Effect of training and feeding on saliva concentrations of cartilage oligomeric matrix protein neo-epitope ($COMP^{664}$) and neuropeptide Substance P in horses

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Swedish University of Agricultural Sciences, SLU
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Abstract

Osteoarthritis (OA) is a low-grade inflammatory disease associated with subsequent lameness and chronic joint pain. Lameness caused by degenerative joint disorders is perceived as one of the most common clinical problems for the equine athlete, advocating the severity of OA as a welfare and clinical issue. Despite an incomplete understanding around onset of the disease and multifactorial aetiology, consensus prevails regarding progression of disease through intensive and repeated loading during training. To this point, diagnostic tools have been inadequate in early detection of reversible biochemical changes within the joint, consequently leading to a late definite diagnosis. Research on equine OA biomarkers have identified promising diagnostic prospects in this field.

Aim: This study aimed to quantify and document the change in saliva concentration of cartilage oligomeric matrix protein neo-epitope OA specific biomarker COMP^{664} and neuropeptide Substance P in response to exercise. The influence of feeding and training intensity was also investigated in these horses. The objective was to study the acute and delayed peaks in saliva biomarker concentration, previously described by others to evaluate the impact of exercise induced load/overload in the joints. The influence of age and presumed OA burden of the joints in the individual horses was also investigated. The results from this study will contribute to develop current knowledge on the subject further guiding the construction of a larger study aiming to enable the interpretation of normal and deleterious responses in healthy joints and distinguish these from pathological responses in diseased joints.

Method: The quantification of biomarkers was carried out using saliva as sampling material. The saliva was sampled from two different cohorts. Cohort 1 included sampling in conjunction with feeding in research horses owned by the university (N=5) and privately owned horses (N=2) to interpret the influence of feeding on biomarkers conducting the following training study. Cohort 2 included sampling in conjunction to a short-term training study (before, during and 2 hours after training) in privately owned horses (N=6). A competitive ELISA was used to determine the levels of COMP^{664} and Substance P concentrations in saliva. Heart rate and speed measurements were recorded during cohort 2 accounting for the effect of training intensity on biomarker levels.

Results: Cohort 1: results obtained from the feeding study showed individual fluctuating differences in both biomarkers, however no significant changes were observed. Cohort 2: results obtained from the short-term training program showed an acute increase of COMP^{664} concentration in saliva at T2 followed by a delayed peak observed after training between T6-T8, however the data was not significantly different. The amplitude, magnitude and time point of these peaks variates when afterwards dividing the horses into young with no lameness history and old horses with known history of lameness and joint treatment. This was done with the hypothesis that the young horses had a healthy joint status and the older horses a presumable OA burden. The concentration of Substance P showed a similar pattern as COMP^{664} in response to training. Pulse measurements: training intensity measured by pulse and speed was interpreted as equal between horses, providing comparable results. Training intensity is increased during the acute peak of biomarkers but not the delayed.

Conclusion: The data presented in this pilot study show an acute concentration peak of biomarkers associated with initiated training followed by an indicated delayed peak with different onset in time depending on the age and presumable joint health status. The different patterns of these peaks suggest the use when evaluating impact of training and early disease changes. This contributes with knowledge guiding future research to further investigate and categorize biomarker response to exercise into physiological and pathological.

Keywords: Osteoarthritis, Horse, Biomarker, Saliva, Exercise, Training, Feeding

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Abbreviations

ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin Motifs
ANOVA	Analysis of Variance
BGN	Biglycan
DAMPs	Damage-Associated Molecular Patterns
COMP	Cartilage Oligomeric Matrix Protein
ECM	Extracellular matrix
ELISA	Enzyme-Linked Immunosorbent Assay
IL-1 β	Interleukin 1-Beta
MMPs	Matrix Metalloproteinase
NGF	Nerve Growth Factor
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PG	Proteoglycan
SF	Synovial fluid
SP	Substance P
SLRP	Small Leucine-Rich Repeat Proteoglycan
TNF-α	Tumour Necrosis Factor Alpha

1. Introduction

Athletic performance is dependent upon a well-functioning musculoskeletal system. For the equine athlete, joints constitute a major element of the musculoskeletal apparatus. Osteoarthritis is therefore one of the most encountered orthopaedic problems in equine athletes causing failure of joint function with subsequent lameness and chronic joint pain (Rossdale *et al.* 1985 see Patterson-Kane & Firth 2014). Considering lameness one of the main reasons for searching veterinary practice and the cause often derived from joint disorders this can be argued a principal clinical and equine welfare problem. Observations of altered natural behaviour when experiencing joint pain further advocates the welfare aspect (Van Loon *et al.* 2010).

Osteoarthritis (OA) is a low grade inflammatory joint disease succeeding with involvement and destruction of the whole joint and tissues within (Skiöldebrand 2004). Essential to the disease is the imbalance between anabolic and catabolic processes eventually unfolds into characteristic lesions. Unfortunately, discovery of these changes is observed in the later stages of disease progression when revision to a healthy joint no longer is possible. The etiopathogenesis of OA is viewed as multifactorial, nevertheless consensus remains about intensive and repeated loading as a predominant cause in equine athletes. Several lines of evidence conclude the biochemical effects of training further emphasized to progress towards degenerative changes when approaching the maximal physiological adaptive capacity (Palmer *et al.* 1995; Murray *et al.* 2001a; b; c). OA is currently diagnosed through orthopaedic clinical examination and imaging techniques in the irreversible stages of disease when the horse is lame. The international research community has stated a consensus requesting biomarkers in serum, synovial fluid or saliva as the ultimate tool for monitoring biochemical destructive changes in the joint disease to make it possible to conclude a diagnose in the reversible stages (Osteoarthritis Research Society International [OARSI] n.d.). Research on biomarkers have shown promising diagnostic prospects and developed our knowledge of disease pathogenesis. Previous work has shown fragmentation of Biglycan (BGN) and cartilage oligomeric matrix protein (COMP) to neo-epitopes during early OA stages (Skiöldebrand *et al.* 2017; Adepu *et al.* 2022, 2023). The neo-epitopes are released into synovial fluid and serum and quantification in saliva is achievable due to the equivalence between serum and saliva. Insights in disease pathology and evaluative

diagnostic methods facilitate opportunities for new therapeutic targets which are up to date, not available (Skiöldebrand *et al.* 2023). Additionally, biomarkers provide an opportunity to evaluate the impact of training and exercise regimes beyond diagnose disease (Frisbie *et al.* 2008; Adepu *et al.* 2023).

The aim of this study is to quantify and document the changes in biomarker concentrations in saliva arising from joints in response to exercise. The intention is to follow the acute and delayed peaks in the respective biomarkers and assess the impact of training and OA burden in the horses. The hypothesis is to comprehend the previously described exercise effects on changes in biomarkers, thus reflecting joint response to loading. This will provide information about the interrelation between joint health and exercise in athletic horses. Additionally, substantiating knowledge to future research interpreting the distinction of normal and deleterious responses in healthy joints and pathological responses in diseased joint to loading. The biomarker levels will also be correlated to exercise intensity, thereby accounting for the individual differences in training intensity when evaluating the results. A feeding study will be performed measuring the biomarker changes during feeding estimating the possible confounding factor of feeding.

2. Literature review

2.1 The Joint

Joints comprise anatomical structures forming a functional unit. Synovial joints, synonymously called diarthrodial or movable joints, are one classification of joints in the musculoskeletal system (van Weeren 2016; Olson & Carlson 2017). Synovial joints are joining adjacent bones covered by articular cartilage and held together by the fibrous capsule lined by the inner synovial membrane that encloses the joint cavity containing synovial fluid (Skiöldebrand 2004; van Weeren 2016; Olson & Carlson 2017). These structures are occasionally, depending on the joint, accompanied by intra-articular ligaments and meniscus. Synovial joints withhold two major mechanical functions, firstly enable movement between bone segments and secondly shock absorbance in response to impact (Wilson & Weller 2011). Primary function is determined by composition and structure further dividing the joints into high motion joints, significantly more affected by osteochondrosis, and low motion joints, more commonly presented with OA. Meeting the functional requirements of robustness from bone, frictionless of cartilage and withstand impact though distribution and attenuation of load comes with the price of limited flexibility and repair capacity (van Weeren 2016).

2.1.1 Articular cartilage

The articular cartilage is an avascular, aneural and alymphatic tissue consisting of a cellular component, chondrocytes and extracellular matrix (ECM) (Caron 2011; van Weeren 2016). Chondrocytes is the predominant cellular component of cartilage and metabolically regulates synthesis and degradation of ECM, highly influenced of both intrinsic and extrinsic factors. A major portion of ECM in articular cartilage consists of water, over 70% and the organic components collagen type II, approximately 50% together with proteoglycans (PG) respectively 35%. Remaining 15% of ECM consists mainly of glycoproteins. Collagen type II is the abundant collagen type, the cross-linked collagen molecules are assembled in collagen fibrils attributing the structure and rigidity of ECM. PG binds directly to collagen fibrils or indirectly through hyaluronic acid. Large and the most profuse

PG is aggrecan, featuring a core protein covalent bound to negatively charged, hydrophilic, glycosaminoglycan sidechains. Small leucine-rich proteins (SLRPs) are another important PG despite minor in both size and fraction. Biglycan (BGN), a SLRP, facilitates interconnection of ECM though regulation of fibrillogenesis. Cartilage oligomeric matrix protein (COMP) is a non-collagenous ECM protein involved in assemblance and stabilizing of the collagen network through binding of collagen molecules. Biomechanically, collagen primarily contributes to tensile forces whereas proteoglycans conduct the resistance of compression through the hydrophilic attraction of water (van Weeren & de Grauw 2010).

