

ATTI DEL CONGRESSO



SEASON FOUR

RESEARCH IS COMING

DEPARTMENT OF VETERINARY MEDICINE - UNIVERSITY OF PERUGIA



4TH JUNE, 2021

Form 9:00 to 17:30

Virtual, Teams platform



SEASON FOUR

4th June 2021

Tendinopathy: the Possible Role of Oxidative Stress, Inflammatory Conditions and Local Anesthetics.

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Tendinopathy is a multifactorial spectrum of tendon disorders. Although its etiopathogenesis remains still poorly defined, mechanical and oxidative stress due to tendon overuse have been described as the major risk factor for tendinopathy [1]. Indeed, oxidative stress, inflammatory mediators, such as cytokines, nitric oxide, prostaglandins and lipoxins, play crucial role in modulating changes in the extracellular matrix during the onset of tendinopathy and in the failure of tendon lesion healing [2,3]. The healing is critical processes that is often also impaired by some drugs, such as local anesthetics which are used to cope the tendon pain [4].

The aim of this study is to define some metabolic and molecular properties of the equine tenocytes in order to better understand the molecular aspects related to their poor responsiveness to the stressors, that have been described as main causes of tendon injuries.

Primary cultures of tenocytes obtained from superficial flexor tendons of horse phalanges will be used. The cells will be exposed to local anesthetics, pro-oxidant (e.g. hydrogen peroxide) and pro-inflammatory (e.g. IL1- β , PGE2) conditions, alone or in co-presence with antioxidants or anti-inflammatory molecules. Cell proliferation, apoptosis, necrosis and autophagy, will be evaluated to test the toxicity of the above mentioned stress conditions. Furthermore, the synthesis of the main molecular constituents of the ECM (e.g. collagen I and III, COMP, etc) and the enzymes (e.g. MMP-2, 9, 13, TIMP-1) modulating its turnover will be evaluated by Western Blotting, qPCR, and immunocytochemistry. The oxidative stress will be evaluated as oxidative protein damage by OxyBlot and by activation of Nrf2 pathways, a transcription factor known as a modulator of oxidative cell response. Moreover, quantitative proteomic analysis will allow to evaluate the changes of tenocyte proteome exposed to different stress.

Function analysis of proteomic data, by bioinformatic tools, will be performed to evidence the pathways that could be affected and/or involved in the stress response to experimental conditions.

The expected results of this PhD project can contribute to extend the knowledge on the molecular mechanisms underlying the onset of tendon injuries and to better understanding the role of oxidative stress, inflammation and local anesthetics in tendon homeostasis and in tendon lesion healing. A better definition of tenocyte metabolism and of the molecular processes underlying the etiopathogenesis of tendinopathies is needed for new therapeutic strategies to better manage the equine and human diseases.

[1] Fu S.C., et al. *J. Orthop. Res.* 36, 3268–3274. 2018; [2] Dakin S.G., et al.. *Vet. Immunol. Immunopathol.* 158, 121–127. 2014. [3] Millar N.L., et al.. *Nat. Rev. Rheumatol.* 13, 110–122. 2017; [4] Yang S.L., et al. *L. Med. Sci. Monit.* 20, 2478–2483. 2014.



SEASON FOUR

4th June 2021

Castration in the standing horse by laparoscopy

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Castration is a common surgical procedure that carries a relatively high complication rate (10-22%) [1]. Castration by laparoscopy was first developed in late '90 in the recumbent horse and it was then modified in the standing horse [2]. It is a fast and minimally invasive technique. Although these advantages, the laparoscopic approach has partially been abandoned due to the possible revascularization of the removed testes by an alternate blood supply from the external pudendal artery and/or the cremasteric artery [3].

The main purpose of our study is to validate the castration in the standing horse by laparoscopy as an alternative to traditional methods, especially for some categories of horses, such as older ones, draft breeds, horses with concurrent problems that would discourage general anaesthesia and monolateral abdominal criptorchids.

Stallions referred to the OVUD for castration are included in the study. Testosterone level is assessed at baseline, then 7 days and 2 months post-surgery. An accurate US examination by B-mode and color doppler of the descended testes is performed.

A pain scale [4] is applied before surgery and every 6h for 48h.

The antibiotic, antiinflammatory and anaesthetic protocols are standardized; two or three laparoscopic portal sites are created for the procedure. Local anesthetic is infiltrated in the spermatic cord and the Ligasure device is used to seal and cut the spermatic cord and the ductus deferens.

An US examination is performed at day 1, 3 and 6 post-surgery and 2 months later to assess the presence of residual or new vascular structures to the testis and to evaluate modifications of the testicular parenchima and the spermatic cord.

The study included 5 horses: 4 had both testis descended in the scrotum and 1 had a normally descended and an abdominally retained testis. One horse was referred for an inguino-scrotal herniation and 2 were draft horses.

Surgery was uneventful in all horses. Animals didn't show surgical-related discomfort and pain scores were below the considered treshold, except for 1 horse which developed a lethal colitis within 48h from surgery.

In the horse with one retained testis, surgery was performed at the side of the descended testis, avoiding the accesses from the other flank.

In the horse referred for the inguino-scrotal hernia at 2 months check laparoscopy the inguinal rings were mostly closed due to the scar tissue formation after transection of the spermatic cord.

The owners were totally satisfied of behavioural changes of their horse, and the serum testosterone level decreased significantly below the stallion threshold within 1 week after surgery in all horses.

The complete US examination was performed in 3 horses and showed the absence of vascularization to the testes since day 1 after surgery and didn't evidence any abnormalities at the following assessments.

Based on these preliminary results, we can consider laparoscopic castration as an easy, fast, minimally-invasive method for orchiectomy, with mild complications. At present, none of the horses showed revascularization of the descended testis with subsequent failure of the technique.

For other considerations, including pain assessment, the anatomical study, and the usefulness of US, more cases are advocated.

[1] Kilcoyne et al. *Eq vet. Edu*, 25, 476-482, 2013. [2] Rijkenhuizen et al. *Pferdeheilunde*, 16, 429-452, 1999. [3] Voermans et al. *EVJ*, 38:35-39, 2006. [4] Dalla Costa et al. *Plosone*, 9, 1-10, 2014



SEASON FOUR

4th June 2021

Gene and protein expression of the ngf and its cognate receptors in different testicle morphotypes of a grey squirrel population (*sciurus carolinensis*) living in central Italy

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Grey squirrel (*Sciurus carolinensis*) is an invasive alien species representing a serious threat to Eurasian red squirrel conservation. In line with one of the tasks of the EU Biodiversity 2020 Strategy, as part of the LIFE Project BIO/IT/000204 U-SAVEREDS, this work aimed at safeguarding the native red squirrel in Umbria, central Italy, limiting the diffusion of the non-native species. Various studies reported that NGF and its receptors (NTRK1, NGFR) play a critical role in the regulation of mammalian reproduction [1, 2] we examined the expression of the NGF system in the squirrel testicles. Seventeen squirrels were captured in the Umbria Region. The animals underwent external or internal orchiectomies and were classified into 3 different morphotypes by histological procedures [2]: active spermatogenesis, pubertal, immature animals. Testicle NGF, NTRK1 and NGFR mRNA were evaluated by Real Time PCR, using BACT as housekeeping. We found that only NGF significantly increased in pubertal with respect to both active spermatogenesis and immature morphotypes [3]. Western Blot revealed the presence of bands matching the expected size for NGF and its receptors. Significant differences among group means were calculated by ANOVA followed by Tukey post hoc test. These data suggest that in the grey squirrel population here investigated the NGF system is involved in the testicle development and function, probably contributing to the reproductive success of this species by a mechanism not yet understood.

[1] Maranesi et al. *Animals* 2020, 10:1558. [2] Maranesi et al. *Biol Reprod.* 2018, 98:634-43. [3] Maranesi et al. *Animals* 2020: 10:738.



SEASON FOUR

4th June 2021

Localization of leptin (Ob) in the abomasum of the sheep: an immunohistochemical study

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Adipokines, mainly produced by adipose tissue, are molecules involved in energy metabolism and represent important links between nutritional status, and neuroendocrine axis [1]. Leptin (Ob) is the first known adipokine [2]. It is a hormone primarily secreted by adipocytes of subcutaneous and visceral fat and it is also produced by several peripheral tissues [3]. Leptin is one of the most important hormones involved in the control of energy homeostasis and feeding behavior. It serves to signal nutritional status to the central nervous system and peripheral organs and, in physiological condition, it acts to reduce appetite [4].

