más adecuada dependerá por tanto de factores como los objetivos del estudio y de las características de los biomarcadores de interés. En muchos casos, el uso combinado de diferentes técnicas dará los mejores resultados. Por ejemplo, es posible identificar una bacteria de biomarcadores potenciales frente a una determinada condición mediante el empleo de ómicas, seguida de una validación clínica y analítica de estos biomarcadores mediante espectrofotometría o inmunoensayos, y finalmente desarrollar inmunoensayos portátiles para su uso en diferentes situaciones.

El **objetivo principal** de esta tesis doctoral fue aumentar el conocimiento sobre la identificación, medición y validación de nuevos biomarcadores de salud y bienestar que pueden usarse en diferentes contextos dentro del concepto de OH. Además, se priorizó el uso de muestras no invasivas como la saliva siempre que fue posible. Bajo esta premisa se utilizaron diferentes especies: humanos; perros ya que en ocasiones son empleados como modelos de enfermedades humanas; vacas que son propensas a sufrir mastitis, que es una enfermedad de gran importancia dentro del concepto OH; y peces y mejillones que aunque son especies filogénicamente alejadas de las anteriores se usan habitualmente en los programas de biomonitorización ambiental y por ello son de gran interés dentro del concepto OH.

Los **objetivos específicos** de la Tesis doctoral fueron:

1. Realizar una revisión del estado del arte sobre la búsqueda y validación de nuevos biomarcadores en diferentes especies y tipos de muestra, bajo las aplicaciones del concepto OH. Este objetivo se alcanzó mediante el Artículo 1, Capítulo de libro 1 y Apéndices I - II.

2. Realizar estudios proteómicos y bioquímicos en diferentes situaciones clínicas o experimentales para el descubrimiento de nuevos biomarcadores. Los estudios en humanos y mejillones se centraron en situaciones de daño muscular, mientras que los estudios en perros y vacas se hicieron en relación con diferentes enfermedades interesantes dentro del concepto de OH por su prevalencia o importancia clínica. Además, el modelo de evaluación medioambiental se realizó en peces y mejillones, ya que estás especies se emplean comúnmente como centinelas de la calidad medioambiental.

   Los modelos experimentales fueron:

   - Modelo 1: daño muscular. Este modelo se estudió en humanos y mejillones y condujo a los Artículos 2-4 y al Apéndice III.

   - Modelo 2: patologías. Este modelo se estudió en perros y vacas y se dio lugar a los Artículos 5-9 y los Apéndices IV - V.

- Modelo 3: condiciones medioambientales como hipoxia o contaminación, que se estudiaron en mejillones y peces y condujo a los Artículos 10-14.

3. Identificar un biomarcador común mediante la aplicación del concepto de OH y desarrollar un sistema rápido para su medición. La identificación de la proteína C-reactiva (CRP) -una proteína de fase aguda mayor en humanos y perros- en mejillones se alcanzó en el Artículo 15, mientras que la creación de un anticuerpo monoclonal anti CRP para el desarrollo de un test rápido y portátil para medir CRP en las tres especies dio lugar al Apéndice VI.

Esta Tesis doctoral cuenta con la aprobación del Comité Ético de la Escuela de Veterinaria de la Universidad de Murcia (CEEA 479/2018; ID: 2038/2018). Todos los estudios fueron realizados teniendo en consideración el principio de las 3R de la experimentación animal (ley 32/2007 y Real Decreto 53/2013), la Directiva 2010/63/UE del Parlamento Europeo y del Consejo relativa a la protección de los animales utilizados para fines científicos y la Declaración de Helsinki de 1964 sobre los principios éticos para las investigaciones médicas en seres humanos.

La **toma de muestras** se realizó de diferentes maneras. En todos los casos, las muestras fueron analizadas inmediatamente tras su recolección o fueron congeladas a -80ºC hasta el día del análisis.

En personas *(Homo sapiens)*, el muestreo de saliva se realizó mediante flujo pasivo. Los participantes no pudieron beber, comer, fumar, lavarse los dientes o hacer ejercicio durante al menos una hora antes del muestro. El suero se adquirió mediante la centrifugación de sangre entera coagulada previamente obtenida mediante venipuntura de la vena cefálica. Las muestras de sangre capilar se obtuvieron mediante punción de la yema de los dedos.

