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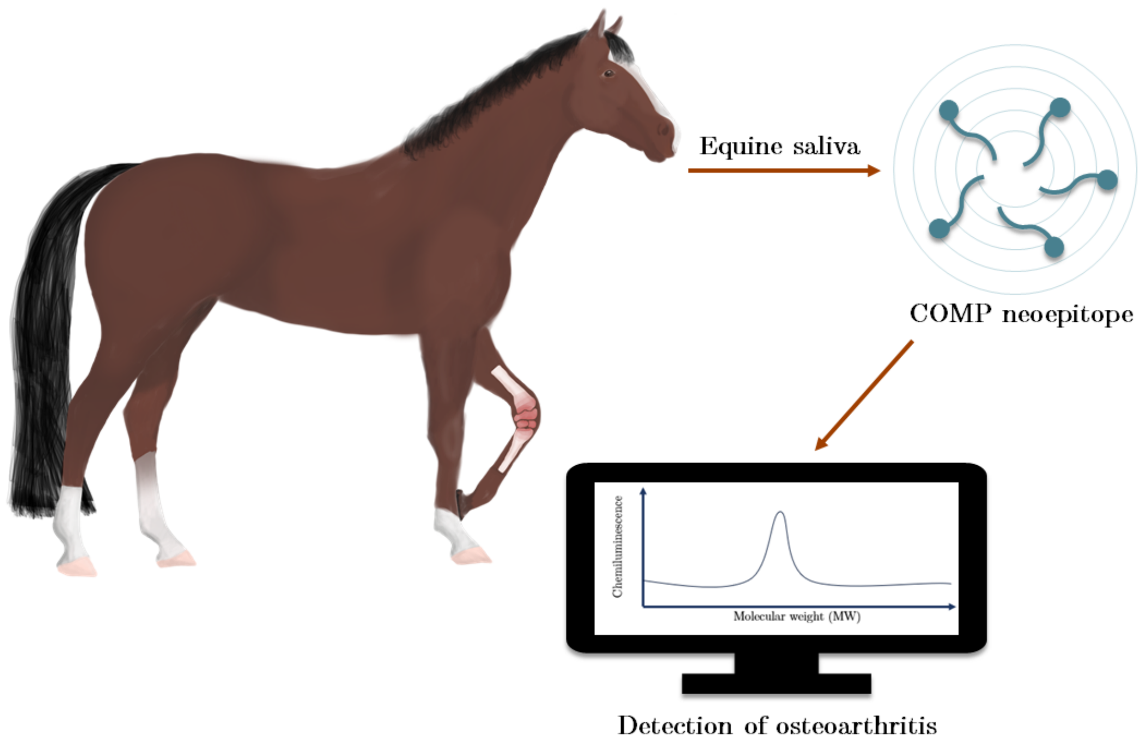

Detection and quantification of COMP neoepitope in equine saliva.

A biomarker for detection of early stages of Osteoarthritis

Master's thesis in Biotechnology

Moa Lord

Department of Physics
CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2021

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Master´s degree Program Biotechnology

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This thesis was performed as a collaboration between Chalmers University of technology, Sahlgrenska university hospital and Swedish University of Agricultural Sciences.

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Cover: Illustration of COMP neoepitope released during osteoarthritic inflammation of equine joint and detected in saliva, used for diagnosis of osteoarthritis in horses. Illustration by Moa Lord.