2.1.2 Subchondral bone

The articular cartilage connects to the underlying subchondral bone through the calcified cartilage (van Weeren 2016). Compact bone is found adjacent to the calcified cartilage providing rigid support and transitioning into elastic trabecular bone further from the joint space increasing the elastic properties. Contrary to cartilage, subchondral bone is richly vascularized and innervated with a pronounced tissue response to physical and pathological stimuli. The non-existent pain perception from cartilage assigns pain in joint disease to the innervated bone and other articular components.

2.1.3 Joint capsule

The joint cavity is surrounded by the articular joint capsule, composed of two layers (van Weeren 2016). The outer fibrous layer withholds a stiff structure, providing stability and mechanical resistance. Inside the fibrous layer, lining the joint cavity, is the inner layer of the joint capsule termed synovium or synovial membrane. The synovial membrane comprises externally well-vascularized and innervated loose connective tissue, the subintimal layer, and internally a thin layer synovicytes without basement membrane, the intimal layer. Synovicytes either conduct phagocytic properties, modulates composition of synovial fluid through production of components or synthesize inflammatory mediators crucial for joint homeostasis.

2.1.4 Synovial fluid

Synovial fluid (SF) is above all an ultrafiltration from blood plasma (van Weeren 2016). The rich vascularized joint capsule and absence of basement membrane enables this free passage between blood circulation and synovial cavity. Exchange between plasma and synovial fluid depends on both hydrostatic pressure at loading and colloid osmotic pressure differences. This transportation mechanism also accounts for provision of nutrients and removal of waste products. Additionally, SF influence joint homeostasis though coordination of communication between articular tissues and distributes pressure at loading (Caron 2011).

2.2 Osteoarthritis

2.2.1 Definition & etiopathogenesis

The proposed standardized definition of osteoarthritis (OA) conclude joint disease characterized by micro- and macro injuries initiating cell stress and matrix degeneration through imbalance in repair responses and simultaneous inflammation (Osteoarthritis Research Society International [OARSI] n.d.). The disease is manifested by a disrupted tissue metabolism and succeeding molecular derangement resulting in anatomic and physiologic derangements. The inflammation leads to cartilage degeneration, bone remodelling including subchondral bone sclerosis, bone necrosis and osteophytes together with inflammation observed as synovitis, capsulitis and located to the ligaments (Skiöldebrand 2004; Osteoarthritis Research Society International [OARSI] n.d.). Clinical presentation variates from mild synovitis to definite destruction and failure of all joint structures. The foregoing accentuates the distinction between biochemical and clinical pathology.

The literature covering OA is segregating the risk factors into normal loading on an abnormal joint or abnormal loading on a normal joint (McIlwraith 2016). Different theories exist in literature regarding the etiopathogenesis of degenerative joint disease but the disease is still not completely understood (McIlwraith 2016; Olson & Carlson 2017). Equine OA can be viewed as "a group of disorders, with many different aetiologies but characterized by a common end stage of degenerative changes within the articular cartilage, bone and soft tissue". Several initiation factors are however recognized including direct damage to articular cartilage, compositional changes of the subchondral bone, inflammation in articular structures including synovial membrane, joint capsule and synovium and finally abnormalities in conformation, congruence between joint surfaces and decreased stability (Olson & Carlson 2017). Evidently, all articular structures can be affected, separately or in combination during OA. The etiopathogenesis of OA is often described as multifactorial but a predominant cause in equine athletes is exposure to intensive and repeated loading (Skiöldebrand 2004).

2.2.2 Pathogenesis

Inflammation of the synovial membrane and joint capsule are among the most common problems in equine athletes and crucial in OA, causing pain and production of inflammatory mediators responsible for biochemical damage in both cartilage and bone (McIlwraith 2016). Matrix metalloproteinases (MMPs), a group of endopeptidase enzymes, are significant mediators of joint disease progression further involved in the degeneration of ECM components collagen, non-collagenous components and in smaller amounts aggrecan (Grässel *et al.* 2021). Other

mediators of considerable importance are aggrecanases, members of the disintegrin and metalloprotease with thrombospondin motifs family (ADAMTS), a group of enzymes engaged in proteolytic cleavage of aggrecans. Moreover, prostaglandins are considered an additional cause of decreased proteoglycan content, produced by cytokine induced enzymes contributing to inflammation and pain (McIlwraith 2016). The production of oxygen derived free radicals from chondrocytes in response to inflammation and mechanical forces are contributing to degeneration through cleavage of hyaluronic acid, degradation of collagen and proteoglycans.

It is now well established from various studies that low grade and innate immunity inflammation from mechanical damage and metabolic dysregulation is crucial in driving OA pathogenesis (Robinson *et al.* 2016). Evidence advocates a primary role in disease, but a secondary inflammatory response to degeneration cannot be excluded. Innate immunity mechanisms include the reaction against cleavage products originating from ECM classified into damage-associated molecular patterns (DAMPs) recruiting inflammatory mediators and cells thereby initiating the immune response (Grässel *et al.* 2021). Up to date, the major considered cytokines implicated in OA are IL-1β and TNF-α released from inflammatory cells or innate immune system stimulated chondrocytes, bone cells and synovial cells. Functionally, cytokines facilitate inhibitory effects on matrix synthesis and stimulate production of matrix degradation enzymes (Svala *et al.* 2015). Inflammation has been concluded to increase chondrocyte response and phenotypic changes during OA and additionally recognized the correlation between inflammation responses and pain mediators (Skiöldebrand *et al.* 2019). Pain sequentially causing lameness is considered a hallmark of OA (van Weeren & de Grauw 2010). Experience of pain requires pain stimuli detected and transduced to the central nervous system. Mechanical damage and inflammatory compounds are considered OA associated pain stimuli, detected by mechanoreceptors and nociceptors. The peripheral transmission holds both afferent transductive properties and efferent neuropeptide mediated functions. Neuropeptides is important in pain transmission and speculated to modulate inflammation further disrupting joint homeostasis and fuelling disease progression (Gatenholm & Brittberg 2019). Proposed mechanisms include the reverse correlation between elevations of proinflammatory cytokines and neuropeptides.

Additionally, besides inflamed synovial membrane and joint capsule, considerable damage can be caused by abnormal stresses directly on cartilage (McIlwraith 2016). Mechanical forces can inflict direct damage to the collagen framework and chondrocytes with subsequent release of MMPs, aggrecanases and prostaglandins primarily or secondary to IL-1. Furthermore, despite the established importance of inflammatory mediated degeneration and damage through direct impact in disease progression, subchondral bone is proposed to hold a substantial role in OA

pathogenesis. Cartilage-bone crosstalk, the cell and mechanical interaction between cartilage and bone, is emphasized important regarding the insufficient adaptation of cartilage to tensile and shear forces protected though force distribution from bone (Goldring & Goldring 2016). OA alterations inflict on this mechanism conducting in shear stresses and cartilage degeneration. Regarding this mechanism, it can be argued that primary changes within subchondral bone, such as early sclerosis, precede cartilage degeneration (Skiöldebrand 2004). However, controversy regarding the chronological order of events prevails, debating OA initiation in cartilage or subchondral bone.

In summary, despite the incompletely understood events during onset of disease, different origin pathways cause a disrupted balance between anabolic and catabolic forces, engaging towards an abundance of degenerative processes compared to restoration proceeding into breakdown of the osteochondral unit (Skiöldebrand 2004; McIlwraith 2016; Grässel *et al.* 2021). Degeneration is initially characterized by loss of proteoglycans and water content followed by disrupted collagen networks (Skiöldebrand 2004; Goldring & Goldring 2016; Grässel *et al.* 2021). Morphologically, these changes are observed as softening and swelling of superficial cartilage extending deeper towards bone. Compensatory phenotypic alterations of chondrocytes predominate in this state associated with direct damage or in mechanosensory responses, engaging in anabolic synthesis of new proteoglycans and collagen type I (Goldring & Goldring 2016). Further development of disease occurs when these compensatory mechanisms become inadequate to counterbalance transition towards amplified cytokine stimulated enzymatic degradation (Skiöldebrand 2004; Grässel *et al.* 2021). This proceeds into cartilage fibrillation and development of fissures extending towards bone leading to delamination of cartilage and exposure of underlying calcified cartilage and bone with simultaneously increased subchondral bone thickness.

2.3 Exercise

2.3.1 Articular response to exercise & loading

Loading effects on articular structures are in relation to the challenges subjected to joint homeostasis during exercise (te Moller & van Weeren 2017). Articular tissue has a proven adaptive response to training and increased loading. However, increased loading will eventually convert the adaptive response to degenerative further alter biochemical composition and resistance and progressively develop into joint pathology. Previous research has established the importance of training intensity transitioning from physiological adaptation to pathological degeneration (van Weeren & Brama 2016). Low to moderate intensity training is perceived

favourable for cartilage whereas high intensity training is deleterious to both cartilage and bone.