This work aimed to investigate the presence and localization of leptin in the abomasum of the sheep in an attempt to shed light on those cells and structures that might locally produce this peptide.

A flock of 15 Comisana x Appenninica adult female sheep in the dry stage was fed with fresh hay from June to the pasture maximum flowering (MxF group). From this period to maximum dryness, the sheep were split into two groups: the control group (Cnt group) was fed with fresh hay while the experimental group (Exp group) was fed with fresh hay supplemented with 600g/day/head of barley and corn (1:1). Samples of the fundic region of the abomasum were collected for each group, fixed in 10% neutralbuffered formalin and processed until paraffin inclusion. Experimental procedures were approved by the Ministry of Health (No. of approval 95/2018-PR). The leptin protein was investigated by using immunohistochemical technique. Histological sections of 5 micrometers were microwaved in 10 mM citric acid (pH 6.0) for antigen retrieval. The endogenous peroxidase activity was blocked with a 3% peroxidase-blocking solution and non-specific binding was blocked with normal horse serum. Sections were incubated with mouse monoclonal anti Ob-antibody (1:150 in PBS, Fitzgerald Industries International, MA, USA) for 24 hours and, successively, they were

incubated with the horse anti-mouse biotin-conjugated antibody. The reaction was detected with the Vectastain ABC kit and visualized with diaminobenzidine.

By observing immunohistochemical treated sections, an intense positivity to Ob was evidenced in the gastric glands of the mucous layer. Immunostaining was mainly localized in the lower half of the fundic glands. The staining for Ob was localized in the cytoplasm of the cells.

To the authors' knowledge, no studies describe leptin localization in the abomasum of the sheep. Its detection in the gastric glands suggests a local activity of this adipokine in the regulation of the digestive function as already attested in other species including humans [3]. This is a preliminary report that introduces leptin investigation in the sheep digestive system and this can be of great importance to improve our knowledge in the biology of the digestive system in farm animals. However, the exact role of this molecule and the influence of diet need further elucidation.

[1] Trayhurn et al. Adipose tissue and adipokines--energy regulation from the human perspective, *J Nutr*, 136(7):1935S-1939S, 2006. [2] Triantafyllou et al. Leptin and Hormones: Energy Homeostasis, *Endocrinol Metab Clin North Am*, 45(3):633-45, 2016. [3] Sobhani et al. Leptin secretion and leptin receptor in the human stomach, *Gut*, 47(2):178-83, 2000. [4] Ahima RS et al. Leptin regulation of neuroendocrine systems, *Front Neuroendocrinol*, 3: 263-307, 2000.



SEASON FOUR

4th June 2021

Role of gene variants in resistance to small ruminant lentiviruses (SRLVS) in flocks reared in central Italy - preliminary results

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Caprine arthritis encephalitis virus (CAEV) and Visna-maedi virus (MVV) belong to the genus *Lentivirus* of the *Retroviridae* family. Recent molecular studies have shown that the two viruses exist in both sheep and goats and they are therefore commonly referred as SRLVs [1]. SRLVs affect animal welfare and productions. They infect the monocyte-macrophage lineage and the classical symptomatology may occur later [1]. Routes of transmission are mainly by respiratory secretions and ingestion of infected colostrum/milk [2]. SRLVs have a worldwide distribution with exception of Iceland [2]. Genetics could represent the most suitable tool to effectively control the disease [3]. Studies revealed that the E35K polymorphism of the TMEM154 protein can modulate the infection [4] as well as the 4-base pair deletion (CCR5-Δ4) in the CCR5 gene [5]. Two other genes, namely TLR9 and its mediator MYD88, could be involved in genetic resistance to SRLVs, especially the G520R polymorphism of TLR9 gene [6]. The aim of this work is to evaluate the association between SRLVs infection and specific variants of TMEM154, CCR5, TLR9 and MYD88 genes. 528 blood samples were collected from sheep reared in Central Italy. To date, 430 samples were subjected to SRLV ELISA testing and genomic DNA was extracted from blood clots. A qPCR allelic discrimination assay [5] was performed to detect the CCR5 polymorphism. PCR and sequencing analysis were used to identify SNPs in exon 1 and exon 2 of the TMEM154 gene and in exons 2 and 3 of the TLR9 and MYD88 genes, respectively [6]. Amplification products were purified and then sequenced using a 3500 Genetic Analyzer. Sequences were analyzed using BioEdit 7.0.9 software. Preliminary results showed that the frequencies of the E35K mutation of TMEM154 protein, the wild type

G520R allele of TLR9, and the deletion of CCR5 gene were 35.2%, 10.7% and 8.2%, respectively. Further analysis is needed to assess the association between serologic status and polymorphisms in target genes. The World Organization for Animal Health (OIE) has recognized the importance of the economic impact of SRLVs, especially on dairy sheep farms, and has included MV and CAE in the list of notifiable terrestrial and aquatic animal diseases. Identifying genetic variants associated with resistance to SRLVs infection could be crucial in ensuring animal welfare and the profitability of farms.

[1] Leroux, C., et al. SRLVs: a genetic continuum of lentiviral species in sheep and goats with cumulative evidence of cross species transmission. *Curr HIV Res*, 2006, 8(1), 94–100. [2] Peterhans, E., et al. Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Vet Res*, 2004, 35(3), 257–274. [3] Molaei V., et al. (2018) First survey on association of TMEM154 and CCR5 variants with serological maedi-visna status of sheep in German flocks. *Vet Res* 49:36. [4] Heaton MP., et al. (2012) Reduced Lentivirus Susceptibility in Sheep with TMEM154 Mutations. *Plos genetics* 8(1): e1002467. [5] White SN., et al. (2009) Common promoter deletion is associated with 3.9-fold differential transcription of ovine CCR5 and reduced proviral level of ovine progressive pneumonia virus. *Animal Genetics* 40:583. [6] Sarafidou, T., et al. Toll Like Receptor 9 (TLR9) Polymorphism G520R in Sheep Is Associated with Seropositivity for Small Ruminant Lentivirus. *PLoS ONE*, 2013, 8(5), e63901.



SEASON FOUR

4th June 2021

Retrospective study on patent ductus arteriosus: surgical ligation in selected dogs not treated by amplatzer occluder

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Patent ductus arteriosus (PDA) is the most common congenital cardiovascular disease in dogs [1]. Left to right PDA can be treated by minimally invasive procedures or open thoracotomic surgery. When the size of the dog and duct's morphology allow it, Amplatz Canine Duct Occluder (ACDO) is the device of choice for closure of PDA because of its effectiveness and reduced invasiveness [2], but when the patient is too small or duct's morphology is not compatible with Amplatz device, thoracotomic PDA ligation is the proper approach [3].

Aim of the work was to evaluate all the intra-(IO) and postoperative (PO) complications related with thoracotomic PDA-ligation, as well as associated risk factors, in a definite category of affected dogs, which were excluded from mini-invasive techniques due to their size or duct morphology and, for this reason, characterized by higher risk factors than the average of the surgery-treated ones. Records of 18 dogs who underwent surgical PDA ligation at Surgery service of the Department of Veterinary Medicine, University of Perugia, between 2011 and 2021 were retrospectively reviewed. All surgeries involved a left thoracotomy through the 4th or 5th intercostal space, followed by standard ligation or Jackson-Henderson technique, according to the peculiarities of the patent duct. All the PDA ligations were performed by using silk suture thread. Complications, either intra- or postoperative, were classified as severe, medium and mild. Associated risk factors were analyzed for each complication. Regarding IO complications, severe hemorrhage occurred in 5,5% of dogs, Branham reflex occurred in 55,5% of dogs, transient aortic aneurysm dilation occurred in 22,5% of dogs while permanent aortic aneurysm dilation occurred in 5,5%; among the PO complications, PDA recanalization showed-up in 5,5% of dogs, while about 28% of dogs showed small

residual shunt and 5,5% showed hypertension. Mortality rate settled at 5,5%, while initial success rate was 94,5%. Patent ductus arteriosus closure confers important survival benefits so, when intravascular PDA occlusion with ACDO is not achievable, thoracotomic PDA ligation must be performed. A number of complications are related to this surgical approach, the risk of which can be reduced through the timeliness of surgery and a clear preoperative awareness of ductal morphology: this allows to predict all the risk factors that can lead to complications, and therefore, to plan the correct surgical procedure and to pick the right tools to carry it out.

