Dyslipidaemia in canine inflammatory diseases: lipoproteins as biomarkers

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Babesia canis induces a marked acute phase response (APR) in dogs and can lead to sepsis and death, but some dogs could remain asymptomatic carriers. It is not known if asymptomatic carriers also could have a subclinical inflammatory response. Also, it is not clear if dogs could completely eliminate *B. canis* without pharmacological treatment. Stratification of APR or subclinical inflammation could be evaluated measuring serum concentrations of acute phase proteins, such as serum amyloid A (SAA) that is also a part of high denisty lipoprotein (HDL) particles. HDL is the dominant class of lipoproteins in dogs that enables revers cholesterol transport. The APR and sublinical inflammation induce alterations in lipid metabolism that can lead to important changes in the concentration of major plasma lipids (cholesterol, phospholipids, triglycerides) and in the concentration and morphology (diameter and/or composition) of lipoproteins. It is known that during APR, SAA replaces apolipoprotein A-1 (ApoA-1). ApoA-1 is a major apoprotein of HDL and is considered as a negative acute phase protein. It is considered that binding of SAA and displacement of ApoA-1 influence HDL steady state roles. We have shown that *B. canis* induced APR lead to several changes: decrease in levels of cholesterol, phospholipid and HDL and increase in HDL diameter, SAA and ApoA-1 concentration. SAA correlates with HDL diameter and ApoA-1. Moreover asymptomatic carrier dogs have similar changes as APR dogs: decrease in HDL and increase in SAA concentration. Asymptomatic dogs without detectable *B. canis* DNA in the blood and seroreactive to *B. canis* antibodies had SAA in reference range, while HDL and cholesterol levels were decreased. Except evidence that lipoprotein metabolism is changed in *B. canis* infection, we also demonstrated that different etiology factors lead to similar lipid and lipoprotein changes during inflammation. In conclusion, this preliminary results show that inflammation, either acute clinical or subclinical lead to alteration in lipid and lipoprotein metabolism and/or changes in apoproteins concentration.

Key words: dogs, inflammation, serum amyloid A, apolipoproteinA-1, high density lipoprotein

Salivary biomarkers in veterinary medicine

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Saliva as a diagnostic fluid has gained interest in the last years since it can reflect the physiological and pathological state of the body and research is readily demonstrating saliva as a viable biofluid for performing detection of different biomarkers (Lee and Wong, 2009; Wang et al., 2016). The use of saliva has many benefits as being cheap, painless, non-invasive and easy to collect. In addition, saliva could be used to sample animals in remote or resource-poor areas and represent a great advantage for people who live far away from veterinary practices or for animals that are not used to being transported (Cantos-Barreda et al., 2017). However, despite all these advantages its use has not been many extended in the veterinary practice, may be due to some drawbacks. The main problems associated to the measurement of any compound in saliva could be: the lack of understanding of the changes that can be found in the different biomolecules that can be present in saliva in relation with the pathogenesis of diseases and also the fact that, although most analytes detected in serum are also found in saliva, their levels are substantially diminished so high sensitive technologies must to be used to detect the low concentrations expected (Parra et al., 2005).

The presentation will be focused in providing updated information about the basics for saliva analysis, including the discussion of some some preanalytical issues that must be considered before using saliva to measure any biomarker, as for example the method of collection, storage conditions or possible presence of circadian patterns. In addition, we will outline our experience with analytes that can be measured in saliva. Therefore this presentation will provide an overview of the potential application of saliva as a non-invasive diagnostic sample and we hope it can stimulate further studies in order to gain knowledge in this field.

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Biomarkers of blood coagulation in canine medicine

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Examination of blood coagulation is indicated, for example, to find possible causes for haemorrhagic diathesis and thrombophilia, to assess the severity of coagulation disorders in diseases regularly associated with coagulation disorders (e. g., sepsis), as part of the preoperative screening, and for monitoring disorders of haemostasis and anticoagulant treatment. Furthermore, coagulation proteins can act as liver function test and are partly minor acute phase proteins. Blood coagulation tests include global tests (viscoelastic measurements, thrombin generation), group tests (prothrombin time [PT], activated partial thromboplastin time [APTT], thrombin time), individual coagulation proteins (fibrinogen, coagulation factors, inhibitors) and activation markers of coagulation.