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Abstract

Osteoarthritis (OA) is a chronic inflammatory joint disease, with painful destructive processes locally in the joint. It is not possible to diagnose the early destructive biochemical events within the joint that leads to pain and subsequent lameness. Many horses with local joint pain due to early OA with a low-grade inflammation are used today in the equestrian industry. This disease develops over time and is usually not diagnosed until the end stages, where irreversible damage and clinical symptoms can be noticed. A biomarker for early matrix degradation of articular cartilage is needed as a diagnostic tool for early diagnosis of OA. A specific neoepitope of cartilage oligomeric matrix protein (COMP) have previously been identified and shown to increase in synovial fluid and serum from horses with acute lameness. The aim of this thesis was to detect and quantify the specific COMP neoepitope in saliva sampled from healthy horses and horses with OA. This aim included evaluation of sample collection and preparation, sample collection throughout the day and validation of the capillary western blot Wes™by inhibition ELISA. Salivette®, a specific collection device developed for salivary collection, was used to collect the saliva. Saliva sampled on the horse tongue, no food intake within one hour, obtained lower background noise, showed higher chemiluminescence signal and more defined peaks compared to saliva sampled close to food intake, when analysed with Wes™. In Wes™, the COMP neoepitope could be detected at the apparent molecular weight of 58 kDa. The concentration of the COMP neoepitope was determined with inhibition ELISA developed for quantification of COMP neoepitope in synovial fluid and serum from horses. The highest concentration of COMP neoepitope was quantified in a horse recently diagnosed with OA, compared to saliva from healthy horses. This indicates that saliva from horses can be used for detection and quantification of COMP neoepitope. However, this thesis included a limited amount of horses and they were not clinically examined by a veterinarian prior to saliva sampling. A larger group of well-defined healthy horses and horses with OA is required to draw statistical conclusions. Quantification of COMP neoepitope in saliva is a step toward the future goal of developing a point of care diagnostics, which can be an accessible tool for diagnosis of OA, monitoring the horse during rehabilitation and also be used in everyday training of athletic horses.

Keywords: Osteoarthritis, Biomarker, COMP neoepitope, Saliva, Wes™, Inhibition ELISA

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Moa Lord, Gothenburg, June 2021

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1 Introduction

Osteoarthritis (OA) is a chronic inflammatory joint disease, where its pathogenesis is described as slow and insidious [1]. OA progresses over time, including repetitive intense inflammation, degradation, decreased function, loss of articular cartilage and remodeling of the subchondral bone [1]. The value of athletic horses is closely related to their soundness, where excessive training and competition can initiate and accelerate the progression of OA, inducing pain and lameness which is a major cause of early retirement and lacking performance [1, 2]. The pathogenesis and progression of OA is similar in humans and horses, thereby research concerning related pain and development of OA in human joints directly benefit from progress made with research on the equine joints [1, 2]. OA in horses is mostly due to repetitive excessive mechanical load to the joint but can also occur after trauma, osteochondrosis or infection in the joint [2]. This disease has a slow progression and develops during months up to years and during the end stages joint deformity, decreased function, and mobility, can be observed [1, 2]. OA can be managed if mechanical stress on the joint is reduced by changing the training regimes, this will not cure the disease, but rather minimise the risk of further disease progression [1]. Therefore, an early diagnosis of OA is required to minimise both the destructive inflammation and the pain for the horse, since the trainer is able to change training regimens for the horse.

Detection of biomarkers in body fluids have for several years been considered as a diagnostic tool for early detection of OA, where a specific neoepitope of cartilage oligomeric protein (COMP) have shown to increase in synovial fluid and serum from horses with acute lameness [3, 4, 5]. However, collection of serum and synovial fluid is a rather complex and invasive process performed by qualified personnel, making this a complicated tool monitoring the disease in everyday work.

Instead saliva have been considered as a medium since its composition reflect the physiological state of the body and contain several proteins, also present in synovial fluid and serum [6, 7]. Salivary collection is non-invasive, non-stressful and can be performed by non trained personnel [6, 7, 8]. Biomarkers related to pain have previously been detected and quantified in saliva from humans and saliva has been used as detection medium in horses with equine acute abdominal disease [6, 8]. The long term goal is to quantify the specific COMP neoepitope in horse saliva with a validated method, which can be used in everyday training of athletic horses. An increase in the biomarker concentration in saliva could indicate a destructive event in the joint.

1.1 Aim

The overall aim of this project was to detect and quantify the specific COMP neoepitope in saliva sampled from healthy horses and horses with OA. This aim includes evaluation of sample collection and preparation, sample collection throughout the day and validation of the capillary western blot analysis Simple Western Wes™(ProteinSimple) by inhibition ELISA.

1.2 Limitations

The horse material available for this thesis was unevenly distributed between healthy horses and horses diagnosed with OA. The healthy horses were considered sound according to the owners assessment, which may not be consistent with a clinical examination performed by a veterinarian. Since the early stages of OA progresses without symptoms such as lameness, a horse considered healthy may have developed OA without noticeable symptoms.
This project was also performed during the pandemic of COVID-19, which included restrictions of personal present in the laboratory. The quantification of COMP neoepitope in saliva by inhibition ELISA presented in this thesis was therefore performed by Kristina Björkman.