[1] Buchanan James W, Patent Ductus Arteriosus Morphology, Pathogenesis, Types and Treatment. *Journal of Veterinary Cardiology*, Vol.3, No. 1, May 2001. [2] Singh M. K., Kittleson M. D., Kass P. H. and Griffiths L. G., Occlusion Devices and Approaches in Canine Patent Ductus Arteriosus: Comparison of Outcomes, *J Vet Intern Med* 2012; 26:85–92. [3] Orton EC, Monnet E, 2017, *Small animal thoracic surgery*, Wiley-blackwell.



SEASON FOUR

4th June 2021

Sheep as an animal model to promote the Apennine territory development via derived products: the case study of adipokines

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The growing summer drought stress given by global warming is pre-empting the moment of the maximum productivity of pasture and shortening the availability period of fresh forage for the animals with an adequate nutritional value to ensure animal productivity [1,2].

The adipokines are molecules mainly produced by adipose tissue related to the individual's nutritional status [3,4].

Since the action of adipokines modulates a series of activities including energy metabolism, reproductive activity, and integumentary system [5,6], the project aims to study the expression of different adipokines and their receptors in the digestive, reproductive and integumentary systems of the sheep under different nutritional level. Specifically, in this work, the apelin system was investigated using immunohistochemistry in the abomasum of 15 Comisana x Appenninica adult female sheep. The flock was free to graze on the pasture from June to the pasture maximum flowering (MxF group) feeding on fresh forage. Throughout the period between the maximum pasture flowering (MxF) and the maximum pasture dryness (MxD), the MxD group has been grazing on pasture feeding only on fresh forage, while the experimental group (Exp) received also a feed supplementation of 600 g/day/head of barley and corn. Preliminary data show the apelinergic system presence in all analyzed samples; the positive cells have been found in the mucous layer, especially in the basal region of gastric glands. Besides, the lining epithelium showed positivity for apelin and its receptor (APJ).

The grastic glands' positive cells have been labeled as the chief cells. The comparisons performed among the three groups evidenced a lower intensity of immunopositivity in

the MxD group for both apelin and its receptor suggesting an influence of diet on the apelin system. The detection of both apelin and APJ in the mucous layer of the abomasum leads us to suppose a role of the apelin system in the digestive function regulation.

The information collected during the project wants to be the starting point to studies aimed to improve and maintain the production performances of sheep kept in rural farms.

[1] Roche et al. Nutrition × reproduction interaction in pasture-based systems: is nutrition a factor in reproductive failure?, *Animal Production Science*, 51(12):1045-1066, 2011. [2] Scocco et al. Effects of summer rainfall variations on sheep body state and farming sustainability in sub-mediterranean pastoral system, *Span J Agric Res*, 14:1-4, 2016. [3] Radin et al. Adipokines: a review of biological and analytical principles and an update in dogs, cats, and horses, *Vet Clin Pathol*, 38(2):136-56, 2009. [4] Trayhurn et al. Adipose Tissue and Adipokines—Energy Regulation from the Human Perspective, *The Journal of Nutrition*, 136(8): 1935–1939, 2006. [5] Fontana R., Della Torre S. The Deep Correlation between Energy Metabolism and Reproduction: A View on the Effects of Nutrition for Women Fertility, *Nutrients*, 11:8(2):87, 2016. [6] Rivera-Gonzalez et al. Adipocytes in Skin Health and Disease, *Cold Spring Harb Perspect Med*, 4(3): a015271, 2014.



SEASON FOUR

4th June 2021

Major Facilitator Superfamily (MFS) transporters, a druggable target for antibacterial therapy

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Major Facilitator Superfamily (MFS) transporters are membrane proteins found in all domains of life. Anomalous functions of MFS proteins in eukaryotic cells have been associated with debilitating diseases, including cancer and degenerative processes. In bacteria, MFS transporters are involved in the uptake of nutrients and extrusion of toxic compounds, including antibiotics and biocides. Because of this function, they were identified as multidrug resistance efflux pumps (MDR EPs) [1]. As MDR EPs, MFS transporters have been a major focus of investigations aimed at developing new drugs against antibiotic efflux systems of pathogenic microorganisms.

The long-term objective of our researches is to elucidate the fundamental mechanisms by which relevant animal and human pathogens extrude antibiotics and biocides and to provide novel therapeutic means to overcome drug resistance by targeting their MFS efflux transporters.

To study efflux-mediated resistance in bacteria, two approaches can be applied: in vitro building up of resistance by stimulation of isogenic strains with increasing concentrations of ethidium bromide, which is a known substrate of cellular efflux systems, [2] and selection of antimicrobial-resistant clinical isolates. The efflux phenotypes are evaluated by fluorometric tests and antimicrobial MIC determination in the presence/absence of chemical molecules known as efflux pump inhibitors (EPIs), while EP gene expression analysis is performed to unveil the molecular mechanisms underlying efflux-mediated resistances. DNA sequencing of promoter regions and adjacent transcriptional regulators is useful to investigate the regulation of MDR EPs expression.

By using the methodology above, exposure of *Rhodococcus equi* to ethidium bromide unmasked an efflux-mediated defence against azithromycin and ciprofloxacin, which

seemingly correlates with the overexpression of the MFS transporter REQ_RS13460. G→A transition that occurred in the transcriptional repressor tetR/acrR adjacent to REQ_RS13460 is likely implicated in the regulation of this MDR EP. These results support the pivotal contribution of this MFS transporter in the survival antibiotic challenge. Finding the involvement of MFS EPs in the antimicrobial resistance of other clinically relevant pathogens could justify the repositioning of EPIs optimized against MSF transporters of *Staphylococcus aureus* [3] and nontuberculous mycobacteria [4] to develop EPIs-based therapies enabling to circumvent antibiotic efflux processes in different bacterial species.

[1] Quistgaard EM et al. Understanding transport by the major facilitator superfamily (MFS): structures pave the way. *Nat Rev Mol Cell Biol*, 17:123-132, 2016. [2] Couto I et al. Efflux-mediated response of *Staphylococcus aureus* exposed to ethidium bromide. *J Antimicrob Chemother*, 62:504–513, 2008. [3] Felicetti T et al. Modifications on C6 and C7 Positions of 3-Phenylquinolone Efflux Pump Inhibitors Led to Potent and Safe Antimycobacterial Treatment Adjuvants. *ACS Infect Dis*, 5:9821000, 2019. [4] Felicetti T et al. 2-Phenylquinoline *S. aureus* NorA Efflux Pump Inhibitors: Evaluation of the Importance of Methoxy Group Introduction. *J Med Chem*, 61:7827–48, 2018.



SEASON FOUR

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Hacking EVs: how to load proteins of interest into MSC extracellular vesicles

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Over the last two decades the study of extracellular vesicles (EVs) has broadened our knowledge about intercellular communication, suggesting new ways through which cells exchange informations in physiological and pathological conditions. EVs are micro and nanoparticles bounded by a lipid bilayer that contain a large variety of biological molecules. They origin from parental cells that “package the cargo” into vesicles and release them in the extracellular space. EVs diffuse in biological fluids and carry out their functions in two principal ways: releasing their cargo into recipient cells by membrane fusion or interacting with surface receptors on target cells.

Mesenchymal stromal cells (MSC) are involved in homeostasis of connective tissues, providing support, nourishment and signals; the ever growing interest on their biology and clinical application is due to their effects on tissue regeneration, immunomodulation, inflammation and angiogenesis. EVs represent an essential system through which MSCs carry out their role, making these cells an excellent model of study for EV biology. Moreover, MSC-derived EVs are considered a possible platform for drug delivery, thanks to their innate pharmacokinetic properties.

The purpose of this work is to deepen the knowledge on mechanisms that regulate EV biogenesis, with special focus on protein loading; the long term goal is to develop a strong method to upload proteins of interest inside EVs from MSCs in order to use them as a tool in drug delivery. Through genetic engineering, we induced expression of fluorescent probe fusion proteins in MSCs to observe and measure the loading index of different EV related proteins.

cDNAs encoding EV related proteins were isolated and cloned from canine total RNA and used to generate fluorescent probe fusion proteins; sequence encoding EV related

proteins were cloned into green fluorescence protein (GFP) expression vector in order to obtain plasmids encoding for chimeric proteins.

Canine MSCs were transfected with plasmids to allow the expression of labeled proteins. Images of fluorescent living cells were acquired 48 hours post-transfection and cells' medium was recovered to isolate EVs. Later, cells were analysed through EM and immunogold labeling was performed on thin sections with anti-GFP antibody to detect chimeric proteins and their fine intracellular localization. Western blotting was performed on lysed cells and isolated EVs to confirm and quantify chimeric proteins expression and concentration.