Articular cartilage has a low tissue turnover rate and therefore limited reparative response to excessive loading (van Weeren & Brama 2016). Several lines of evidence from previous research acknowledge the effects of training on cartilage composition. Significantly increased total cartilage and calcified cartilage thickness have been observed following intensive exercise compensated by softening of hyaline cartilage and rigidity of subchondral bone (Murray *et al.* 1999). Further speculated to predispose for pathological changes. On a biochemical level, molecular cartilage changes include training intensity dependent variations in amount and distribution of COMP (Murray *et al.* 2001b; Skiöldebrand *et al.* 2010). Studies investigated training effects on PG provide evidence of exercise induced increase in newly synthesized PG (Palmer *et al.* 1995). The latter is in line with observed higher levels of glycosaminoglycans in horses undergoing highly intensive training (Murray *et al.* 2001a). Collagen content and composition are perceived not affected by exercise but recognized to decrease in predilection sites for OA correlated to high intensity training. Subchondral bone responds to increased pressure according to Wolff's law, explaining the changeable characteristics of bone tissue compared to cartilage (Wolff 1892 see van Weeren & Brama 2016). High intensity training is suggested to increase subchondral bone thickness and sclerosis, contributing to progressive structural stiffness and consequently risk a reached threshold of adaptive changes further converting into pathological changes (Murray *et al.* 2001c, 2007).

2.3.2 Exercise physiology

Based on the previous, assessment of intensity is of great value estimating exercise effects on articular structures. Cardiovascular measurements can be used to estimate training intensity (Hodgson 2014). The utility of heart rate has been evaluated in several studies, further providing evidence supporting usefulness of the method (Williams *et al.* 2019).

2.4 Biomarkers

2.4.1 Definition & recent knowledge

Lameness is a common clinical problem in horses as previously described and up to 60% urges from OA in the joints (Hunter *et al.* 2014 see Mcllwraith *et al. 2017*). OA is currently diagnosed through an orthopaedic examination and imaging techniques reflecting the functional and structural status of the locomotor apparatus

unable to assess the altered underlying metabolic processes contributing to the disease (Frisbie *et al.* 2016). The inability of early screening and detection results in a delayed definitive diagnosis consequently passing the early restorable OA stages. An inquiry for non-invasive, sensitive and specific diagnostic methods to characterize early OA and prevent irreversible changes are required (McIlwraith *et al.* 2018; Osteoarthritis Research Society International [OARSI] n.d.). The definition of biomarkers is diverse but suggested as objectively measured characteristics with indicative capacity of normal and pathological processes and evaluative qualities of pharmacological effects (McIlwraith *et al.* 2018). OA related biomarkers are molecules or molecular fragments released into SF and serum during joint degeneration and inflammation enabling quantification (Frisbie *et al.* 2016).

Research on equine biomarkers have shown promising results in diagnostics and developed current knowledge of disease pathogenesis. Previous work have observed fragmentation of cartilage oligomeric matrix protein in the articular cartilage during inflammation creating fragments with new cleavage sites (neo-epitopes) (Skiöldebrand *et al.* 2017). COMP neo-epitopes have been shown to reflect OA associated changes in articular cartilage (Skiöldebrand *et al.* 2017; Ekman *et al.* 2019). Foregoing studies have emphasized the diagnostic capacity thought documentation of the release of COMP during acute lameness and early OA changes (Skiöldebrand *et al.* 2017). Significantly higher concentrations were observed in acute lame horses compared to healthy controls and chronically lame horses. The predominantly higher concentrations of the neo-epitope in acute cases were argued correlating to lower concentrations of COMP in degenerated cartilage with more developed diseased cases. Confounding factors of age, circadian rhythm and short-term training effects were implied insignificant (Ekman *et al.* 2019). However, the proposed absence of the biomarker response to short-term training has afterwards concluded uncertain with contradictory results in later studies declared depending on fragment analysed (Mazor *et al.* 2019; Lisee *et al.* 2023). Another promising biomarker for degenerative joint pathology corresponding to bone lesions is the proteolytic cleavage fragment of native biglycan, i.e., the biglycan neo-epitope biglycan262 (BGN^{262}) (Adepu *et al.* 2022, 2023). Data presented in recent studies fortifies BGN^{262} as a predictive biomarker for early changes in bone and subchondral bone sclerosis. Significant elevated concentrations have been observed during training but no correlation with age, sex and breed has been proposed (Adepu *et al.* 2022). In general, a diversity of different biomarkers is advocated in evaluation of joint health, implying the value of pain and inflammatory biomarkers of equally importance as the structural ones (Frisbie *et al.* 2016). Nerve growth factor (NGF) has recently been studied with significantly higher concentrations in OA joints compared to healthy controls (Kendall *et al.* 2021). These results predict the importance of pain-mediators in the disease and the

relevance of pain-related biomarkers. A recent study aimed to evaluate neuropeptide Substance P (SP) in OA progression emphasize similar conclusions (Shirakawa *et al*. 2021). SP was established mainly in the articular cartilage and subchondral bone and secondly, decreased concentration correlated with severity of disease.

2.4.2 Sampling material

It is constitutive to consider sampling material when investigating a putative biomarker (Frisbie *et al.* 2016). The interference of metabolism, contribution, production and elimination must be taken into consideration. Despite overcoming these effects when sampling from SF, joint punctuation carries a great risk of infection and confounding concentrations assigned to active inflammation. Sampling serum minimizes infection risks but is still considered invasive. Various studies have proposed saliva to have the potential of a non-invasive sampling material mainly because of the ease of the procedure, limited discomfort and availability (Meleti *et al.* 2020). Fragmentation of molecules from the articular cartilage and subchondral bone during the degenerative and inflammatory processes in joints generates components further to be released into SF and serum. Equivalent properties between saliva and serum allows quantification of these components in saliva, supporting the diagnostic potential of saliva as sampling material (Lee & Wong 2009). The molecular characteristics of different components influence the route of entry into saliva conducting either a paracellular or transcellular route. Lipophilic molecules are considered highly membrane permeable with rapid transcellular diffusion through acinar cells. Molecules with limited lipophilic properties have low membrane permeability and enter saliva through tight junctions between cells, paracellularly. The molecular characteristics of different components differentiate in saliva flow dependency (Tasaka *et al.* 2014). Lipophilic molecules are independent on saliva production rate reflecting their fast diffusion contrary non-lipophilic molecules evidently depending on saliva production (Jusko & Milsap 1993). Therefore, a non-lipophilic molecule concentration will significantly decrease with increased salivation. Previous studies emphasize the molecular aspect of the compound as crucial when interpreting saliva concentrations of molecules.

2.4.3 Biomarkers at exercise & loading

Biomarkers enables interpretation regarding articular exercise effects and joint health (van Weeren & Brama 2016). Consensus prevails concerning training intensity as essential for initiation of changes in biomarker concentrations, reflecting the metabolic response to strenuous training. Prior work has emphasized the importance of differentiating physiological adaptation and pathological destruction when interpreting loading responses (Frisbie *et al.* 2016). This is

proposed to be accomplished through detection of concentration differences or diverse time patterns, prior to classification of the response as healthy contrary diseased. Comparison between changes in biomarkers during exercise and biomarker changes in OA horses undergoing training has been studied in previous work (Frisbie *et al.* 2008). The results conclude concentration variations of specific biomarkers depending on joint status, furthermore implying the importance of diagnostically enabling differentiation between physiological and pathological responses. A recent review evaluating the potential use of biomarkers interpreting loading effects on joint health concluded the diagnostic potential of several promising biomarkers (Roberts *et al.* 2019).

Most of the recent research has been focused on short-term evaluation on different mechanical loading on total COMP in humans. Results from several studies agrees that loading and type of impact causes changes in total COMP and perhaps fragmentation of the same, resulting in neo-epitopes (Niehoff *et al.* 2010; Dreiner *et al.* 2020). A recent review summarize the previous research on biomarkers and exercise effects (Mazor *et al.* 2019). The article comprehends current knowledge of native or total COMP as elevated immediately after exercise and directly regressed to baseline with no significant magnitude differences regarding healthy or OA joints, advocating an unrelated OA change of the biomarker. However, indications of a delayed increase of COMP have been observed and hypothesized reflecting the degenerative processes within the joint associated with training and not the immediate confounding release of already existing fragments. These results were supported in a study observing significant total COMP elevations as a delayed and not immediate response to loading, insinuating a later time point for reliable assessment of metabolic activity (Lisee *et al.* 2023). Recently, BGN^{262} has been evaluated in equine short-term training studies and shown increased concentrations during training advocating utility in measuring exercise effects (Adepu *et al.* 2022). Observed differences of BGN^{262} comparing different riding surfaces with a trend toward elevations dependent on firmness and cushioning emphasize the importance of measuring biomarkers sensitive to impact of different exercise regimes when searching for potential exercise related biomarkers (Adepu *et al.* 2023).

3. Material and methods

3.1 Study protocol

3.1.1 Horses and study design

The aim of the study was to search for osteoarthritis biomarkers in equine saliva. The saliva was collected using a commercial saliva collection swab and analysed with ELISA for quantification of biomarkers ($COMP^{664}$ and Substance P). The saliva samples were collected from two different cohorts. Cohort 1 in conjunction with feeding to determine the impact of feeding on the biomarker concentration. Cohort 2 was a short-term training study including sample collection before, during and after exercise to determine the acute and delayed response of the biomarkers concentrations. Heart rate measurements were implemented during exercise to correlate individual biomarker levels to training intensity.