2 Background

The following sections provides background information necessary for further understanding of: the anatomy of synovial joint, osteoarthritis and its progression of stages, biomarkers used as a detection molecule for diseases and the specific COMP neoepitope, how saliva have previously been used as a medium in a diagnostic method and how the capillary western blot analysis Simple Western operates.

2.1 Anatomy of the synovial joint

The synovial joint, also known as the diarthrodial joint, enables smooth movement between the adjoining bone ends, which is achieved when its component has a normal anatomy and functions on the cellular level [1, 9]. An illustration of the synovial joint can be seen in Figure 2.1. The synovial joint is considered an organ and is composed of the fibrous joint capsule, articular cartilage, synovial membrane, synovial fluid and subchondral bone [1, 9, 10]. The fibrous joint capsule is a stiff fibrous tissue composed of collagen type I, elastin fibers and proteoglycans (PG), providing the synovial joint with structural support and stability [1, 9].

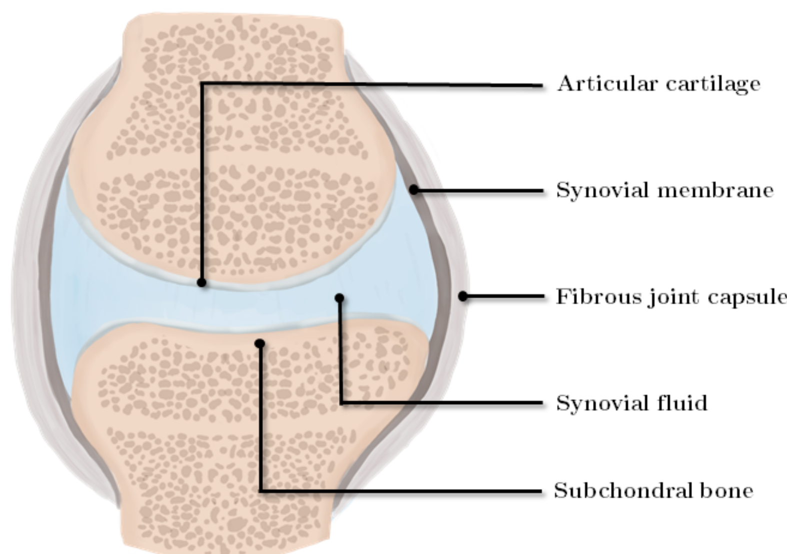

Figure 2.1: A cross section of the synovial joint, showing the main components. Illustration by Moa Lord.

The synovial membrane encapsulates and regulates the composition of the synovial fluid, which is highly viscous and transports nutrients to the articular cartilage, together enabling shock adsorption and a nearly frictionless motion of the joint [1, 2, 9]. The articular cartilage is aneural and avascular hyaline cartilage covering the epiphysis of the subchondrial

bone, which provides the shape and stability of cartilage [2, 9]. The articular cartilage is composed of chondrocytes and the extra cellular matrix (ECM), which includes collagen type II, collagen type I, PG, glycoproteins and water [9, 11]. Glycoproteins are different growth factors, COMP, proteinases, minerals, lipids and miscellaneous substances [9]. The chondrocytes of the articular cartilage is responsible for the synthesis of ECM and degradation by enzymes, described as matrix metabolism [2, 11].

The biochemical characteristics of the articular cartilage been shown to differ between horses of different ages [2]. The articular cartilage of horses less than 2 years of age had higher cell numbers, total collagen, DNA content and lower PG content compared to mature horses of 2 to 20 years of age [2]. A certain amount of mechanical load on the articular cartilage is necessary to sustain a functional adaptation which occurs during 5 months postpartum [2]. The mature normal articular cartilage of an adult horse has limited healing capacity and slow turnover of the matrix metabolism [2, 9]. The state of cartilage can be used for defining the progression of joint diseases [9].

2.2 Osteoarthritis

Osteoarthritis (OA) is a chronic inflammatory joint disease describing the degradation of the articular cartilage's ECM and later in the disease progression involves the majority of all tissues in the joint [9]. An illustration of an osteoarthritic joint can be seen in Figure 2.2. In a healthy joint, a dynamic equilibrium is maintained between the synthesis and degradation of the components of the articular cartilage [11].