EV related proteins distribution in cultured MSC confirmed the complexity of the mechanism involved in EV biogenesis. Different paths could be hypothesized with the extensive involvement of the endosomal system; the use of GFP tags allowed to describe the localization in living cells, without the perturbation of antibody labeling, and facilitates recognition in immuno-TEM analysis. This pilot study aims at clarifying the complex biology of EVs and at developing a strong method to bioengineer EV cargo of MSCs as a new tool in drug delivery.

[1] Fitzsimmons R. Mesenchymal Stromal/Stem Cells in Regenerative Medicine and Tissue Engineering, Stem Cells International, eCollection 2018. [2] Crivelli B. Mesenchymal stem/stromal cells extracellular vesicles: From active principle to next generation drug delivery system, Journal of Control Release, 262:104-117, 2017. [3] Corso G. Systematic characterization of extracellular vesicle sorting domains and quantification at the single molecule – single vesicle level by fluorescence correlation spectroscopy and single particle imaging, Journal of Extracellular Vesicles, eCollection 2019.



SEASON FOUR

4th June 2021

A case of diffuse idiopathic skeletal hyperostosis in a young cocker spaniel

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Diffuse idiopathic skeletal hyperostosis (DISH) is a common, systemic disorder of the axial and peripheral skeleton. It results in ossification of soft tissues such as ligaments or entheses. DISH was also differentiated from ankylosing spondylitis by differences in clinical, pathological, and radiological features. Various etiologies have been postulated however is often linked to obesity [1-2]. The purpose of these case description is to describe a severe form of DISH in a young Cocker Spaniel dog. A 3-year-old female Cocker Spaniel presented for a history of progressive stiff gait and a hind limb left lameness since 2weeks. Physical examination revealed a stiff, pacing gait in all four limbs with lameness of the left hind limb, body condition score was 7/9. Mild pain was elicited on palpation of the lumbosacral spine with limited cervical range of motion. Lateral and ventro-dorsal radiographs of the cervical and thoracolumbar spine revealed proliferative osseous lesions along the entire length of the spine with varying degrees of severity and distribution resulting in a complete fusion of the turaco-lumbar tract. CT images of the spine revealed that the cortical margins and architecture of affected vertebral structures were maintained, and ossification appeared to be extracortical. The T1-L7 tract was completely fused; a severe amounts of new bone surrounded the ventral aspects of T1-T11 tract with irregular margins. L7-S1 osseous lesions were distributed along ventral and lateral aspects of vertebral bodies, forming an irregular and bulky bony bridge across intervertebral disc space, deforming the spinal canal, with increased sclerosis.

Based on the imaging findings, presumable diagnoses of DISH was made. Complete blood count and biochemistry profile (unremarkable results) were performed. The dog was medically managed with oral meloxicam for approximately 1month and a balanced diet to reduce weight, but without any improvement. A 6months later follow up was made, but the condition remain stable. In DISH, involvement of the innervated

periosteum may contribute to spinal pain. The massive new bone formation may dislocate and compress surrounding soft tissues as ventral lumbar muscles, nerve roots or also spinal cord, producing more pain. As in humans, dogs with spinal stiffness and/or pain of the caudal lumbar spine could benefit from conservative treatment with NSAIDs and weight loss [3]. Surgery may be necessary if conservative treatment doesn't provide adequate symptom relief or if severe neurological deficits or vertebral fractures are present. DISH should be suspected in any case of widespread extracortical ossification affecting entheses and soft tissues of the axial skeleton. Veterinarians should be aware of the occurrence and the possible clinical relevance of DISH, which may cause stiffness and back pain.

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SEASON FOUR

4th June 2021

Ultrasonographic, computed tomographic, ct-arthrographic description of normal intra-articular anatomy of the canine stifle: a cadaveric comparative study.

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Cranial cruciate ligament (CCL) rupture is one of the most frequent orthopedic diseases in the canine species. The rupture of the CCL is diagnosed in most cases through a special orthopedic physical examination; however, for the complexity of the disease, sometimes the clinical examination alone does not provide a clear diagnosis. In case of uncertain findings, it is necessary to rely on imaging [1-2]. Considering the limited diagnostic sensitivity of X-ray for soft tissues, the poor distribution of medical centers provided with nuclear Magnetic Resonance Imaging (MRI) and the invasiveness of an arthroscopic examination, in the last few years the focus has been shifting to two diagnostic tools rarely used in veterinary orthopedics before: the ultrasound examination and computed tomography (CT) [3-4]. This comparative Ex-Vivo study allowed us to improve our knowledge of ultrasound anatomy of the main intra-articular structures of the dog's healthy knee, with the aim of analyzing the strengths and weaknesses of this diagnostic method. We performed at first an ultrasound examination focusing on the CCL, the patellar tendon and the menisci; then we performed a CT scan and CT- arthrography (CTA) examination on the same joint. The CT images were read using both hard and soft tissue algorithms, and the image quality was carefully evaluated depending on which CT algorithm was used. For CTA images, the distribution pathway of the contrast agent was described. Furthermore, in order to adequately understand the CCL sonoanatomy, a comparison between ultrasound images, CT and CTA scans has been performed. In order to prove the ultrasound identification of the CCL, the ligament was stained with methylene blue by means of a spinal needle inserted into the joint crossing the CCL fibers via ultrasound guidance; the right identification of

the ligament was confirmed by the execution of an arthrotomy. Aware of the limitations of performing an Ex-Vivo study, an ultrasonography examination and comparison with healthy awake dogs was performed. Finally, an innovative ultrasound approach to the stifle joint has been tested and described for the first time in veterinary medicine: the caudal femoral- tibial scan. The results obtained indicate that in dogs weighing more than 25 kg, ultrasonography is a valid diagnostic method for visualizing the cranial-distal part of the CCL rupture, with a clear and well-defined manner. Similarly, ultrasonography examination is a diagnostic technique capable of assessing the structure of both menisci with a high anatomical detail.

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SEASON FOUR

4th June 2021

Neutrophils and macrophages in canine melanocytic tumors

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Despite promising immunotherapy strategies in human melanoma, there are few studies on the immune environment of canine melanocytic tumors [1]. Many studies conducted on humans highlight the key role of innate immunity in the development, growth and prognosis of human malignant melanoma, through the release of pro- and/or anti-inflammatory cytokines, and tumour growth factors [2,3]. Numerous studies focused on the evaluation of the infiltration of lymphocytes and T cells [4], also in dogs [5], but few studies did focus on the role of other immune system cells.

This study aims at the characterization of the innate immune system in canine melanocytic tumors, by the investigation of tumor-associated macrophages (TAMs) and tumor-associated neutrophils (TANs); moreover, the role of these cellular populations in predicting the prognosis has been assessed.

Sixty-five cases of melanocytic tumors were retrospectively selected from our archive. After diagnosis confirmation and histopathological evaluation, immunohistochemistry for MAC387, IBA1, and MPO was performed.

Results from our study showed that both neutrophils (MPO and MAC387) and macrophages (IBA1 and MAC387) were more numerous in oral melanomas, when compared to cutaneous melanomas and melanocytomas ($P < 0.001$). Moreover, an increased number of IBA1⁺ cells was associated with macroscopic and histological negative prognostic factors, such as the major diameter ($P < 0.05$), the tumor thickness ($P < 0.05$), the mitotic count ($P = 0.001$), and the nuclear atypia ($P < 0.05$). These associations were not observed instead with the number of MAC387⁺ cells. The number

of MPO⁺ cells was associated with the mitotic count ($P=0.001$), the nuclear atypia ($P<0.05$), the major diameter ($P<0.05$), and the tumor thickness ($P<0.05$).

None of the three markers was associated with survival times or death for melanoma. Further studies should focus on the role of the innate immune system in canine melanomas, since it could represent a feasible target for future immunotherapeutic strategies.

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SEASON FOUR

4th June2021

Colistin resistant Escherichia coli and spread of ESBL E.coli strains in samples collected from organic, antibiotic-free and conventional broilers in farms and at slaughterhouse.