En perros *(Canis lupus familiaris) y vacas (Bos taurus)*, las muestras de saliva se obtuvieron mediante la introducción de una esponja en la boca del animal hasta que ésta estuviera mojada, introduciéndola a continuación en un tubo Salivette (V-Bottom, Sarstedt, Aktiengesellschaft & Co, Nümbrecht, Germany) y para su centrifugado y separación de la saliva (Contreras-Aguilar *et al.*, 2017). El muestreo de sangre se realizó vía yugular, cefálica o safena,

colocando la sangre en tubos con activadores de la coagulación y centrifugando una vez coagulada para obtener el suero.

En mejillones *(Mytillus galloprovincialis)*, la hemolinfa se recolectó mediante punción y aspirado del músculo aductor posterior y posterior centrifugación en la que se desecha el pellet. Tras el sacrificio de los animales se procedió a la recogida de branquias y glándula digestiva, que fueron pesadas, homogenizadas 1:4 en tampón fosfato y centrifugadas.

En peces *(Sparus aurata* y *Oncorhynchus mykiss)* se siguieron los métodos descritos por Guardiola et al. (2016) para la obtención de moco cutáneo. En resumen, se realizó un raspado cutáneo suave en la superficie dorso lateral, evitando lesionar la piel. El moco cutáneo fue entonces homogenizado 1:1 en tampón Tris salino y centrifugado. La sangre fue recogida de la vena caudal mediante el empleo de jeringas heparinizadas y posteriormente centrifugada para la obtención de plasma.

Se emplearon diversas **técnicas analíticas** durante la consecución de los objetivos de esta Tesis doctoral, los cuales se resumen a continuación:

Estudios proteómicos. Los estudios proteómicos realizados incluyen técnicas basadas en gel y técnicas que no requieren el uso de geles. En todos los casos se realizó inicialmente una determinación de la concentración de proteínas presentes en las muestras mediante la técnica de Bradford (Bradford, 1976). Las técnicas basadas en gel consistieron en electroforesis de dos dimensiones (2-DE), en las que las proteínas se distribuyen en el gel atendiendo a su peso molecular (eje vertical) y su punto isoeléctrico (eje horizontal), y ensayos tipo Western Blotting (WB), en el que las proteínas primero se ordenan atendiendo a su peso molecular en un gel, para a continuación transferirlas a una membrana de nitrocelulosa con el objetivo de detectar si un determinado anticuerpo tiene afinidad por alguna proteína presente en la muestra. En el caso de técnicas no basadas en gel, se emplearon ensayos utilizando Tandem Mass Tags (TMT, Thermo Fisher Scientific) siguiendo las recomendaciones del fabricante o precipitación de proteínas y digestión con tripsina, siguiendo protocolos descritos anteriormente (Martínez-Subiela *et al.*, 2017) y (Villar *et al.*, 2015), respectivamente.

Análisis bioquímicos, el listado de análisis bioquímicos empleados en esta Tesis doctoral puede verse en la Tabla 1.

Cromatografía de afinidad. Se utilizó esta técnica para la purificación de la proteína C-reactiva (CRP) en homogenizados de glándula digestiva de mejillón. En resumen, los homogenizados de glándula digestiva de mejillón fueron filtrados y concentrados hasta un volumen de 4 mL. La columna de afinidad con O-phosphoryletanolamine como ligando fue preparada siguiendo las instrucciones del fabricante (GE Healthcare), y la técnica se desarrolló utilizando un sistema automático de cromatografía líquida (ÄKTA pure, GE Healthcare).

Esta Tesis doctoral incluye estudios de **validación analítica** de diversos analitos. En concordancia con protocolos previos (Kjelgaard-Hansen y Jacobsen, 2011), la validación analítica consistió en la evaluación de la precisión, exactitud, límite de detección y estabilidad.

Precisión. Se calculó el coeficiente de variación (CV) intra- e inter-ensayo utilizando muestras con valores altos, bajos y medios que fueron medidas 5 veces en el mismo momento (intra-ensayo) o en días consecutivos (inter-ensayo).