When an inflammation starts in the tissue, an increase in both synthesis and degradation of tissue takes place, which eventually shift and the catabolic pathways become more predominant and an overall degradation of cartilage begins [9, 11]. Inflammation of the tissue is the hallmark of the disease [9, 11]. In most cases during pathogenesis of OA, the contents of the synovial fluid change and the fluid becomes less viscous, due to degradation by inflammatory enzymes of the high molecular weight hyaluronic acid present in the synovial fluid [9]. The pathogenesis of OA is affected by both metabolic turnover, the disruption of equilibrium, and increased abnormal mechanical load [9]. Abnormal mechanical loads overwhelm the normal repair process, causing microdamages and initiates inflammation, which accumulate over time and eventually lead to degeneration of tissue after failed reparation [9]. A metabolic turnover, will also cause proliferation of chondrocytes, exaggerating production of inflammatory cytokines and matrix-degrading enzymes, which degrade the components of ECM including collagen, aggrecan and COMP into fragments [2, 11]. These matrix-degrading enzymes, specifically proteinases, has shown to have increased activity in equine osteoarthritic joints [2]. The damage of articular cartilage is repaired by fibrocartilage containing collagen type I, consequently leading to the repaired cartilage not having the same biomechanical qualities as the original tissue, which is mainly

composed of collagen type II [1]. Production of inflammatory cytokines, dysregulation of chondrocytes and degradation of ECM causes intense inflammatory activity, subsequent pain and physical disability such as lameness in the horse [1, 11]. The osteoarthritic joint have entered a vicious cycle of relapsing inflammation, where the disease is between bouts largely asymptomatic and the joint can function normally until the inflammation relapses and the ECM is continuously degraded [1]. In an inflammation of the joint, the cells in the synovial membrane can contribute by producing inflammatory mediators, which makes it a central role in degenerative diseases such as OA [1, 9].

The progression of OA can be managed if mechanical stress on the joint is reduced, however this will not cure the disease, but rather minimise the risk of further development [1]. Early diagnosis would therefore prevent degenerative inflammation and minimise the pain of the horse.