Ethical approval for the sampling of privately owned horses was obtained according to current legislation concerning clinical trials on animals (permission number SLU-ID 4.6.18-08892/2019). All owners concerned were informed orally and in writing about the aim of the study, arrangement and content. A written consent form was included and signed by the owners. Sampling was also performed on university horses belonging to Swedish University of Agricultural Science and housed at the centre for Veterinary Medicine and Animal Science. The university horses were included according to the teaching permission (permission number 5.8.18-15533/2018). In total 13 horses were included in the study, eight privately owned and five from the university.

3.1.2 Cohort 1: Feeding study

Saliva samples were collected from university-owned horses (N=5) and privately owned horses (N=2) before, during and after morning feeding. From 22.00 the night before sampling the horses were not fed. The saliva collection was carried out according to the following time schedule: TP1) 2 hours before morning feeding at 05.00 and thereafter in 30-minute intervals until feeding, TP2) at 05.30, TP3) at 06.00, TP4) at 06.30, TP5) before feeding at 07.00. Then T6) 15 min into feeding at 07.15 and T7) by the end of feeding at 08.00, T8) and continuous collection in 30-minute intervals 3 hours after feeding 08.30, T9) 09.00, T10) 09.30, T11) 10.00, T12) 10.30, T13) 11.00. The two privately owned horses and four university owned horses were analysed for quantification of COMP[664]. Five university owned horses were analysed for Substance P.

Table 1. Age, gender and breed of the horses included in cohort 1: feeding study

Horses	N	Age mean value ± SD (min - max)	Gender (n = M/G/S)	Breed (n = SWB/STB/HANN)
University horses	5	17,4 ± 4,8 (10-26)	(5/0/0)	(0/5/0)
Privately owned horses	2	12 (8-16)	(2/0/0)	(1/0/1)

Horses: holder/owner. N: number of horses. Age min: minimum, max: maximum. Gender n: number of horses of different gender; M: mare, G: gelding, S: stallion. Breed n: number of horses of different breeds; SWB: Swedish warmblood, STB: Standardbred horses, HANN: Hannoveraner Verband.

3.1.3 Cohort 2: Short-term exercise study

A short-termed training study was performed using privately owned riding horses (N=6). During the study, the horses were not fed 2 hours before the first sampling until the sampling was finished. The horses were exercising according to a standardized training program on the same riding surface (fibre sand). The training program carried out as followed: Warmup) 10 minute walk in straight lines followed by 10 minutes of all gaits in straight lines, Intensive training) 20 minutes trot and canter, equivalent among direction of laps and performed circles (the same amount and size) finishing with Cooldown) 10 minute walk. The saliva collection was carried out accord to the following time schedule: T1) 1 hour before training, in the stable resting, T2) post warmup, T3) post intensive training, T4) post cooldown, thereafter in 30-minute intervals post complete training T5) 30 minutes

post-training, T6) 60 minutes post-training, T7) 90 minutes post-training, T8) 120 minutes post-training. The owners were asked to fill out a survey compiled by the author estimating training level and orthopaedic health status (Appendix 3). Training intensity (pulse and speed) were measured at T1-T5 including resting pulse.

Table 2. Age, gender, breed, training level and health status of the horses included in cohort 2: training study

Horses	N	Age mean value ± SD (min - max)	Gender (n = M/G/S)	Breed (n = SWB/KWPN)	Training level (n = L/M/H)	Health status (n = LT/ NLT)
Privately owned	6	9,6 ± 5,9 (4-21)	(4/2/0)	(5/1)	(2/4/0)	(4/2)

Horses: holder/owner. N: number of horses. Age min: minimum, max: maximum. Gender n: number of horses of different gender; M: mare, G: gelding, S: stallion. Breed n: number of horses of different breeds; SWB: Swedish warmblood, KWPN: Dutch warmblood. Training level n: number of horses within the training level; L: low training level, M: medium training level, H: high training level. Health status n: number of horses included in one of the two major groups LT: previous lameness history and treatment or NLT: no previous lameness or history of treatment.

3.2 Saliva collection and preparation

The saliva samples were collected using a commercial collection device (Equisal® Saliva Collection Kit, Austin Davis Biologics Ltd. UK) according to the manufacturer's instructions (Appendix 1). Instructions included exhortation of restriction feed intake and exercise 1 hour prior to sampling, deliberately omitted in this study. The swab was held by the plastic handle and the absorbent portion was placed between the interdental space against the tongue, moving it along the tongue. The swab was inspected after 30 seconds to check for change in the colour of the volume indicator to bright pink. If incomplete collection, i.e less than 500 µl, the collection continued. Food debris was removed after finishing sampling. The device was carefully placed in the tube with 1 ml preservative solution with the absorbent portion immersed in the preserving solution and labelled with the date, time and horse name. The samples were transported at -20°C to the laboratory. At the laboratory, the samples were thawed and the swabs were positioned upside down in the tubes and centrifuged for 5 min at 4°C 3000 rpm. After centrifugation, the solution was carefully aliquoted in smaller volumes and kept at -80 until use. The collected saliva was analysed for the presence of COMP[664] using quantitative

competitive ELISAs and a commercial competitive ELISA kit for the quantification of Substance P.

3.3 Enzyme-linked immunosorbent assays (ELISA)

3.3.1 COMP^{664} ELISA

A competitive ELISA was used to analyse COMP$^{\mathbf{664}}$ in saliva samples (Appendix 2). The saliva was thawed and diluted 1:8 in Sample Xtra buffer (Kementec, DK). The anti-COMP^{664} primary monoclonal antibody (0.445 mg/ml) was diluted in Sample Xtra buffer to reach a final concentration of 30 ng/ml. The synthetic peptide COMP^{664} was used to construct a standard curve of 12 points ranging from 2000 ng/ml to 0,000 ng/ml by a 1:2 serial dilution in Sample Xtra buffer. A volume of 60 µl/well of both standards and sample dilutions was transferred to a Sterilin Clear Microtiter^{TM} (Thermo Fisher, Sweden) and mixed with 60 µl/well of the primary antibody dilution. The plate was incubated overnight in a plate shaker at 37°C 40 rpm, in the dark. A Nunc MaxiSorp ELISA plate (Invitrogen) was coated with 100 µl/well of COMP^{664} synthetic peptide at a concentration of 1 µg/ml in Carbonate buffer (100 mM. pH 9.6). The plate was incubated overnight at 4°C. After incubation and washing of the ELISA plate four times with PBS-Tween 20 0,05%, the plate was blocked with Synthetic blocking buffer (Kementec, DK) (200 ml per well) at 37°C 40 rpm for 1 hour. Then, the plate was washed once and both pre-incubated standards and samples were transferred to the ELISA plate (100 µl/well) and incubated for 1 h at 25°C 600 rpm. After incubation, the samples were discarded and the ELISA plate was washed 4 times and 100 µl/well of the secondary antibody (polyclonal goat anti-rabbit IgG HRP 1 mg/ml, Abcam) diluted 1:50 000 (10 mM PBS, 1% Bovine Serum Albumin and 0,1% Tween-20) was added to the ELISA plate incubated for 30 min at 25°C 600 rpm. After washing 8 times, 100 µl/well of TMB was added and incubated for 3 min at room temperature. The reaction was stopped by adding 100 µl/well of SO_4H_2 2.8 mM solution. The absorbance was read at 450 nm (Tecan-Spark-10M_V1_1 Multimode plate reader with Magellan software version 3.1) and the best-fit line was determined by a 4-parametric logistic regression analysis.

3.3.2 Substance P ELISA

A commercial competitive ELISA was used for the evaluation of Substance P levels in saliva samples (Appendix 2). A standard curve of 7 points was prepared (10.000; 2500; 625; 156; 39.06; 9,8 and 0,00 pg/ml) by diluting the Substance P standard (100 000 pg/ml) 1:10, and thereafter 4 times serial dilutions using the kit Assay buffer. Saliva samples were diluted 1:2 in the kit Assay buffer. Fifty µl/well of

standards and samples, 50 ml/well of the substance P Alkaline Phosphatase conjugate and 50 ml/well of anti-substance P antibody (final volume 150 ml) were added to the pre-coated plate ELISA (anti-Substance P antibody), The ELISA plate was incubated on a plate shaker at 25°C, 500 rpm for 2h. After incubation, the plate was washed 3 times, 200 ml/well of pNpp Substrate was added and further incubated for 1 h at 25°C 500 rpm. At the end of the incubation, the reaction was stopped and the absorbance read at 405 nm with correction between 570 and 590 nm. (Tecan-Spark-10M_V1_1 Multimode plate reader with Magellan software version 3.1) and the best-fit line determined by a 4-parametric logistic regression analysis.

3.4 Statistical analysis

The program GraphPad Prism 10.1.0 was used for the statistical analysis and the results are presented with mean value and standard deviation. One-way Analysis of Variance (ANOVA) was performed to determine significant differences among sampling points in both feeding and training data. T-test was used in statistical validation of changes in mean values between sampling points if conducted ANOVA showed statistical significance. Statistical significance was set at p-value <0,05.