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The increase in antibiotic resistance is a global concern for human and animal health. The use of antibiotics in poultry production increases the selection pressure for antibiotic-resistant bacteria [1]. *Escherichia coli* is a commensal bacterium, ubiquitous in animals and humans and extended-spectrum β -lactamase (ESBL)/AmpC *E. coli* is frequently found as contaminant in broiler meat [2]. Furthermore, ESBL-producing *E. coli* are often resistant to fluoroquinolones and aminoglycosides. Colistin also, which is an antimicrobial previously widely administered for prevention, treatment, metaphylaxis and growth promotion [3], has recently been reassessed as a critically important antimicrobial for human therapy [4]. We aim to highlight the susceptibility of *E. coli* towards colistina and the spread of ESBL *E. coli*, isolated in the different typology of farming (conventional, antibiotic-free, organic) and in slaughterhouses located in Central Italy. A total of 174 *E. coli* strains were isolated in farms and at slaughterhouse. To assess the antimicrobial susceptibility, all *E. coli* isolates were analyzed by the minimum concentration of the antibiotic (MIC) using Euvsec plates. The susceptibility to colistin was evaluated using the FRCOL Plates (0.12-128 $\mu\text{g}/\text{mL}$). All results were evaluated through EUCAST guidelines. The presence of ESBL producing *E. coli* was confirmed by the combined disk test with cefotaxime and ceftazidime alone and in combination with clavulanic acid and by the microdilution method using Sensititre ESBL plates, according to EUCAST guidelines. Our results

demonstrate the importance of the type of farming. The number of ESBL E. coli strains was higher in slaughterhouses (OR 2.72; IC95%: 1.23-6.02) than in farms and samples from conventional carcasses presented the highest percentage of ESBL E. coli (18.27%). No statistical differences were found between organic and antibiotic free sample's carcasses (4.30%; 9.67%). This could be supported by the large use of β lactams against C. perfringens infection in conventional farming, especially ampicillin. The highest number of colistin-resistant E.coli strains was found in the organic (OR 8.27; IC 95%: 3.26-20.96) and antibiotic-free (OR 4.73; IC 95%: 1.86-12.05) rearing systems. The vertical transmission associated with the absence of antibiotic treatment in these typologies of farming, in comparison to conventional one, could have favored the increase of the resistant E. coli strains in an intestinal microbiota "less pressured" by antimicrobials use. It should be also highlighted that the therapeutic protocol in conventional management is based on the use of other antimicrobial classes being the use of colistin in derogation. Furthermore, the role of the external environment contaminated also by antimicrobial resistant bacteria shed by wild animals should not be neglected in organic farms.

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SEASON FOUR

4th June 2021

Bovine lymph nodes as a source of Escherichia coli contamination of the meat

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Ground beef contamination with *Escherichia coli* is usually a result of carcass faecal contamination during the slaughter process [1]. Carcasses are contaminated when they come into contact with soiled hides or intestinal leakage content during dressing and the evisceration processes [2]. A more recent and compelling hypothesis is that, when lymph nodes are present in manufacturing beef trimmings, they can be a potential source of Enterobacteriaceae contamination of ground beef [3]. The aim of this study was to investigate the occurrence of *E. coli* in lymph nodes from beef carcasses used for ground meat production, in six slaughter plants situated in central Italy. A total of 597 subiliac (precrural) lymph nodes were obtained from 597 cattle carcasses and screened for *E. coli* by culture. Furthermore, *E. coli* isolates (one per positive carcass) were tested for *stx1*, *stx2*, *eaeA* and *hlyA* genes that are commonly used to identify and characterise shiga toxin-producing *E. coli* (STEC). In addition, the *E. coli* isolates were profiled for antimicrobial susceptibility.

A proportion of 34.17% (204/597) carcasses were positive for *E. coli*. PCR revealed that 28.92% (59/204) of *E. coli* possessed *stx1* or *stx2* which corresponded to 9.88% of the cattle sampled. More than 95% of isolates were susceptible to gentamicin,

ceftriaxone, ciprofloxacin and cefotaxime while high rates of resistance were recorded for cephalotin, ampicillin, tetracycline, tripe sulfa and streptomycin. Animal age at slaughter was significantly associated with the presence of generic *E. coli* and STEC in bovine lymph nodes.

In conclusion, subiliac lymph nodes represent a source of *E. coli* for ground beef. These results are of major importance for risk assessment and improving good manufacturing practices during animal slaughter and ground meat production.

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SEASON FOUR

4th June 2021

Development of research in methodologies that improve the use capacity of preserved canine semen. Preliminary results

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Artificial insemination is an important tool in livestock, although in recent decades, its use in companion animals and conservation programs has had an unexpected growth. However, cooling and cryopreservation are known to induce unregulated capacitation and pathological modifications of sperm in numerous domestic species [1]. The objective this line of research is to evaluate new alternatives either from the management and conservation of canine semen after it is obtained that promote flexibility in its use, preserving its fertility capacity, like others previously initiated, through administration to animal products that would improve the formation and conservation of semen within the male reproductive organs. Semen activators (SA) have been produced to provide the molecules necessary to maximize the sperm survival and quality with the aim to enhance fertility and prolificacy. In this study, the effect of SA was recorded by daily evaluation of chilled semen for 14d. For this experiment, 6 adult healthy Neapolitan Mastiff dogs, were used as donors and the semen was manually collected. Spermatozoa-rich fractions of each subject was chilled using a new generation extender for long periods of time (d0) starting from the d1 to d14, different aliquot, with (experimental trial) and without SA (control trial), were evaluated daily for motility, vigor [2], morphology [3] and membrane integrity [4]. The initial sperm concentration of extended semen was $417.3 \pm 170.4 \times 10^6/\text{mL}$ (mean \pm SEM) with $85.89 \pm 4.76\%$ of MNS (morphologically normal sperm), $84.47 \pm 5.22\%$ vital sperm and a pH of 6.2 ± 2.8 . The initial vigor was 3.83 ± 0.48 , but after one min with SA, it rose to 4.45 ± 0.45 ($P < 0.001$). The semen motility parameters increase significantly ($P < 0.05$) in experimental trial, respect to control trial, starting to d2 at finish, except for the d7. The vigor analysis

significantly increase in experimental trial ($P < 0.05$) during the most day of the study with exclusion of d3 and d14. For evaluate the semen characteristics over time, the experiment was divided into T1 (d0-d5), T2 (d6-d10) and T3 (d11-d14) ($P < 0.001$) in evaluation of morphology and membrane stability. The MNS reached 70% at d10 and finally 65% at d14, being considered normal and fertile. With Host-s, 65% of MNS were also achieved at d14. The presence of glucose and fructose in the diluents used for refrigeration can exert very important effects given the fact that metabolic routes have been found in both sugars, providing both different and complementing effects. It can be concluded that the use of SA prior to artificial insemination improves the quality of chilled semen significantly, although it does not reverse the effects of deterioration due to cellular metabolism over time.

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SEASON FOUR

4th June 2021

Comparison of pharmacological methodologies for termination of pregnancy in bitches (2): relation to protocols and adverse effects

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The reproductive clinic requires that, in relation to possible events that imply unwanted pregnancies and that may involve situations that threaten the quality of life of pets, the intervention of veterinarians to evaluate the eventuality of interrupting the advance of pregnancy. For this, there are surgical and pharmacological methods, in recent years, have advanced substantially. Most of the methods currently proposed for the interruption of pregnancy in dogs and cats act by interrupting or interfering with the action of progesterone on the uterus and the adherence of the placentas. The objective of the work was to determine the adverse effects of 4 different pharmacological protocols to interrupt pregnancy in bitches. In the Veterinary Reproductive Service of Uruguay (SRVI_UY), 129 non-sterilized female dogs of various breeds and crosses were received, and ages 4.5 ± 0.7 years (mean \pm SEM) and weights of 16.7 ± 2.9 kg that had been copulated by males during reproductive cycle, and that for different reasons it was not desired to continue with the possible pregnancy, but to continue with the reproductive potential. All the animals attended the veterinary service 2 times/week for control and on other occasions, according to possible effects appreciated by the owners. In this work, the treatments were compared: corticosteroids/dexamethasone [1] (n = 32) every 12 hours at 200 ug/kg (DEX) for 10 days (30-32 gestation); dopamine antagonists and prostaglandins [2] (CAB/PG): cabergoline ug/kg/ day for 10 days, and cloprostenol injections 1 ug/kg, on days 1 and 5 (n = 35) (28- 30 gestation); lotrifen [3] at a single dose of 2.5 mg/kg (LOT; n = 31) (7-15 gestation) and aglepristone [4] 10 mg/kg (n = 31) every 24 hours for 2 days (AGL) (25-35 gestation). The adverse effects

recorded were the following: DEX 90.6% polyuria/polydipsia during treatment and up to 7 days later, non-infectious vaginal discharges in 6.3% between 6 and 15 days after the start of treatment, 3.1% decline and loss of appetite for 1 week without other signs; CAB/PG 80.0% agitation with increased heart rate 5-15 min after PG, 60.0% salivation and emesis between 10-120 minutes PG, 20.0% loss appetite for 4-6 days and shortening of the interval interstral at an average of 120 ± 25 days; LOT caused 6.5% of neck pain between 10-12 days post administration, 13.0% of localized muscle pain for 3-5 days, 13.0% of cystic glandular hyperplasia, vomiting, diarrhea, anorexia and/or dysorexia in 18.5%. AGL 3.2% pyometra at 25 days, 48.4% anorexia 24-48 hours, 35.5% local pain, 16.1% excitement, 19.4% depression and shortening of the interestrum of 45 ± 15 days in 48.4%. The DEX and AGL groups had greater efficacy ($P \leq 0.05$) in their target, but DEX had less serious adverse effects.