Exactitud. Se evalúo la exactitud de los ensayos mediante el estudio de la linealidad bajo dilución, en la que una muestra de valor conocido se diluye secuencialmente desde 1:2 a 1:64 en agua ultra pura, suero salino fisiológico o tampón, y mediante estudios de recuperación, en los que dos muestras de valores conocidos se mezclan entre sí en diferentes concentraciones.

Límite de detección. Se calculó como el valor medio de medición 10 medidas de agua, suero salino fisiológico o tampón más dos desviaciones estándar.

Estabilidad. Los efectos del almacenamiento fueron evaluados mediante la medición de al menos 5 muestras en fresco y tras su almacenamiento a -80ºC durante 7, 15, 60 y 120 días.

Para la consecución del **objetivo 1** se realizaron dos revisiones de la literatura. En el Artículo 1 se describen las potenciales ventajas de la adopción del concepto OH desde el punto de vista de un laboratorio de análisis clínicos, así como consejos para su aplicación. Por su parte, en el Capítulo de Libro 1 se describen las principales técnicas analíticas usadas en el estudio de saliva tanto en humanos como en animales. Además, se incluyen dos Apéndices resultados de la consecución del objetivo 1. En el Apéndice I se describe el nivel de conocimiento sobre OH en alumnos de 2º de Veterinaria de la Universidad de Murcia antes y después de la realización de un módulo sobre

OH basado en el aula invertida. El Apéndice II describe los cambios que se producen en algunos biomarcadores tras el empleo de algunas técnicas comunes en los estudios con saliva humana, como son la filtración de partículas y la depleción de la proteína alfa amilasa.

Para el abordaje del **objetivo 2** se realizaron estudios bioquímicos y proteómicos en suero y saliva de diversas especies con el fin de detectar biomarcadores de utilidad en diversas situaciones.

El primer modelo evaluado consistió en daño muscular. Éste se basó en el estudio de un modelo de daño muscular inducido en mejillón (Artículo 2) para determinar si podría utilizarse como modelo animal, y en el posible daño muscular producido tras el ejercicio en deportistas. En mejillones, se identificaron cambios en la expresión de diversas proteínas tanto mediante proteómica 2DE como mediante análisis bioquímico, lo que podría sugerir la utilidad del mejillón como modelo animal de daño muscular. En personas, se estudiaron los cambios en la composición de la saliva después de ejercicios de resistencia (Artículo 3) y de esfuerzo explosivo (Artículo 4), observando cambios en más de 20 proteínas en comparación con los valores basales, las cuales podrían tener potencial para la monitorización de la actividad física. También se identificaron 65 proteínas que cambiaban significativamente entre hombres y mujeres en reposo así como 16 proteínas que cambian diferencialmente en respuesta al ejercicio entre hombres y mujeres, lo que demuestra diferencias asociadas al sexo que deben ser tenidas en cuenta por deportistas e investigadores. En el Apéndice III se desarrolló y validó un método automático para la medición de adenosine deaminase (ADA) y sus isoenzimas ADA1 y ADA2 en saliva humana, además de evaluar si estos marcadores pueden ser de utilidad en los dos modelos de ejercicios anteriores. El método permitió la medición automática de ADA y sus isoenzimas en saliva humana y mostró correlación con otros biomarcadores usados tradicionalmente en las ciencias del deporte como la inmunoglobina A o la alfa amilasa, por lo que podría ser un biomarcador válido en estos contextos.

El segundo modelo comprende enfermedades de gran importancia tanto clínica como dentro del concepto OH en perros y vacas. Todos los estudios emplearon técnicas de proteómica sin gel y siempre que fue posible se optó por el uso de saliva al tratarse de una muestra no invasiva. Las enfermedades a estudiadas en esta Tesis doctoral fueron leishmaniosis (Artículos 5-6), parvovirosis (Artículo 7), piometra (Artículo 8), tumores mamarios (Artículo 9) y

diabetes mellitus (Apéndice IV) en perros, así como mastitis en vacas (Apéndice V). Estos estudios permitieron la identificación de más de 6700 proteínas, observando diferencias estadísticamente significativas en más de 400 proteínas en relación a su grupo control. Además, se realizaron estudios bioinformáticos para identificar los procesos biológicos y rutas metabólicas más comúnmente afectados, y los cambios observados en algunas proteínas como la albúmina y la haptoglobina fueron validados en muestreos independientes. Muchas de estas proteínas diferencialmente moduladas en estos estudios fueron descritas por primera vez en estas enfermedades, lo que abre las puertas a diversas vías de investigación sobre el desarrollo de nuevos biomarcadores para su diagnóstico, pronóstico o monitorización. Finalmente, estos estudios ponen de manifiesto el gran potencial de la saliva como alternativa no invasiva a los análisis sanguíneos u otras técnicas invasivas, ya que se observaron cambios en un mayor número de proteínas en comparación con el suero.