4. Results

4.1 Cohort 1: Feeding study

4.1.1 The concentration of COMP^{664} in saliva during feeding

The concentration of COMP^{664} in saliva exhibited individual (N=6) fluctuations but were not feeding dependent. Values above the detection limit were allocated a value of 8000 mg/ml. Statistical analysis concluded no significant change between time points in conjunction with feeding (Fig. 1. Table 3).

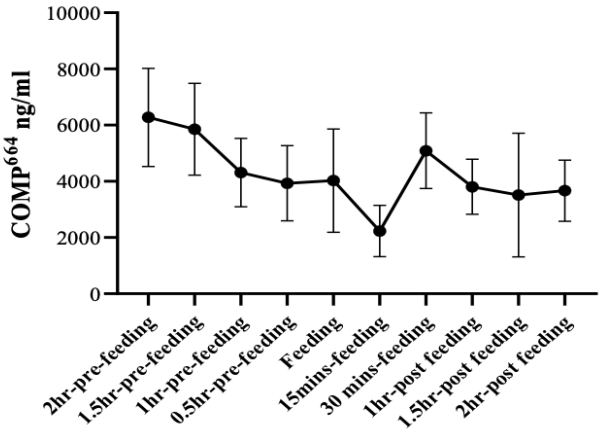

Figure 1. The concentration of COMP^{664} (ng/ml) from four university and two privately owned horses before, during and after feeding (mean values ± SD).

Table 3. The concentration of COMP^{664} (ng/ml) from six horses before, during and after feeding (time point 1-10). Time points are numerically labelled in the table and correspond to the exact time specified in the graph (Fig. 1). All values are showed as mean ± standard deviation (SD).

Time point	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
COMP^{664} (ng/ml)	6276	5852	4309	3930	4028	2232	5089	3808	3515	3668
± SD	4277	4006	2980	3275	4497	2234	3300	2398	5389	2670

4.1.2 The concentration of Substance P in saliva during feeding

The concentration of Substance P in saliva varied between horses (N=5) with individual peaks and concentration differences. However, there was no statistically significant difference in concentration of Substance P in saliva before and after feeding (Fig. 2. Table 4).

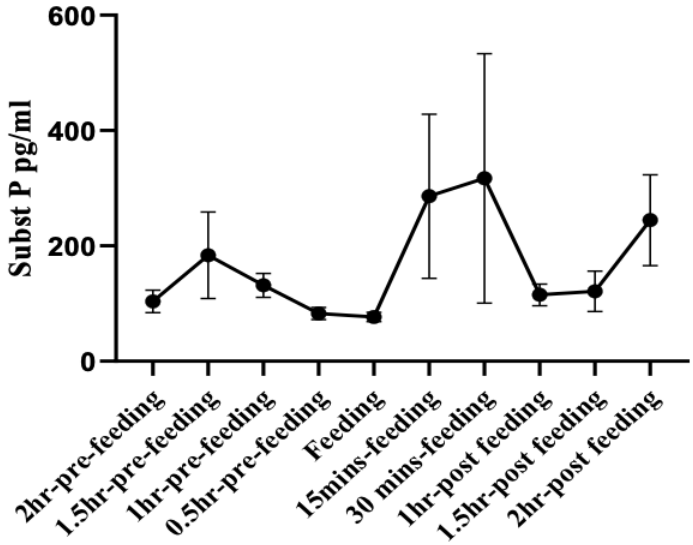

Figure 2. The concentration of Substance P (pg/ml) from five university owned horses before, during and after feeding (mean values ± SD).

Table 4. The concentration of Substance P (pg/ml) from five horses before, during and after feeding (time point 1-10). Time points are numerically labelled in the table correspond to the collection times in the graph (Fig. 2). All values are shown as mean ± standard deviation (SD).

Time point	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
Substance P (pg/ml)	104	184	132	83	77	286	317	115	121	245
± SD	44	168	46	24	18	318	483	42	79	176

4.2 Cohort 2: Short-term exercise study

4.2.1 The concentration of COMP^{664} in saliva during short-term exercise

The concentration of COMP^{664} in saliva in all horses (N=6) changed in response to exercise presenting a trend with an acute peak at T2 followed by a delayed peak observed post-training between T6-T8 (Fig. 3. Table 5), however the differences observed in the results were not statistically significant. Thereafter, the horses were divided in two groups depending on age and previous history of joint health (Fig. 4. Table 6). Group 1= young horses (N=2) (age 4, 5 with no history of previous lameness and osteoarthritis). Group 2= old horses (N=4) (age 6, 9, 13 and 21 with a known history of lameness and joint treatments). In the group with older horses, the concentration of COMP^{664} was higher at T2 compared to the young horses. Additionally, a delayed peak is observed in both groups either at T6 in group 2 or at T7 in group 1, not completely included during the sampling period. The differences between young and old horses were not statistically significant.

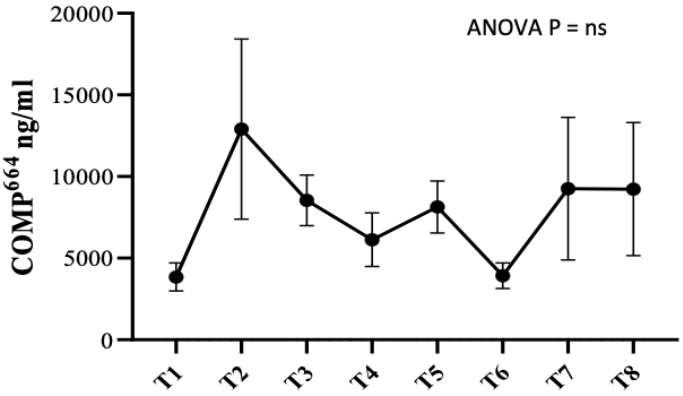

Figure 3. The concentration of COMP^{664} (ng/ml) in saliva from six horses before, during and after training (mean values ± SD). Collection time points T1) 1h before training resting in the stable, T2) post warmup, T3) post intensive training, T4) post cool-down, thereafter in 30 minutes intervals after complete training at T5) 30 min post-training, T6) 60 min post-training, T7) 90 min post-training, T8) 120 min post-training.

Table 5. The concentration of COMP^{664} (ng/ml) in saliva from six horses during short-term training by each sampling point. All values are shown as mean ± standard deviation (SD).

Time point	T1	T2	T3	T4	T5	T6	T7	T8
COMP^{664} (ng/ml)	3848	12907	8539	6132	8138	3931	9256	9232
± SD	2095	13500	3792	4009	3893	1921	10683	9983

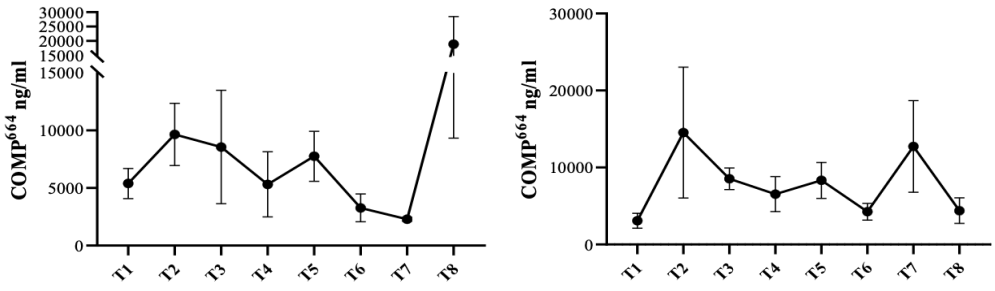

Figure 4. The concentration of COMP^{664} (ng/ml) in saliva from group 1 (left) and group 2 (right) before, during and after training (mean values ± SD).

Table 6. The concentration of COMP^{664} (ng/ml) in saliva from group 1 and group 2 by each sampling point. All values are shown as mean ± standard deviation (SD).

Time point	T1	T2	T3	T4	T5	T6	T7	T8
Group 1								
COMP^{664} (ng/ml)	5391	9648	8563	5319	7759	3276	2294	18913
± SD	1848	3818	6955	3999	3074	1703	237	13559
Group 2								
COMP^{664} (ng/ml)	3077	14537	8526	6539	8328	4259	12737	4391
± SD	1949	16979	2799	4560	4687	2181	11904	3329

4.2.2 The concentration of Substance P in saliva during short-term exercise

The concentration of Substance P in all horses (N=6) in response to exercise showed a trend towards increased concentration post-training starting from T4 till T8, although not statistically significant (Fig. 5. Table 7). Individual variations at baseline (T1) values were observed. As previously described, the data was divided into two groups (Fig. 6. Table 8). A second delayed peak is observed and varied in onset of time, early at T5 in group 2 and later at T7 not completely included in group 1.