Viscoelastic measurements on whole citrated blood have the advantage of assessing the interaction of blood coagulation factors and cells (platelets) and to inform about the clot quality. The generation of thrombin is a fundamental part of the clotting cascade and, thereby, the estimation of endogenous thrombin potential, i. e. the concentration of thrombin in clotting plasma, may individually help to predict possible risks of bleeding or thrombosis.

Conventional coagulation tests PT and aPTT have disadvantages (artificial system, very early endpoint, when only 3–5 % of thrombin is generated). Nevertheless, PT and aPTT are the standard tests to efficiently examine the whole coagulation system. Supplemented by platelet count and – where appropriate – capillary bleeding time (reflecting platelet count, platelet function and von Willebrand factor activity) they provide a rational screening profile of haemostasis. PT should be measured using a modified test system with diluted plasma and fibrinogen supplementation. Coagulation factor measurements with human deficient plasmas require higher predilution of canine sample plasma than quoted for human sample material. Antithrombin can be measured using human test systems. Amidolytic protein C assays require modifications due to limited activation of canine protein C by Protac.

Activation markers of coagulation such as D-dimers, soluble fibrin, thrombin-antithrombin-complex, fibrinopeptide A, and prothrombin fragment 1 + 2 can help to diagnose DIC (as part of advanced DIC scoring systems) and to exclude thrombosis and possibly to test the efficacy of anticoagulant treatments.

Proteomic and metabolomic platforms to identify biomarkers of equine arthropathies

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The presentation will cover our use of mass spectrometry proteomics and NMR metabolomic platforms to distinguish osteoarthritis (OA) synovial fluid within equine arthropathies and stratify OA in the horse.

Osteoarthritis (OA) is characterised by loss of articular cartilage, abnormal bone proliferation, synovial membrane dysfunction and subchondral sclerosis. The pathogenesis of OA is not fully understood with no disease-modifying treatments available. Few studies have used a global approach to stratify the equine synovial fluid (SF) proteome or metabolome according to OA severity. The identification of differentially expressed proteins to distinguish arthropathies or OA severity will aid our understanding of underlying disease pathogenesis, determining markers of early OA to allow timely management interventions and aid in discovering potential therapeutic targets.

I will describe the next generation omics methods used to interrogate SF in different equine arthropathies. Methodological descriptions will include how to identify lower abundance proteins in a complex medium such as synovial fluid or serum, proteolytic digestion options, the use of high mass resolution LC-MS/MS systems (such as the UltiMate 3000 Nano LC System coupled to a Q Exactive Quadrupole-Orbitrap), data analysis and follow on bioinformatics analysis. Additionally neopeptide analysis as biomarkers will be described.

Methods including sample preparation and data analysis we have employed using 1D ^{1}H NMR spectroscopy will be introduced. The use of univariate and multivariate statistical analyses as well as identification software such as Chenomx will be discussed.

The result of a number of our published and unpublished work will be presented. We have identified a panel of proteins, neopeptides and metabolites which alter in accordance with OA severity or the type of arthropathy. These potential biomarkers hold promise in early disease identification and the stratification of equine OA and as indicators of early OA. This may allow for timely interventional management and help identify further therapeutic targets.