El tercer modelo del objetivo 2 se centró en la biomonitorización ambiental mediante el desarrollo de nuevos biomarcadores en peces y mejillones. Los estudios en mejillones se centraron en validar diversos métodos automáticos para la medición de biomarcadores y observar si éstos podían ser de utilidad para detectar los efectos provocados por contaminantes (Artículos 10-11) o hipoxia (Artículo 12). En estos estudios se validaron con éxito 9 métodos automáticos para su uso en muestras de mejillón, y se observaron cambios en EA, TOS, TAC, AChE, FRAP, AOPP, CUPRAC y tioles en branquias, glándula digestiva o hemolinfa en los grupos expuestos a contaminación por metales pesados o hipoxia con respecto a los grupos control. En peces, se validaron EA, TOS y TAC y se utilizaron para analizar los efectos de contaminantes derivados de la industria farmacéutica (Gemfibrozil) (Artículo 13), así como también se validó con éxito un kit diseñado para la medición de cortisol en humanos por métodos de quimioluminiscencia (CIA) como alternativa a los radioinmunoensayos (RIA) para su uso en plasma y moco cutáneo (Artículo 14). Todos los biomarcadores estudiados mostraron una correcta validación analítica en muestras de branquias, plasma o moco cutáneo de peces. Los animales expuestos a gemfibrozil mostraron cambios en sus niveles de TOS, además de otras alteraciones. Finalmente, cuando se compararon los valores de cortisol obtenidos por CIA con los obtenidos mediante RIA, se observó una alta correlacione entre ellos ($r= 0,93$ ($p<0,001$) en plasma y $r=0,95$ ($p<0,001$) en moco cutáneo), aunque CIA era capaz de detectar aumentos significativos de cortisol seis horas tras la inducción del estrés que no fueron detectados con RIA.

Los resultados de estos estudios sugieren el gran potencial de la aplicación de métodos actualmente usados en medicina humana y veterinaria para la biomonitorización ambiental, así como el gran beneficio económico y ecológico que puede suponer la adaptación de unos métodos a otras especies y tipos de muestras.

Para la consecución del **objetivo 3** de esta Tesis doctoral se realizó en primer lugar un estudio con el objetivo de comprobar si existía la proteína C-reactiva (CRP) en mejillón (Artículo 15), ya que es una proteína ampliamente utilizada para detectar inflamación en humanos y perros. En este estudio se consiguió identificar y purificar CRP en glándula digestiva de mejillones por primera vez. Además se validó analíticamente el uso de un método comercial para la medición de CRP humana en muestras de mejillón, y se demostró su potencial aplicabilidad en el campo de la biomonitorización ambiental al mostrar incrementos en animales expuestos a hipoxia o contaminantes. En el Apéndice VI se muestran los resultados de la creación de una batería de anticuerpos monoclonales para la detección de CRP, con el objetivo posterior de desarrollar un sistema rápido de detección de CRP portátil que pueda ser empleado en diferentes especies. La creación de los anticuerpos monoclonales anti-CRP se realizó siguiendo protocolos previos (Yokoyama, 1999), consistiendo principalmente en el aislamiento de la proteína, inoculación en ratones, fusión con células de mieloma y selección de aquellos hibridomas que producen anticuerpos que reconocen CRP purificada. Tras tres ciclos de selección de hibridomas, un total de 17 líneas celulares que reconocen CRP canina purificada mediante ELISA se proponen como posibles candidatas para la generación de sistemas de detección rápidos y portátiles de CRP.

De la discusión de los resultados obtenidos en esta Tesis doctoral se pueden derivar seis **conclusiones**.