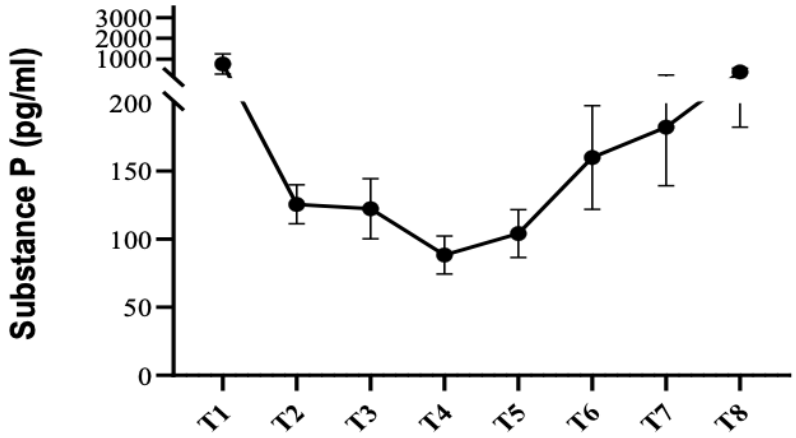

Figure 5. The concentration of Substance P (pg/ml) in saliva from six horses before, during and after training (mean values ± SD). Collection time points T1) 1h before training resting in the stable, T2) post warmup, T3) post intensive training, T4) post cool-down, thereafter in 30 minutes intervals after complete training at T5) 30 min post-training, T6) 60 min post-training, T7) 90 min post-training, T8) 120 min post-training.

Table 7. The concentration of Substance P (pg/ml) in saliva from six horses during short-term training by each sampling point. All values are shown as mean ± standard deviation (SD).

Time point	T1	T2	T3	T4	T5	T6	T7	T8
Substance P (pg/ml)	757	126	123	88	104	160	182	369
± SD	1187	35	54	34	43	93	105	457

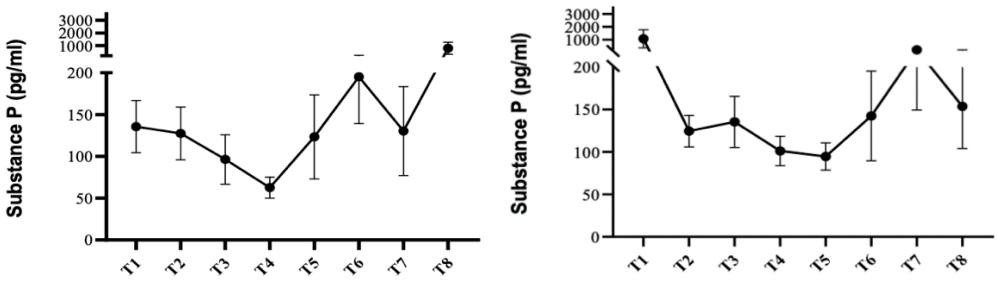

Figure 6. The concentration of Substance P (pg/ml)in saliva from group 1 (left) and group 2 (right) before, during and after training (mean values ± SD).

Table 8. The concentration of Substance P (pg/ml) in saliva from group 1 and group 2 by each sampling point. All values are shown as mean ± standard deviation (SD).

Time point	T1	T2	T3	T4	T5	T6	T7	T8
Group 1								
Substance P (pg/ml)	136	128	96	63	123	195	130	799
± SD	44	44	42	18	71	79	75	676
Group 2								
Substance P (pg/ml)	1067	125	135	101	95	143	208	154
± SD	1401	37	60	35	32	106	118	99

4.2.3 Heart rate and speed measurements during short-term exercise

The heart rate and speed as measure for training intensity showed an increase at T2 and thereafter a sequential decrease which correlated to the amount of training (Fig. 7). The peak at T2 follow to the acute response of biomarkers but not the delayed response at T6-T8. Two of the horses pulse measurements were not included because of lost contact with the pulse watch.

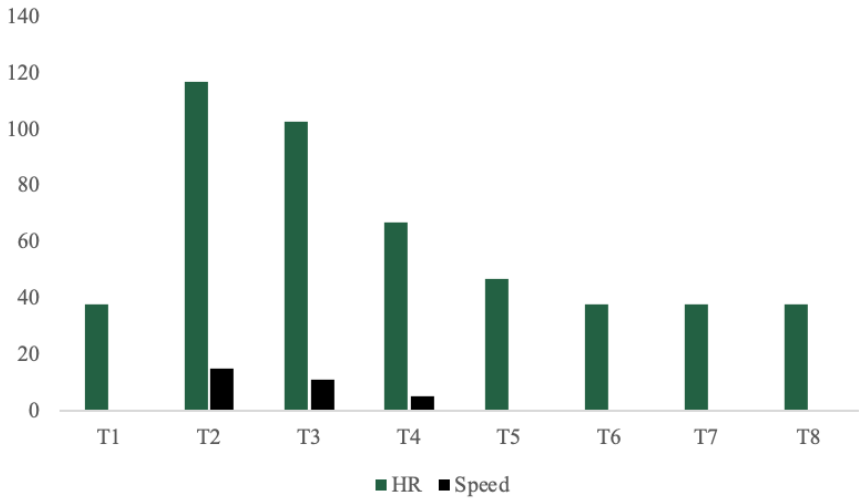

Figure 7. Heart rate and speed measurements from six horses included in cohort 2 before, during and after training (mean values). Time points correlate to saliva collection time points described above.

5. Discussion

Biomarkers reflective of the mechanical load endured by the joints during training could be used in the future to prevent/detect injuries or deleterious effects of performance on the joints. The aim of this current study was to measure the concentration of a neo-epitope OA specific biomarker COMP^{664} and neuropeptide Substance P in terms of acute and delayed response to short-term training. The previously described acute peak are suggested to correlate to already pre-existing biomarkers in the joint and the delayed peak correlate to the breakdown of the cartilage tissue in response to the impact of joint load. Training intensity measured by heart rate and speed was determined during the training study accounting for the effect of training intensity on biomarker levels when comparing results. The influence of the confounding effect of feeding was also investigated. Furthermore, this pilot study will aid in defining a larger study aiming to enable the assessment of normal physiological and deleterious training responses in healthy joints and pathological responses in diseased joints.

Concentrations of COMP^{664} and Substance P in saliva during feeding

Data from the feeding study measuring COMP^{664} concentrations in saliva revealed fluctuating values and individual differences between horses but no obvious changes were observed during feeding. Further confirmation of this interpretation was established performing statistical analysis with no significance. A trend towards decreased concentrations of COMP^{664} was observed in the middle of feeding and in the end of training associated with increased saliva production. Previous literature has established that horses start to produce saliva during chewing and the production increases with prolonged chewing time and intensity (Ermers *et al.* 2023). The observed trend implies a need to consider the impact of saliva production and its possible influence on molecular transportation from serum to saliva when evaluating biomarker concentrations. It is believed that lipophilic molecules are independent of saliva production. In contrast, non-lipophilic molecules are speculated to decrease in concentration with increased salivary flow rate. The biochemical composition of the molecule determines how transportation of a biomarker into saliva is facilitated (Jusko & Milsap 1993). Lipophilic molecules are highly membrane permeable, and a majority enter saliva transcellularly

through rapid diffusion in comparison to molecules with none or limited lipophilic solubility, which are unable to pass through cells thus entering saliva through a paracellular route. Therefore, increased saliva production could influence the concentration of the biomarker and should be taken into account when interpreting quantification of biomarkers in saliva (Tasaka *et al.* 2014). Theoretically, due to biochemical composition of $COMP^{664}$, this molecule might use a paracellular route from serum to saliva which would be dependent on the salivary flow rate leading to a decrease in concentration with an increase in salivation. Substance P is considered a molecule with low transmembrane diffusion resulting in a similar pattern. Analysis of Substance P in accordance with feeding showed individual differences which were not observed or statistically interpreted feeding dependent suggesting the changes to be related to other contributing factors i.e., medical history or other. External validation of the feeding study is low considering few included individuals and the extent of unknown confounding factors in the horses. The general conclusion from this pilot study cannot confidently be stated further, but according to previous works, it is advisable to implement feeding restrictions before the quantification of exercise-induced biomarkers (Lisee *et al.* 2023). With these results and previous knowledge, the horses were decided to be sampled two hours after feeding conducting the short-term training study.

The concentration of $COMP^{664}$ in saliva during short-term exercise

There is only limited data in literature describing the effect of training especially in equine saliva biomarkers. This study was designed as a pilot and the results will be used for designing a larger exercise study. Native COMP and its fragments have recently been proposed as a valuable biomarker for equine OA in synovial fluid and serum (Skiöldebrand *et al.* 2017; Ekman *et al.* 2019). Additionally, BGN^{262} a biomarker arising from the subchondral bone has been shown to be a promising mechanosensitive biomarker in saliva with an acute peak appearing immediately during training and increases when the horses are trained on harder footing (Adepu *et al.* 2022, 2023). The acute peak has recently been re-evaluated to be more informative in a retrospective perspective and the indicated delayed peak after exercise has therefore been proposed as more relevant in evaluating joint cartilage catabolism produced during the preformed training (Mazor *et al.* 2019; Lisee *et al.* 2023). In this study, saliva COMP was measured before, during and after training with the aim to follow these biomarker changes. Results obtained from these measurements revealed both the acute and delayed peak of $COMP^{664}$ associated with exercise, further supporting its mechanosensitive properties. The statistical analysis of mean saliva $COMP^{664}$ in all horses showed no statistical significance probably due to the small group size of horses with probable delayed peaks beyond the time points included in the study.