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SEASON FOUR

4th June 2021

Wildlife as health indicator and biosentinel: Zoonotic bacteria and antibiotic-resistance

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Wildlife do not naturally come into contact with antimicrobial molecules; however, they can become infected with antibiotic-resistant bacteria through acquisition from human sources, agriculture and contaminated environments. Wild animals can act as reservoirs, vectors and bioindicators of bacterial pathogens, with zoonotic power and antimicrobial resistance (AMR) genes in the environment [1]. The emergence and spread of zoonotic bacteria and AMR in natural environments is a major global health concern, leading to serious implications for both human and animal health[2]. Wild species can also reveal the quality of the environment acting as sentinels for zoonotic diseases. Biological monitoring of these species is difficult to perform due to various determinants: legislative provisions, characteristics related to the species and the environment, both climatic and pedological, and the discovery of deceased animals in an advanced state of decomposition [3]. In this project we have the opportunity to explore and improve the know-how in AMR and diseases shared between animals and humans. This is a fundamental for the development new environmental monitoring systems.

The aim of the work is twofold: the application of species-specific health protocols will allow the development of a system for monitoring the phenomenon of antimicrobial resistance on bacteria isolated from wild animals, using some species as bioindicators (european hedgehog, raptors, roe deer). These animals will be part of those recovered on the regional territory in difficulty by the WildUmbria Wildlife Rescue center and delivered to the first aid service

of the Department of Veterinary Medicine. The animals that will die, or will be euthanized, will be transferred in a short time to the laboratories of the Experimental Zooprophyllactic Institute of Umbria and Marche. Health protocols will be applied to diseases of relevance to public health. The second part of the project will be focused on the study of some wild species, particularly suitable for their ecology and distribution in the habitat, in the role of environmental bioindicators, such as: i) the European hedgehog (*Erinaceus europaeus*), for its characteristics of cohabitation with human activities; ii) birds of prey, both diurnal and nocturnal, due to their apical position within the food chain and iii) roe deer (*Capreolus capreolus*), an increasingly widespread animal present in the area. On these species it will be possible to evaluate antimicrobial resistance by identifying indicator bacteria resistant to antimicrobial classes used in veterinary and human medicine. In detail, *Escherichia coli* resistant to third generation cephalosporins and carbapenems will be also investigated. Furthermore, the prevalence and genomic characterization of zoonotic bacteria, such as *Salmonella*, will be described.

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SEASON FOUR

4th June 2021

Evaluation and validation of an immunohistochemical panel for feline melanomas

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Melanocytic tumors are uncommon in cats, with a lower prevalence of melanocytomas than their malignant counterpart. Diffuse iris melanomas are the most common neoplasms, followed by those originating in the oral cavity and the skin [1]. Histologically, melanocytic neoplasia may have a variable melanin content and amelanotic tumors often represent a diagnostic challenge for the pathologist. Currently, the definitive diagnosis of amelanotic melanomas relies on the use of antibodies such as Melan-A, S100 and PNL2 [2], [3]. S-100 has been shown to have high sensitivity but low specificity, since other neoplasms may be immunoreactive to this marker.

Tyrosinase-related protein 2 (TRP-2), an enzyme involved in melanocyte differentiation, has been tested in dogs and found to be a promising marker for melanomas [4]. Moreover in human medicine, Sry-related HMG-Box gene 10 (SOX-10), a nuclear transcription factor involved in the differentiation of neural crest progenitor cells to melanocytes, has been validated for both primary amelanotic melanomas and their metastases [5].

The aim of this study is to evaluate SOX10 and TRP-2 immunolabeling in feline melanocytic tumors in comparison to other validated markers (Melan-A, PNL-2 and S100) to establish a valid IHC panel for diagnostic purpose.

Thirty-five cases of feline ocular and non-ocular melanomas were retrospectively selected from the histological database of the University of Perugia. These included: i) heavily pigmented tumors, easily diagnosed on routinely H&E stained slides, ii) poorly pigmented tumors, in which the presence of melanin pigment in a few cells allowed to confirm their melanocytic origin, iii) amelanotic tumors, for which immunohistochemistry with PNL-2 and Melan-A was positive. All cases underwent immunohistochemistry by the use of the following markers: S100, PNL-2, Melan-A, TRP-2 and SOX-10.

All the cutaneous, oral and ocular neoplasms demonstrated nuclear and cytoplasmic S100 expression (100% sensitivity). PNL-2 was found to be positively correlated with the degree of tumor pigmentation and to be mostly expressed in ocular tumors, compared to cutaneous and oral neoplasms, with sensitivities of 91.7, 50, and 37.5%, respectively. Melan A was as sensitive as S100 only in ocular melanomas (100%), while showed less sensitivity than S100, but more than PNL-2 for cutaneous (93.75%) and oral (87.5%) tumors.

The use of the two additional markers (TRP-2 and SOX-10) are under investigation and the results will be compared with sensitivities obtained in the same cases with the other tested markers.

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SEASON FOUR

4th June 2021

Equine papillomavirus-associated genital tumors: a possible animal model for human cancers

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Equine genital squamous cell carcinomas (SCCs) are common tumors in older horses, with poor prognosis mostly due to local invasion and recurrence.¹ These tumors are thought to be mainly caused by *Equus caballus* papillomavirus type 2 (EcPV-2).²

The aim of this study is to better characterize the tumor immune environment (TIME) in equine genital SCCs.

Twenty-three equine genital epithelial tumors were retrospectively selected; immune infiltrate was assessed by histology and immunohistochemistry; RT-qPCR tested the expression of selected chemokines and EcPV-2 DNA and RNA.

The results confirmed EcPV-2-L1 DNA in 21/23 (91%) samples and E6 DNA in 21 out of 23 cases (91%). L1 expression was instead retrieved in 18/23 cases (65%), whereas 14 cases (60.8%) tested positive for E6 and 8 (34.7%) for E2. The samples showed an increased infiltration of CD3⁺lymphocytes, macrophages, plasma cells, and FoxP3⁺lymphocytes in the intra/peritumoral stroma when compared to extratumoral tissues ($p < 0.05$). Only MAC387⁺neutrophils were increased in EcPV-2^{high} viral load samples ($p < 0.05$). IBA1 and CD20 were intratumorally increased in cases where interleukin (IL)-10 gene expression was detected ($p < 0.005$). receptor activator of nuclear factor-kappaB ligand (RANKL), nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB)-p50, NFkBp65, IL6, IL17, IL23p19, IL8, IL12p35, IL12p40, β -catenin (BCATN1), FOS like 1 (FOSL1), and lymphoid enhancer binding factor 1 (LEF1) showed a significant increased gene expression in tumor samples compared to healthy tissues. Moreover, IL12p35 was differentially expressed in EcPV^{high} and EcPV^{low} groups

($p = 0.007$). A significant decrease of IFNG and IL2 expression was highlighted in TGFB1-positive samples ($p < 0.05$).

Our results describe an inflammatory environment similar to human counterpart^{3,4} and characterized by the activation of RANKL/RANK and IL17 with the relative downstream pathways, and a positive modulation of inflammatory cytokines genes such as IL6 and IL8. Moreover, the increase of BCATN1, FOSL1, and LEF1 gene expression suggests an activation of both canonical and non-canonical Wnt signaling pathway that could be critical for carcinogenesis and tumor progression.

Equine genital squamous cell carcinomas may represent a good spontaneous model for the human counterpart. Further prospective studies are needed in order to confirm these preliminary results.

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SEASON FOUR

4th June 2021

Evaluation of anatomical variations of equine lumbosacroiliac region: ex vivo comparison between ultrasonographic and computed tomographic findings and correlation with abnormal finding and poor performance on clinical cases

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Pathological acquired changes involving lumbosacral vertebrae and sacroiliac joints have been identified as causes of chronic poor performance, back stiffness and lack of hindlimb engagement in athletic horses [1]. In addition, several variations of the lumbosacral vertebrae have been described [2].