Making veterinary test for acute phase proteins: from biomarker discovery to commercial product

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Acute phase proteins (APPs) are blood proteins which change its circulating concentration in response to inflammation caused by tissue injury, infection or stress. The APP response is rapid and usually reflects disease evolution, correlating with the severity of the underlying condition. APPs are highly sensitive indicators of inflammation, increasingly used in veterinary medicine as biomarkers in the diagnosis, prognosis and monitoring of the efficacy of treatments of infectious and inflammatory diseases. Differences exist in the pattern of APP response among species. In the pig main APPs include CRP, haptoglobin, SAA, and pig Major Acute Phase protein (pig-MAP). Pig-MAP was described by first time in 1994 by researchers of the University of Zaragoza in a study aimed to characterize the acute phase response in the pig. This novel protein was further characterized as a new member of the inter-alpha-trypsin inhibitor family, although it is not a proteases inhibitor. Pig-MAP has proved sensitive biomarker of inflammation and stress conditions/welfare in the pig. An overview of the story of this protein and the different methods used for its quantification in connection with the biomarker development pipeline will be presented. The second part of the presentation will deal with the development and validation of a turbidimetric immunoassay for canine CRP. The assay is a particle-enhanced method which uses antibodies and standard specific for the canine species. It is a precise, accurate and robust method which is not affected by hemolysis, lipaemia or bilirubinemia. Special mention will be done of the importance of an adequate validation and standardization of the assays, and the necessity of common reference materials for the calibration of biomarkers assays in veterinary medicine, in order to harmonize the measurements.

Development and Commercialisation of Point Of Care Diagnostics

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Discovery of a biomarker's association with a disorder is just the start of a journey to potential commercialisation, and there are many stages that it must go through before it becomes an on-market reality. An initial assessment of the commercial viability should encompass a review of the intellectual property status, the technical and clinical utility as well as potential market size. Most proposed new markers will fail at least one of these assessment criteria, but the most common reason for markers not being fully commercialised is that whilst the analyte is associated with the particular disorder it is not providing any additional information and is telling the veterinarian or clinician what they already know.

If a marker passes these initial assessment criteria, then development of the assay will proceed through multiple stages, typically Proof-of-Concept/Feasibility, Optimisation, Scale up, pilot lot manufacture, design freeze, production of validation and verification batches, verification and validation testing and then preparation of launch materials such as 'Instructions for Use'. In some cases regulatory clearance may be required before a product can be sold. Developing assays for 'Point-of-Care' use can have particular challenges as these are often intended for use by non-laboratory staff and therefore they need to be developed to be as easy to use and robust as possible.

Even after passing the initial assessment criteria and following successful assay development, challenges remain in gaining market acceptance, and considerable effort is generally needed to generate scientific papers demonstrating the utility of the new test and to make veterinarians aware of its utility and advantages.

New Homogeneous Luminescence Immunoassays Reveal More About Acute Phase Protein Levels In Dogs With Lymphoma Than Previously Thought

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Despite widespread use of acute phase protein (APP) testing in human medicine and a reasonable amount of peer-reviewed veterinary literature on the importance of APPs their methods by the wider veterinary community is limited; except in Japan and South Korea. A range of tests for APPs in dogs, cats and horses are available as either reference laboratory methods or on point of care devices, yet the only method that has so far seen anything like wide-spread use is serum amyloid A in horses.

We have recently developed a range of novel tests for APPs using Spatial Proximity Analyte Reagent Capture Luminescence (SPARCL™) technology. SPARCL™ uses two specific antibodies: one conjugated to horse radish peroxidase (HRP), the other to acridan, a chemiluminescent substrate. When HRP and acridan conjugated antibodies bind to their target biomarker they are brought into proximity. Upon addition of hydrogen peroxide, HRP catalyzes oxidation of proximal acridan molecules causing a flash of chemiluminescence that is proportional to biomarker concentration. Acridan molecules distant from HRP are not oxidized and therefore produce no luminescence. The technology therefore facilitates a simple “mix and measure” one-step protocol, free of repeated wash and incubation steps required by conventional ELISA tests.

Using unique antibody pairs, we have developed high sensitivity SPARCL™ assays for APPs that are of improved clinical relevance for detection and monitoring canine lymphoma. Tests are available in 96-well microplate and single-tube formats for reference laboratory and in-practice use respectively.

Data will be provided on the use of SPARCL™ assays for APPs and other inflammation related biomarkers. The relevance of these data to potentially wider-spread application of SPARCL™ tests in veterinary medicine will also be discussed.