Division of the horses into two groups depending on age and joint health status was based on the hypothesis that the young group had a healthy joint status while the older group had a presumable OA burden and this revealed different biomarker response patterns. These results are supported by earlier work evaluating changes in total COMP during training in horses (Helal *et al.* 2007). Exercise results in an increased clearance of degradation products from synovial fluid. The observed acute peak measuring $COMP^{664}$ could be a result from effusion of already existing ECM fragments derived from previous processes within the joints flushed out during training. Theoretically, the amplitude and magnitude of this peak represent the extent of recent degeneration of cartilage or/and bone thus showing a small or non-existing peak in healthy joints, compared to a distinct and profound peak in diseased joints which originates from pre-existing accumulated degradation products in SF. The delayed peak extent and onset in time could be a result of an already working degenerative machinery i.e., chronic degeneration and inflammation resulting in rapid degeneration time and amount in response to training. The previous referred study supports these arguments and discusses high concentrations found in response to training either originating from upregulated proteolytic fragmentation in diseased joints or increased tissue turnover in healthy joints. Therefore, the delayed peak could additionally be argued to mirror the response of healthy cartilage to training, interpreted as concentration differences depending on strenuosity of the performed loading resulting in differences in the extent of initiated inflammation and degeneration. Observations from previous research further strengthen this line of reasoning where they found greater loading responses of total COMP in symptomatically affected OA knee patients, explaining results in this study showing greater loading responses in the group with older horses presumably more affected by OA (Hay *et al.* 2023). Although further work is needed to confirm these assumptions, the data presented here could reflect health status and loading stress.

The concentration of Substance P in saliva during short-term exercise

Substance P has been regarded as a potential OA biomarker (Shirakawa *et al.* 2021). Interestingly, this data delineates Substance P concentration changes in response to exercise, theoretically explained by the loading induced inflammation that stimulates the neuropeptide release (Gatenholm & Brittberg 2019). This defines both inflammatory and mechanoresponsive properties of the biomarker. In the supposedly less healthy group, we observed higher initial concentrations of Substance P during training compared to the healthy group, but that is not sufficient to state to which extent there is an ongoing inflammation promoting degeneration within these joints. The data are further not clear enough to assess an immediate peak. Both groups present a delayed peak pattern similar to $COMP^{664}$. The initial high baseline concentrations are proposed to be caused by other confounding

factors i.e., medical treatment, pain, etc. Previous research has shown stem cell recruitment in response to increased Substance P levels (Kim *et al.* 2016), which allows us to speculate the opposed scenario can be responsible for the high baseline values in one of the horses recently treated with stem cells. Further work and surveys over influential factors on Substance P release are needed to understand and interpret these results, emphasizing the need for continued sampling from different groups of horses.

Association between COMP^{664} and Substance P

Comparison between the two biomarkers reveal a similarity in terms of their response to exercise. A mechanosensitive increase in Substance P seems to be associated with an increase of COMP^{664}. The inflammatory biomarker also remains elevated to a greater extent compared to the degenerative one. This pattern is supported by recent studies delineating the inflammatory and degenerative processes in response to loading (Hutcherson *et al.* 2023). The authors aimed to investigate the mechanisms involved in abnormal loading altering the metabolism within joints with sequential metabolic dysfunction including inflammation and degeneration. The study results indicated that loading or stress initiate changes in COMP associated with increased pro- and anti-inflammatory mediators simultaneously driving the inflammatory response. The study further emphasized the sequential biochemical alterations following these events, referring to the disrupted joint homeostasis conducting to the cycle of inflammation and degeneration. Loading evidently induces the release of COMP from degeneration of matrix further stimulating the onset of low-grade inflammation. It can be speculated that the release of COMP^{664} in response to exercise in this study is simultaneously associated with the release of pro- and anti-inflammatory mediators results in elevations of Substance P concentrations. The release of COMP^{664} in response to exercise and the association with low-grade inflammation further supports the prognostic potential of the biomarker in the assessment of cartilage degeneration and inflammation within joints.

Training intensity during short-term exercise

Training intensity is considered a crucial factor for the transition from physiological adaptation towards pathological degeneration. Previous studies evaluating COMP levels in horses undergoing different training protocols have shown higher concentrations of this biomarker with increased training intensity (Helal *et al.* 2007). In this study, an association between the acute peak and elevated training intensity was also observed. The delayed peak might not have a direct association with intensity since the elevated biomarker peaks were observed after the performed training, though the relationship between training intensity and the

delayed peak cannot be excluded. Since intensity of exercise imposed was equivalent among the horses, changes in biomarker levels could be considered a true reflection of the joint status, highlighting the importance of including exercise intensity measurements in training studies both for standardization purposes and to limit the effects of different intensities.

Study limitations

Another aim of this project was also to identify timepoints and cohorts designing a larger study. The feeding study results are insufficient to draw conclusions on, partly because of the study group of different ages and medical history. In the future, it would be appropriate to have two matched cohorts (sex, age and breed) including different health status (healthy and OA diagnosed horses) to reduce confounding factors in the results. This also concerns future training studies where inclusions and exclusion criteria should be used dividing the study group into a healthy control group and an OA affected group. Additional improvement through lameness control before performing the training study will contribute with knowledge about the individual status before assessment of biomarker changes. A longer sampling time to follow the complete delayed peak is also desirable. Furthermore, more horses need to be included and sampled in order to collect and better describe the baseline values and patterns for development of standardized measurements. When standardized measurements and baseline values are established, sampling under different circumstances should be performed. Finally, most of the recent studies of biomarkers reflecting OA in horses are cross-sectional. For further investigation and to prove the importance of biomarkers in equine orthopaedics prospective longitudinal studies are needed. The prospective longitudinal studies provide a reference or baseline on how the markers vary on an individual level. Up to date, there are no normal reference values when interpreting a single sample, and consequently resulting in confounding effects of other factors.

Conclusion

In summary, this study provides knowledge about changes in biomarkers in response to training. This can be categorized into a pilot study and a successful project contributing to the knowledge needed for succeeding studies aimed to investigate changes of biomarkers during training. Future research in this field can potentially distinguish normal physiological and deleterious responses from healthy joints and pathological responses in diseased joints further developing our understanding of joint response to exercise and to the early detection of OA.

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Popular Science Summary

Both humans and horses are dependent on movement and exercise for welfare and wellbeing. Failure of this fundamental need have consequences, especially for horses that are known active and athletic animals. Lameness is therefore considered a major clinical and equine welfare issue. A variety of reasons might explain arising lameness, though originating from joints and underlying degenerative joint diseases such as osteoarthritis, commonly termed arthrosis, is not unusual. Osteoarthritis is characterized by inflammation and breakdown of the joint, consequently resulting in loss of function and pain, referred to as lameness. The original cause is rarely determined when osteoarthritic changes are discovered but there are several known risk factors. Concerning horses, one of the main considerations is strenuous and intensive training with sequential overloading and altered joint health.

Unfortunately, the disease is often diagnosed too late in the irreversible stages when there is no treatment available. The lack of early screening and detection methods is an urging problem facing both veterinary and human medicine. Consensus regarding the need for alternative diagnostic methods has prevailed in recent years and revealed the potential use of biomarkers. Briefly, a biomarker is a component originally from the joint and can be used as an indication of deleterious physical or pathological activity. Detection of biomarkers has been investigated and proven feasible at the original location in the joints or distantly in blood or saliva. The potential of biomarkers as a new diagnostic tool has repeatedly been proven and further contributed with knowledge of disease mechanisms guiding the search for new pharmaceuticals. Evaluation of training impact using biomarkers has become part of this research field, enabling investigation of disease mechanisms and exercise effects in joints.

The aim in this study was to analyse biomarkers in horses during training using saliva as sampling material. Previous research has observed an acute response in biomarkers immediate to training and noted a late increase of biomarkers after training. This late increase is proposed to be more truthful in reflection of the effect of training performed and potentially more informative differentiating these effects from early disease changes. Before performing the training study, horses were measured during feeding to evaluate if the biomarkers concentrations change with feed intake which can interfere with training results. No evident effects of feeding

were seen but based on the small number of horses measured and previous literature, feeding restrictions were applied. The horses were measured before, during and after training simultaneously as measurements of heart rate and speed were performed to take the intensity into the overall assessment of results. The results showed an acute response of biomarkers and a later delayed and theoretically more informative response. Dividing the horses by age and presumable joint health status, different patterns of responses were detected.

In conclusion, this project engaged in understanding how joints react during training to enable future investigation on harmful or favourable training of horses regarding joint health and early detection of joint disease. The disease is not only a common problem encountered in veterinary medicine but also viewed as both a welfare and economic burden in human medicine. Considering the above, this research field develop our knowledge of the disease and will be of great importance and valuable in human medicine diagnosing and treating osteoarthritis.