1. El concepto One Health puede ser aplicado a las condiciones de laboratorio mediante el uso de un mismo biomarcador en diferentes especies, tipos de muestra y enfermedades.
2. Las muestras no invasivas como la saliva y el moco cutáneo son biofluidos de gran valor analítico ya que son capaces de mostrar cambios en su composición tras condiciones de daño muscular, enfermedades o hipoxia.

3. Los estudios relacionados con el daño muscular permitieron la identificación de un potencial panel de biomarcadores para su diagnóstico en muestras de saliva de humanos y en hemolinfa de mejillón, incluyendo lactato, actina, tropomiosina y cistatina-B.
4. Los estudios proteómicos realizados en perros y vacas mostraron que tanto el suero como la saliva pueden proporcionar una valiosa información clínica en diversas enfermedades como parvovirosis, leishmaniosis, piometra, diabetes mellitus, tumores de mama y mastitis. Más de 6700 proteínas fueron identificadas en estos estudios y más de 400 fueron diferencialmente moduladas en estas situaciones, proporcionando una gran batería de posibles biomarcadores para estas enfermedades.
5. Ciertos biomarcadores empleados en medicina humana para la evaluación del estrés oxidativo pueden aplicarse en otras especies como peces y mejillones para la biomonitorización de los efectos fisiológicos asociados a contaminación ambiental. Las características de estos biomarcadores (rápidos, económicos, sensibles, accesibles, automáticos y requieren poco volumen de muestra) los hacen viables para su uso en programas de biomonitorización ambiental relacionados con contaminación o hipoxia.
6. La proteína C-reactiva ha sido identificada y descrita en mejillones por primera vez en esta Tesis doctoral. Además, se han producido una serie de anticuerpos monoclonales con afinidad con la proteína C-reactiva canina, que podrían dar pie a la creación de un sistema rápido y portátil de medición de CRP en distintas especies para la detección de inflamación.

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Appendices

It was considered appropriate to include as appendices the abstracts from data of experiments carried out during the thesis. This data is pending for its publication in JCR journals or planned to be submitted for possible publication in the future.

Appendix I

Teaching the One Health concept to undergraduate veterinary students: a pilot study.

The "One Word, One Health" (OH) concept emphasises the need for collaborative multi-disciplinary approaches for the assessment of human and animal health and the environment. Although the OH approach is being increasingly acknowledged among the professionals of different fields, it is unclear if the undergraduate veterinary students receive adequate education about OH and its importance.

The present study evaluates a module of activities created to teach the OH concept which was given in the course of General Pathology to second-year veterinary students. The module consisted of three different activities: theoretical classes, teamwork for the preparation of PowerPoint or video presentations, and expositions of the students of a practical case. For this, two questionaries' consisting of online surveys, were fulfilled before (questionnaire 1, Q1, n=55) and after (questionnaire 2, Q2, n=45) a course about OH. Each questionnaire consisted in 3 groups of questions related to (1) personal data (sex, age), (2) the OH concept and applications, (3) their opinion about the OH course in Q2.

The pre-course questionnaire Q1 showed that the majority (62%) of the surveyed students had heard about OH and acknowledge its importance, although their definitions of OH did not include humans, animals or the environment in 71% of cases. After the course, the general knowledge of the students about OH was improved (from >80% indicating poor or very poor knowledge in Q1 to >95% indicating normal, good or very good in Q2). Most students classified the course positively, although the lack of time and the high number of students per groups were the main drawbacks.

In conclusion, the active-learning OH-based course was fruitful for the increase of awareness and knowledge of OH by veterinary students. The survey system provided with an overview of the perception of OH by the students allowed detecting gaps in their knowledge about the topic such as the consideration of the environment or socioeconomic concerns as important topics in OH. This report could serve as a basis for the inclusion of OH in the development of future curricula designs by the teaching institutions.

Appendix II

Effects of filtration and alpha-amylase depletion on salivary biochemical analysis.

Saliva has several advantages over other invasive and non-invasive specimens such as high acceptance of repeated collection by the subjects, easy to collect from people of all ages, and pain and stress-free sampling. On the other hand, the use of saliva as diagnostic specimen possesses a series of challenges such as high variability between and within subjects, high viscosity, or the presence of some components such as alpha-amylase than could interfere with the measurement of other analytes.