Our main purpose is to describe the incidence of pathological changes and anatomical variations of this region through an ex vivo comparison between ultrasonographic (US) and computed tomographic (CT) findings and to correlate US findings and clinical presentation in horses referred to our Veterinary Teaching Hospital for back pain and poor performance.

Lumbosacroiliac spines were dissected from horses euthanized for reasons unrelated to the study. US examination mimicking a transrectal US examination [3] of L4-L5, L5-L6 and L6-S1 discs, intertrasversary joints (ITJ) and sacroiliac joints (SIJ) and CT examination were performed.

Once removed the soft tissues, the vertebrae were disarticulated, boiled in water for around 15h to remove any remaining soft tissue. They were then boiled with water and H₂O₂ to whiten them and air-dried before being evaluated.

A correlation of US and CT images and anatomical findings was made considering breed, age, weight and attitude of the euthanized horses.

26 specimens were included at this time in the study. The most represented breeds were Italian Warmblood, Anglo Arabian and Italian Trotter. Mean age was 17 years and average body weight was 482.

The most common US findings were heterogeneity and mineralisations within L6-S1 disc, reduced ITJs articular space, periarticular bony proliferation of the sacral or iliac wings often associated with reduced SIJ space.

Most common CT findings include heterogeneity within L6-S1 disc, periarticular ventral bony proliferation from L4 to S1, L6-S1 spondylolisthesis, reduced ITJs space with marginal sclerosis, facets joint ankylosis and/or arthropathy, ankylosis of lumbar trasverse process or lumbar and sacral dorsal process, dorsal and ventral periarticular bony proliferation of the sacral or iliac wings.

Anatomical dissection of the specimens allowed to better understand the diagnostic findings, performing an overall comparison of the data.

We also found a rare lumbosacral transitional vertebra consistent with an incomplete sacralisation of L6 characterized by the fusion of the left trasverse process of L6 and the left sacral wing, simulating the normal ankylosis between the sacral vertebrae.

This study highlights transrectal US examination as a valuable diagnostic tool to detect lumbosacroiliac pathologies and anatomical variations in horses. The correlation of US and CT findings with anatomical findings in ex vivo specimens has the purpose of making easier the interpretation of the clinical significance in horses with US abnormalities of the lumbosacroiliac region. However, CT examination allow us to detect some abnormalities that cannot be identified on US examination and this limit should be considered in the evaluation of a horse with back pain and poor performance.

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SEASON FOUR

4th June 2021

Computed tomographic diagnosis of fracture of the caudal branch of the pubis in a Thoroughbred foal

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In horses, hindlimb lameness caused by pelvic fractures is far more common than previously documented. Actually, radiography, ultrasonography, computed tomography (CT) and nuclear scintigraphy are the most commonly used imaging modalities for their diagnosis [1]. However, CT can be a very effective diagnostic aid in foals [2]. The aim of this report is to describe the clinical and diagnostic imaging findings in a 15 days old Thoroughbred foal, with fracture of the left pubis, presented at our VTH for a left hindlimb lameness of 8 days duration.

At admission, the foal was bright and alert; the patient showed severe left hindlimb lameness at walk and at trot in straight line under the mare. No swellings were founded at physical exam. Haematological and biochemistry analyses showed hypochromic normocytic anaemia, increased serum amyloid A and fibrinogen. A radiographic screening of the left hindlimb from the foot to the stifle was considered unremarkable as well as ultrasonographic examinations of the pelvis and umbilicus; small and diffuse comet tail artefacts in the cranial portion of the lungs were identified. Considering the risk for presence of occult infection on the base of blood works and age of the foal, a CT examination of the pelvis and hindlimbs was performed under general anaesthesia. CT examination showed a simple slightly displaced fracture involving the caudal branch of the left pubis; no changes consisting with a septic process were founded. The foal was restricted to box-rest for 3 weeks and a small paddock for additional 3 weeks.

At 3 years follow-up by telephone interview with the trainer, the horse is in full athletic activity and there are no residual gait changes related to the fracture of the pubis.

In the case presented here, CT examination was extremely useful to detect the fracture of the left pubis, which was impossible to identify through the standard transcutaneous ultrasound examination.

CT examination revealed the exact configuration of the fracture of the caudal branch of the left pubis, allowing a correct diagnosis to establish the more appropriate treatment and prognosis.

One disadvantage of CT is the increased cost, which may be considered acceptable in specific cases to get an early diagnosis and avoid delay in appropriate treatment and prognosis in foals [3,4].

In conclusion, CT examination is a valid diagnostic tool that allows to detect pelvis fractures due to its sensitivity; in this particular case, CT allows to identify the cause of the lameness and to ruled out the presence of a septic process, which is a major problem in young foals with severe lameness. The equine practitioners should not overlook the pelvis as a possible site of fractures in cases of acute onset of hindlimb lameness in foals. The use of advanced diagnostic imaging techniques, as CT, is recommended when ultrasonographic and radiographic examinations of the pelvis are unremarkable.

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SEASON FOUR

4th June 2021

Bioelectrical Impedance Analysis in dogs with right congestive heart failure before and after diuretic therapy: a pilot study

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Congestive heart failure (CHF) is a complex clinical syndrome characterized by fluid retention due to severe heart disease. Sign of CHF are commonly evaluated and monitored by physical examination, medical imaging and biomarkers detection. Bioelectrical Impedance Analysis (BIA) is a simple, non-invasive, real-time diagnostic technique, validated and routinely used in human medicine, to assess body composition and hydration status [1]. BIA is based on measuring the electrical resistance that tissues oppose to the flow of a low-intensity alternating current applied to the body. Body resistance is primarily and inversely affected by body water content [2]. Therefore, we hypothesized that BIA could be helpful to detect and quantify fluid retention due to CHF. The aim of this study was to evaluate if BIA was able to detect changes in body water content in dogs with right CHF before and after diuretic therapy.

Six client-owned dogs with ascites of cardiac origin were referred to the Veterinary Teaching Hospital of Perugia University and prospectively recruited in a longitudinal study design. Three consecutive whole-body BIA measurements were performed before and after resolution of the ascites in each dog, using a bi-frequency (50-100KHz) bioelectrical device (Biosmart ®), and bioelectrical variables of impedance, reactance, resistance and phase angle were recorded. All measurements were obtained in non-sedated dogs, gently restrained in standing position using a standard tetrapolar electrode configuration with two emitting electrodes applied by crocodile clips on the skin dorsal to the right elbow and dorsal to the patella of the right hindlimb. Two receiving electrodes were positioned 3.5cm dorsally to their respective emitting electrodes [3]. For each dog morphometric measurements including body weight, length, rib cage circumference and abdominal circumference were also

recorded. Bioelectrical and morphometric variables of each dog obtained before and after resolution of the abdominal effusion were averaged and compared using a Student's t-test for paired data.

All bioelectrical variables except reactance and phase angle measured at 100kHz showed a statistically significant increase ($p < 0,05$), in accordance with an increase of whole-body impedance, while both body weight and abdominal circumference decreased ($p < 0,05$). We concluded that BIA is effective in detecting decreases in total body water in dogs with right CHF after diuretic therapy and could be a useful monitoring tool during treatment. Our preliminary results need to be confirmed by larger studies.

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SEASON FOUR

4th June 2021

Flame retardants residues in commercially important freshwater species from Lake Trasimeno, Italy: risk characterization and benefit–risk analysis

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The term flame retardants refers to a diverse group of chemical compounds which are added to manufactured materials in order to prevent or delay flaming [1, 2]. Since the early 1970s, the most widely used flame retardants were polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs) [1, 2]. These compounds might separate or leach from the products into the environment and due to their potential adverse health effects, their widespread global distribution, and their ability to bioaccumulate and biomagnificate in food chain, some restriction on their use have been introduced since 2003 [3, 4]. However, extensive and frequently unrestricted use of these compounds in the past has resulted in their ubiquitous diffusion in various environmental matrices. Consequently, humans can be exposed to both PBDEs and HBCDs through numerous routes although food consumption appears to be the main one for general population, especially regarding “Fish and other seafood” product category [3, 4]. The study aimed to define the dietary exposure to several specimens of PBDEs and HBCDs for central Italy population in relation to the consumption of freshwater fish species from Lake Trasimeno (Umbria region, central Italy), to characterize the consumers’ risk was and to assess benefit-risk ratio. The maximum average incidences of positive samples ranged the value of 54.5% for *Procambarus clarkia* and 43.7% for *Perca fluviatilis*, for \sum HBCD and \sum PBDE respectively. The maximum UB concentration was of 965.98 pg/g and 341.21 pg/g for \sum HBCD and \sum PBDE respectively, both for *Procambarus clarkia*. The overall risk characterization reveal

a minor concern for consumers. The benefit–risk quotients indicated that the health benefits of consumption of fish from Lake Trasimeno outweigh the potential risks related to flame retardants.