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Appendix 1

Saliva collection and preparation with Equisal saliva collection device

1. The horse is not allowed to eat or be exercised 1 h prior to collection. Even small morsels of hay or other type of food on the ground should be avoided. Grooming is allowed during this time.
2. Open the foil package containing the saliva swab, hold the plastic handle. Collect the sample from the horse by inserting the swab through the interdental space (the space between the front and back teeth).
   Position the swab on the tongue and move it up and down along the tongue, allowing the horse to move its tongue under the swab. This movement minimise the risk of the horse getting hold of the swab and biting it.
   After 30 seconds, remove the swab and inspect the colour indicator, on bottom under part of the collection swab. The collection is done when the indicator has changed colour from white to bright pink. If the indicator has not completely changed colour, return the swab into the mouth, and continue sampling and checking the indicator until it has changed colour. If your horse's mouth is dry, then sample collection may take several minutes.
3. When the indicator has turned bright pink, remove food debris carefully with a tissue.
4. Place the swab in the tube with preservative solution. Make sure the cotton/foam end is placed into the preservative solution first. Screw on the cap.
5. Write the horse's name and date of collection on the tube.
6. Place the tube in -20°C (-4°F) if the sample can't be taken care of directly. If the sample is needed to be transported from the place of sample collection to the laboratory, keep the equisal with freezer packages.
7. Fill out the form about the horse and collection.
8. Continue saliva sample preparation in the instructions
9. Thaw the tube containing Equisal collection swab or used the cooled sample collected within 2 hours.
10. Open the tube and carefully take out the collection swab and turn it upside down, now with the plastic handle first. Screw on the cap again.
11. Centrifuge 3000g 5min.
12. Gently remove the collection swab. Take out the solution with a pipette, do not touch the pellet (food debris), it is generally quite fragile.
13. Add the solution to a falcon tube and vortex carefully.
14. Add the solution to Eppendorf tube/s and place in -80°C (-112°F) freezer. Clearly annotate the tubes. Include date (year-month-day) of collection and horse's annotation.

Appendix 2

COMP^{664} ELISA

Material

Plates:

- Nunc MaxiSorp™ Clear Flat-Bottom 96-Well Plates (Invitrogen, Cat. No. 44-2404-21. For the coating of peptide, the ELISA-plate.
- Thermo Scientific™ Sterilin™ Clear Microtiter™ Plates (Fisher Scientific, Cat. No. 11319163. For the incubation of standard and samples overnight 37*C with slow shake (39-40 rpm).

Buffers:

- Coating buffer: 100 mM Carbonate buffer, pH 9.6
- Blocking buffer: Synthetic Blocking Buffer (Kementec, Cat. No. 4520)
- Buffer for dilution of standard, sample and primary antibody: Sample Xtra buffer
- Buffer for dilution of secondary antibody: 10 mM PBS with 1 % Bovine Serum Albumin (BSA) and 0,1 % Tween-20
- Substrate: TMB One (Kementec, Cat. No. 4380)
- Stop solution: 0,2 M H_2SO_4
- Wash buffer: 10 mM PBS with 0,05 % Tween, pH 7,4

Peptide and primary antibody:

- Peptide COMP II- Neo reconstituted according to GeneScript protocol to 2mg/mL and thereafter aliquoted . 20µL/ tube and thereafter stored in -80*C in aliquots prior to use.
- Primary antibody anti COMP II- Neo (0.445mg/mL) thawed and aliqouted into micro-tubes. 10µL/ tube and stored in -20*C freezer prior to use

Secondary antibody:

- Polyclonal Goat anti rabbit (IgG) HRP 1mg/mL (Abcam, Cat.No ab 97051)

Method

Day 1:

- Coat the Nunc MaxiSorp plate with COMPII- Neo peptide diluted to 1µg/mL in Carbonate buffer (100mM. pH 9.6) thereafter placed in the ELISA-plate; 100µL/ well, seal with adhesive film for microplates as well as parafilm around the plate and incubated at 4*C overnight. OBS! The sealing must be done properly to avoid evaporation during the incubation.
- Dilute the standard i.e., peptide COMPII- Neo (2mg/mL) to 2000ng/mL in Sample Xtra buffer and thereafter in 1:2 series using the same buffer. Use 1mL of 2000ng/mL and dilute 1:2. The standard here was chosen 15.mg-2000ng/mL in duplicates, 100µL/well, see table 1 and Figure 1 for examples.
- Dilute the samples in Sample Xtra buffer (Saliva 1:8, Serum 1:4, Synovial fluid 1;4)
- Dilute the primary antibody Anti COMP II Neo (0.445mg/mL) to 30ng/mL in Sample Xtra- buffer.
- Add Standard and serum samples (60µl/each and per well) in to the Sterilin™ Clear Microtiter™ Plate and incubate with primary antibody (60µL/well/ std and sample) overnight at 37*C incubator with slow shaking (40rpm) in the dark and in a humid chamber the plate should be sealed properly and the wells in the outer positions of the microplate should be filled with water and not be used for standard or samples due to a risk of "volume dry out" in the outer wells. The pre incubation and temperature is performed to give the primary monoclonal antibody of IgG type enough time and optimal reactivity (at 37*C) and thereby be able to find their antigen more efficient.

Day 2:

- Wash the peptide coated wells ELISA-plate - 4 times in wash buffer (10 mM PBS with 0,05 % Tween, pH 7,4) and thereafter block with 200ul/well Synthetic Blocking Buffer for 1hr in the 37*C incubator with shake (40rpm). Thereafter wash once in wash buffer before loading with standard and sample.
- Transfer the overnight incubated standard and samples with primary antibody (100µL/well) to the peptide coated and blocked plate, ELISA-plate. Incubate in dark on a plate shaker at 25°C for 1 hour with 600rpm shaking.
- After incubation wash (4X) the ELISA-plate with standard and samples with washing buffer.
- Dilute the secondary antibody, goat anti rabbit HRP, in 10 mM PBS with 1 % BSA and 0,1 % Tween-20 first to 1:10 000 and then to 1:50 000 (held dark in RT prior to use). Add 100µl/well of the secondary (1:50 000) the ELISA-plate. Incubated in the dark on the plate shaker at 25°C for 30minutes with shaking set at 600rpm.
- After the secondary antibody incubation Wash the plate 8X with wash buffer.
- Bring the TMB to RT to use. Add TMB-one (50µl/well) and incubate in the dark at RT for 3min.
- Add Stop solution (100µL/well) and read the absorbance at 450nm with the standard set at 4-parametric curve with the x-axis set as logarithmic. OBS! To analyse 4-parametric standard curve with logarithmic x-axis the zero standard point cannot be set as zero but should be set to 0,001 (even though it is 0).

Substance P ELISA

Method

Let all reagents warm to room temperature before using them in the assay

- Prepare the standard curve and sample diluations:
    - Dilute SP Std (100.000 pg/ml) 1:10
      (100 in 900 ul sample buffer= 1000 ul 10.000 pg/ml)).
      Make a six points, 4X serial dilutions in a final volume of 1000 ul (i.e.: 250 ul dilution + 750 sample diluent = 2500, 625, 156,2, 39,06 and 9.8 pg/ml)
    - Prepare dilutions in a final volume of 200 ul :
      Sinovia: 1:2 dil (110 ul synovia + 110 ul Sample buffer)
      Serum:
      Saliva : Neat (in Equisal 1 or 2 ml)
- Add 50 µL of standard diluent (Assay Buffer) into the Non-Specific Binding (NSB) and the B0 (0 pg/mL) standard wells. TA & BLK (blank) empty.
- Add 50 µL of Standards #1 through #6 into the appropriate wells.
- Add 50 µL of the Samples into the appropriate wells.
- Add 50 ul Sample diluent to NSB control wells.
- Add 50 µL of blue Conjugate into each well, except TA & BLK wells.
- Add 50 µL of yellow Antibody into each well, except the for the TA, Blank and NSB wells.
- Seal the plate. Incubate at room temperature (RT) on a plate shaker for 2 hours at ~500 rpm.
- Empty the wells and wash with ~400 µL of 1x Wash Buffer to each well for a total of 3 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.
- Add 5 ul of conjugate to TA wells
- Add 200 µL of the pNpp Substrate solution to every well. Incubate at RT for 1 hour with shaking.
- Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.
- Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm.
- Calculation
    - Several options are available for the calculation of the concentration of Substance P in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic (4PL) curve fitting program.

- The concentration of Substance P can be calculated as follows: Calculate the average net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

Average Net OD = Average OD - Average NSB OD

Using data analysis software, plot the Average Net OD for each standard versus Substance P concentration in each standard. Samples with concentrations outside of the standard curve range will need to be reanalysed using alternative dilution(s).

Appendix 3

Horse information
Date:_____
Horse name:_____
Breed:_____
Gender:_____
Age:_____
Owner/Rider:_____
Trainer:_____

Area of Use

Competition branch: Dressage □ Showjumping □ Eventing □ Other _____

Training level: Low* □ Medium** □ High*** □

Is the horse in training?: Full training level/competition season □ Commissioning □ Resting □

Time of last training?: Same week □ 2 weeks ago □ 3 weeks ago □ 4 weeks ago □ > 4 weeks ago □

Time of last competition?: < 1 month ago □ 6 months ago □ 1 year ago □ Don't compete □

Medical History
Short summary of medical history: _____

Has the horse been locally treated/injected within the last 3 months? Yes□ No□
If yes: which date, anatomical structure and pharmaceutical used?_____

Has the horse received systemic anti-inflammatory treatment last month? Yes□ No□
If yes: which date, pharmaceutical and dosage used?_____

Other treatment i.e., herbal medicine, laser…? Yes □ No□
If yes, which date and treatment? _____

**Low: horses in early training or horses easily ridden without increased intensity.*
***Medium: horses competing in dressage, showjumping and eventing. Trained with increased intensity 1–2 times per week.*
****High: horses competing in trot or gallop. Trained with increased intensity more than 2 times per week.*

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