In the present study, the effects of saliva (n=14) filtration and alpha-amylase depletion were assessed. Each saliva sample was split into three aliquots that were treated as follows: (1) No additional treatment; (2) passed through a 0.45 µm commercial filter (Millipore, PAIS); and (3) passed through a 0.45 µm commercial filter (Millipore, PAIS) and by a home-made column with potato starch for the affinity depletion of alpha-amylase, as described by Deutsch et al. (2008). A battery of biochemical biomarkers consisting on amylase, ALT, AST, GGT, ALP, lipase, CK, calcium, phosphorus, total protein, albumin, urea, creatinine, cholesterol, triglycerides and uric acid were measured in all aliquots in the same run.

Statistically relevant differences after filtration in comparison to pre-treatment samples were observed for ALT, AST, GGT, lipase, CK, cholesterol and triglycerides. These biomarkers plus amylase, ALP, calcium, phosphorus, total proteins, urea, creatinine, and uric acid showed significant changes after the amylase depletion of the samples. Besides, all the analysed biomarkers except albumin and cholesterol were statistically different between filtration and amylase-depleted measurements. Although the changes in alpha-amylase and total protein were expected after depletion, the causes of the differences in the rest of biomarkers are unknown and should be further evaluated.

In conclusion, the salivary filtration and amylase depletion methods employed in the present study, which are among the most used in literature, caused significant changes in a variety of biomarkers. Although these changes should be confirmed in future studies, it may be recommended for researchers to analyse the possible influence of these protocols in their biomarkers of interest and consider these biases when interpreting results.

References: O. Deutsch, Y. Fleissig, B. Zaks, G. Krief, D.J. Aframian, A. Palmon, Electrophoresis. 29 (2008) 4150–4157. DOI:10.1002/elps.200800207.

Appendix III

Adenosine deaminase (ADA) and its isoenzymes in saliva: analytical validation of an automated assay and applications in physical and psychological stress.

The present study aimed to validate an automated assay for the measurement of total adenosine deaminase (tADA) and its isoenzymes (ADA1 and ADA2) in saliva using a low sample volume in a fast and accurate way. Also, this assay was applied to evaluate if tADA and its isoenzymes could change in different situations of physical and psychological stress and its possible relations with biomarkers of the immune system such as immunoglobulin A (IgA) and total white blood cell (WBC) count, biomarkers of anaerobic metabolism such as lactate and biomarkers of psychological stress such as salivary alpha-amylase (sAA).

The high- throughput automated method was accurate and precise, with intra- and inter-assay coefficients of variation below 9.43%, linearity under dilution linear regression equation with R2 close to 1, and percentage of recovery between 80 and 120 in all cases.

Resistance exercise did not produce significant changes in tADA; however, tADA and ADA2 showed correlations with blood WBC and salivary IgA. In the situation of explosive effort sequences, tADA in saliva was decreased in untrained individuals after several 60m sprints, and correlated with blood lactate. In a situation of psychological stress, ADA did no differ nor correlated with salivary alpha-amylase.

In conclusion, tADA and its isoenzymes ADA1 and ADA2 can be measured accurately and precisely in saliva samples in a rapid, economical, and reproducible way. In addition, they correlated with biomarkers of the immune system (WBC and IgA) in a resistance exercise and they showed a short-term decrease after an intensive exercise in non-trained individuals. However, they did not change after psychological stress. These data contribute to making saliva analysis of ADA more widely used, increase the knowledge about changes of ADA in saliva in different situations such as exercise or psychological stress.

Appendix IV

The serum and saliva proteome of dogs with diabetes mellitus.

This study aims to evaluate the changes in salivary and serum proteomes that occur in canine diabetes mellitus type-1 (DM) through a high-throughput quantitative proteomic analysis. The proteomes of 10 paired serum and saliva samples from healthy controls (HC group, n=5) and dogs with untreated DM (DM group, n=5) were analysed using Tandem Mass Tags (TMT) – based proteomic approach. Additionally, 26 serum samples from healthy controls and untreated DM were used to validate haptoglobin in serum.

The TMT analysis quantified 767 and 389 proteins in saliva and serum, respectively. Of those, 16 unique proteins in serum and 26 in saliva were differently represented between DM and HC groups. The verification of haptoglobin in serum was in concordance with the proteomic data.