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SEASON FOUR

4th June 2021

OMIC characterization of cow, donkey and goat milk extracellular vesicles reveals their anti-inflammatory and immune-modulatory potential

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Other than being a valuable nutrition source, milk represents a sophisticated signaling system that delivers maternal messages. This property seems to be mostly mediated by signaling molecules enclosed in micro/nano sized membrane-bound structures called Extracellular Vesicles (EVs) [1]. EVs act as signal mediators between distant cells and/or tissues, exerting biological effects as immune modulation, anti-inflammatory, anticancer, and pro-regenerative activity [2-4]. Moreover, milk is a unique, scalable and reliable source of EVs. Our aim is to characterize the molecular content of cow, donkey and goat EVs derived from mass milk (MEVs) through RNA and metabolites omic analysis in view of prospective applications as a nutraceutical in inflammatory conditions. After the assessment of morphological MEVs features by transmission electron microscopy and nanoparticle tracking analysis (mean diameter of 142.7 nm for bovine, 150.5 nm for donkey and 124.1 nm for goat) RNA sequencing of MEVs was carried out for both mRNA and smallRNA libraries, highlighting over 10,000 transcripts and 2,000 smallRNAs in each species. Concerning smallRNAs, donkey was found to be the most differing species with 57% of the RPKM total amount referring to micro RNA (miRNA) and a conspicuous component of miscellaneous RNA (42%), mostly Y-RNA and Vault. For cow and goat, miRNA was the main represented type with over 99%. Among the 50 most expressed smallRNA, 11 were shared by the three species (all miRNAs), 18 between cow and goat only while donkey had 3 other common miRNAs with cow and 4 with goat. As regards mRNA, a comparison between MEV cargos was carried out

selecting orthologous genes and ranking by relative expression level. Within the 10% of the most expressed orthologous genes in all three species (1223), 110 were shared. Donkey and goat were the most similar species with 335 shared genes while cow had only 170 genes in common with donkey and 155 with goat. Functional analysis on the 110 core genes revealed enriched GO terms related to translation and protein processing and potential involvement in innate and acquired immunity. These terms were also confirmed in analysis on species pairwise shared genes with a further possible function on energy metabolism. Concerning the most abundant genes for each species, donkey and goat MEVs displayed additional terms relative to the immune system and amino acids metabolism.

For the metabolomic analysis of MEVs of three species, mass spectroscopy (MS) coupled with Ultrahigh-performance liquid chromatography (UHPLC) was performed. Metabolites, both common or specific of a species, were identified and enriched metabolic pathways were investigated. Results are in particular accordance with our transcriptomic analysis and identify common pathways among the three species involving metabolites with immunomodulating effects such as arginine, asparagine, glutathione and lysine.

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SEASON FOUR

4th June 2021

Transcutaneous ultrasound guided removal of a wooden stick in a dog

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Superficial foreign bodies are a fairly common cause of clinical problems in companion animals [1], especially in hunting breeds during the flowering period of grasses. They are often associated with complications such as abscesses, empyemas or granulomas [2,3], so progressive instrumental investigations are necessary to better plan the surgical approach [3].

A 9-year-old female Border Collie was presented at the Veterinary Teaching Hospital of Perugia University for depression and a sudden onset of a swelling on the left neck region. Physical examination showed hyperthermia (39,9 °C) and a painful tumefaction localized immediately behind the left mandible branch. A preliminary ultrasound examination of the region was performed, revealing a neoformation characterized by air bubbles trapped inside. A CT scan was then performed and pointed out a lesion compatible with the presence of a thin, elongated, hyperdense foreign body surrounded by an abscess, communicating with the pharynx through a fistula. Endoscopic examination revealed an oedematous area in the left laryngeal-pharynx region, close to the epiglottis, but it was not possible to identify the entrance of the foreign body. During the general anesthesia, it was possible to reach this oedematous area with fingers and feel the foreign body through the soft tissues.

By ultrasonographic guidance the foreign body was successfully removed with a mininvasive approach: one of the key point of the extraction was the pressure applied from the inside of the oral cavity on the soft tissues, which allowed the foreign body to get closer to skin surface.

This clinical case once again confirmed the importance of diagnostic imaging methods, in particular ultrasound, in the diagnosis and intraoperative guidance for the removal of foreign bodies. The combination of these methods allowed the quick detection of the foreign body, reduced surgical procedure times and a mininvasive

and targeted approach to the lesion. Furthermore, the patient's recovery time and the morbidity of the method have been drastically reduced.

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SEASON FOUR

4th June 2021

Outer membrane vesicles (OMVs) as antibiotic resistance carriers

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Most bacteria release membrane vesicles (MVs) with sizes ranging from 20 to 400 nm in diameter. These MVs contain different cargo molecules and have specific functions that influence different biological processes, including virulence, horizontal gene transfer, export of cellular metabolites, phage infection and cell-to-cell communication. MVs were first found to be produced in Gram-negative bacteria, therefore they are often referred as Outer Membrane Vesicles (OMVs) [1]. OMVs are small, spherically bilayered blebs, released into extracellular milieu from the outer membrane of Gram-negative bacteria [2]. Since no studies have been conducted on *Salmonella Infantis*' OMVs yet and on their role in antibiotic resistance, a preliminary study has been carried out on this topic. The aim of the study was to phenotypically evaluate antimicrobial susceptibility of several strains of *S. Infantis* isolated from broiler chickens and humans, to collect the OMVs and to quantify the amount of β -lactamase enzyme in both the bacterial strains and the OMVs. To assess the antimicrobial susceptibility and the presence of ESBL, *S. Infantis* isolates were analysed by disk diffusion test and by microdilution method. OMVs isolation was performed on one β -lactam *Salmonella Infantis* resistant strain. *Salmonella* was grown on Luria-Bertani broth until the late exponential phase. The sample was centrifugated and the supernatant was filtered through 0,45 μ m pore size filters to remove residual bacterial cells and cellular debris. The OMVs were concentrated by ultracentrifugation of filtered culture supernatants and then suspended in phosphate buffered saline. β -lactamase enzyme was quantified by Nitrocefin test. Transmission electron microscopy was used to investigate OMVs morphology. Proteins both from cell lysate and from OMVs were quantified by Bradford assay and separated by SDS-PAGE. Statistical analysis was applied to calculate the β -lactamase activity value, expressed as milliunit per milligram (mU/mg).

Our results demonstrated the presence of β -lactamase activity in OMVs isolated from *S. Infantis* resistant strain and confirmed that also in *Salmonella Infantis* the enzyme gets packaged in OMVs from bacterial periplasm during vesicles biogenesis. Further studies should be carried out to characterize OMVs' content, in order to understand how OMVs can regulate and serve as vehicle of β -lactam resistance in bacteria.

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SEASON FOUR

4th June 2021

Toll-like receptor expression in avian heterophils

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Toll-like receptors (TLRs) are a major class of innate immune pattern recognition receptors that have a key role in immune homeostasis and defense against infections [1]. Assessment of innate immune indices, such as cytokine mRNA expression by avian heterophils, may be a useful tool that will indicate the overall immunological responsiveness of a particular line of commercial poultry [2].

The aim of the present study was to investigate the expression of TLR-4, TLR-7 and TLR-15 in heterophils of four different slow-growing avian genetic lines.

Four distinct slow-growing chicken lines, designed as A, B, C and D were enrolled in the present study. Venous blood in EDTA was collected from wing vein of 24 birds for each avian breed. Heterophils were isolated according to previous work [3] and total RNA was extracted, treated with DNase I and reverse transcribed into cDNA, before qPCR reaction. The expression ratio of the TLR-4, TLR-7 and TLR-15 was normalized relatively to GAPDH and β -actin reference genes.

All TLR under investigation have been amplified from heterophils of all the lines. In particular, TLR-7 and TLR-15 showed no significant differences between the four genetic lines. Only TLR-4 is differentially up-regulated in heterophils of line A when compared with line B ($P= 0.034$) and line D ($P= 0.033$).

The production of cytokines by heterophils may be useful biomarkers for breeders to consider when developing new immunocompetent chicken lines. Further studies are needed to find the correlation between TLRs expression level and avian genetic lines.

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