In conclusion, our results pointed out changes in saliva and serum proteomes that reflect physiopathological changes occurring in DM in dogs. While some of the proteins identified here were previously described in relation with DM in dogs, most of the proteins found to be modulated in serum and saliva are described here for the first time and can be potential biomarkers of the disease.

Appendix V

Changes in saliva and serum proteins in cows with mastitis: a proteomic approach

Currently, bovine intramammary infection or mastitis is an endemic disease among the most important problem in the global dairy industry causing decreased welfare of affected animals, as well as important economical loses. Label-free proteomics has been increasingly used for the detection of biomarkers of health and welfare in a variety of species and sample types. We hypothesize saliva and serum proteomes would change in cows with mastitis in comparison to healthy ones, and these changes may reveal potential biomarkers of diagnosis in this disease.

In the present study, the salivary and serum proteomes of healthy cows (n=18) and cows with clinical mastitis (n=18) were studied using Tandem Mass Tags based proteomics approach.

More than 2000 and 1200 proteins were identified in saliva and serum proteomes, respectively, being 63 in saliva and 29 in serum differentially modulated between the two groups.

In conclusion, alterations in saliva and serum proteome are present during mastitis in cows. The proteins which change in mastitis might serve as an initial step for the identification and validation of novel early biomarkers of this disease that could improve animals' health, welfare and increase the economical savings in the dairy industry.

Appendix VI

Development of a novel Point of Care assay for the measurement of C-reactive protein in different species.

According to the One Health concept, the advances achieved in one species could be applied to others, promoting the increased knowledge and resources. One of the possible applications of this concept is the use of the same assay in different species, which may be especially relevant in highly conserved pathways such as stress or inflammation. Acute-phase proteins such as C-reactive protein (CRP) are among the most employed biomarkers of inflammation due to its high sensitivity.

The present study attempted to produce a monoclonal anti-CRP antibody for the subsequent development of a fast and portable point-of-care assay for the measurement of CRP in a variety of species. For this, canine CRP was purified using commercial agarose and monoclonal antibodies for this protein were developed following previous protocols. The affinity of the monoclonal clones against canine CRP was assessed by ELISA for three times during the process, selecting those clones which showed higher affinity for further development.

A total of 17 cell clones produced monoclonal antibodies that recognized CRP with higher affinity than other commercial antibodies. For practical reasons, all cell lines were frozen and three were proposed for further research. The affinity of anti-CRP monoclonal antibodies produced by these three cell lines were tested against human CRP by ELISA and western blotting.

The development of a monoclonal anti-CRP antibody produced in the present study entails the first step for the production of point-of-care assays that would be able to the early detection of inflammation on a variety of species.

Appendix VII

Journal Impact Factor.

Table 2. Journal impact factor and quartile of the articles published in the present PhD thesis, according to Journal Citation Reports (JCR). *Last available data (2019). Q: quartile, D: decile.

Journal	Year of publication	Impact factor*	Quartile	Category
Animals	2020*	2.323	Q1	Veterinary Sciences
Frontiers in Veterinary Science	2020*	2.245	Q1	Veterinary Sciences
Transboundary and Emerging Diseases	2019	4.188	Q1 (D1)	Veterinary Sciences
Journal of sports medicine and physical fitness	2019	1.432	Q4	Sport Sciences
Comparative Immunology, Microbiology and Infectious Diseases	2018	1.871	Q1	Veterinary Sciences
Ecological Indicators	2018 2019	4.49 4.229	Q1 Q1	Environmental Sciences
Fish and Shellfish Immunology	2018	3.298	Q1 (D1)	Veterinary Sciences
Research in Veterinary Science	2020*	1.892	Q1	Veterinary Sciences
Veterinary Parasitology	2019	2.157	Q1	Veterinary Sciences
Journal of Proteomics	2020*	3.509	Q2	Biochemical Research Methods
Comparative Biochemistry and Physiology Part - B: Biochemistry and Molecular Biology	2018	1.99	Q1	Zoology
Marine Pollution Bulletin	2016	3.146	Q1 (D1)	Marine & Freshwater Biology
Environmental Science and Pollution Research	2016	2.741	Q2	Environmental Sciences