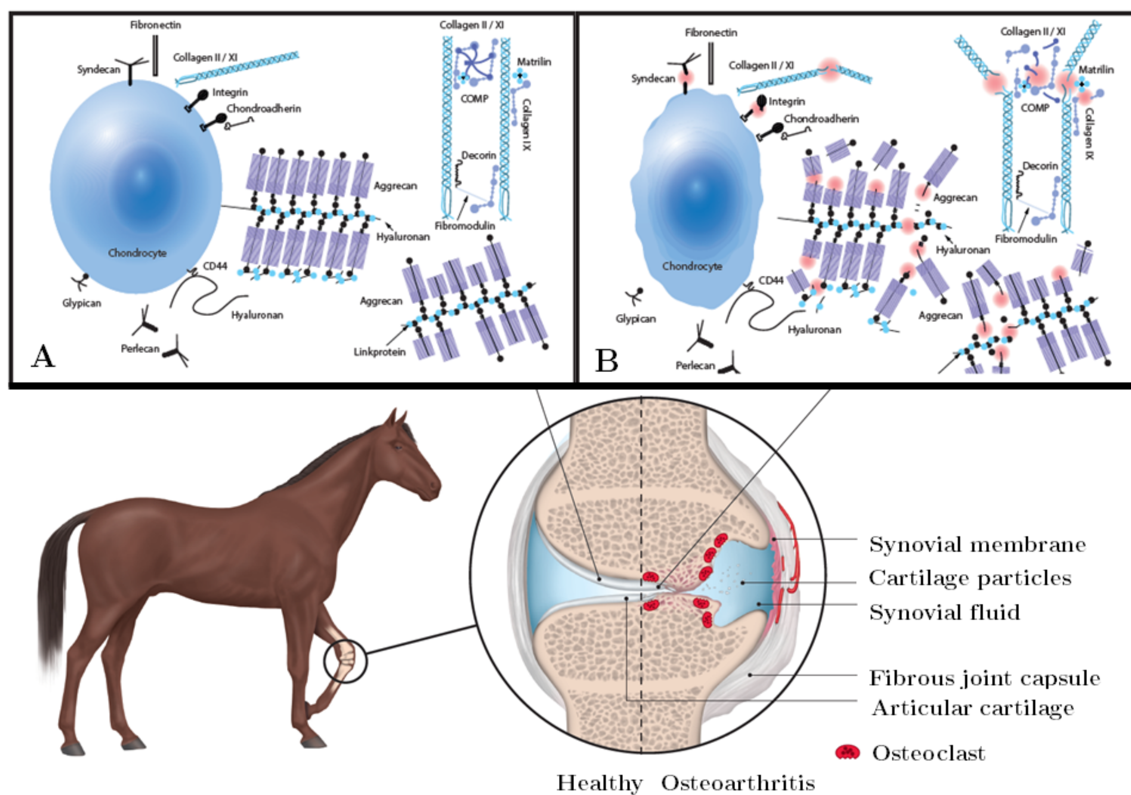

Figure 2.2: A comparison between normal/healthy joint and a joint with structural changes, cartilage destruction and remodeling of the subchondral bone resulting from OA. (A) the composition of normal articular cartilage. (B) showing the destructive process the ECM and degraded components, which leaks into the surrounding synovial fluid. Illustration by Pontus Andersson, modified by Moa Lord.

In the early stages of OA, erosion of the articular cartilage occurs without noticeable symptoms since the cartilage is both aneural and avascular [1]. Over time, the structural changes of articular cartilage become severe and involving the subchondral bone, which changes shape and eventually causes sclerosis and osteophytes [2]. In these end stages of OA, the horse has developed irreversible structural changes in the joint and clinical symptoms as lameness, bone damage and pain can be observed [1, 3]. Once the irreversible

structural changes have been developed, there are limited possibilities to heal or stop the further progression of the disease and the treatment options are mainly palliative [3]. OA developed into the later stages can be diagnosed with radiology, computed tomography, magnetic resonance imaging and ultrasonography. Radiology is used as a standard tool, however the diagnostic tools differs between veterinarian clinics [9]. However the clinical signs for both humans and horses are not representing the severity of the disease, radiology of the joint does not show the destructive cartilage and structures of soft tissues which are usually responsible for the pain [9]. Identification of biomarkers reflecting the fragmentation of ECM components have for several years been wanted since an early diagnosis can help to identify new pharmacological substances that can inhibit or slow down the progression of disease [3].

2.3 Biomarkers and specific COMP neoepitope

Biomarkers are proteins or other molecules that can be detected in body fluids, such as saliva, synovial fluid or serum. Specific proteins can be associated to different diseases and their presence in body fluids can be used as a diagnostic tool. Different prognostic factors have been evaluated as potential biomarkers for OA and in which bodily fluids these proteins can be detected [3].

The cartilage oligomeric protein (COMP) is a large glycoprotein complex that is normally found within the cartilage, binding in to collagen type II network and thereby cross binding fibers [3]. COMP is a calcium binding protein, also known as thrombospondin 5, composed of five identical subunits linked at their N-terminal via a coiled coil domain forming a pentamer with the molecular weight of 524 kDa [3, 12]. Each identical subunit has the molecular weight of 100 kDa [13]. The synthesis of native COMP and thereby its concentration in synovial fluid decreases in horses with repetitive high load on their joints during a long-term strenuous training programme of young horses [14].

The low-grade inflammation resulting from OA, releases proteinases which degrades COMP together with the other ECM components in an osteoarthritic joint, forming fragments, neoepitopes, with different cleaving sites creating new N- and C-terminal ends [4, 12]. These COMP neoepitopes, together with the intact pentamer, are released into the synovial fluid, as the articular cartilage undergoes remodeling and repair [4, 12, 15]. These specific neoepitopes of COMP have shown potential as biomarkers for cartilage breakdown [4, 12]. A specific COMP neoepitope (SGPTHEGVG) has been identified in serum and synovial fluid, where it had increased concentration in horses with acute lameness compared to healthy horses, and in the later case also compared to horses with the diagnosis of chronic lameness or structural OA [4, 5]. The COMP neoepitope concentrations detected in serum were not influenced by time of day, short-term exercise or age [5]. The increased concentration of COMP neoepitope in horses with acute lameness suggests that

this specific neoepitope is a suitable biomarker for the early stages of matrix degradation within the early progression of OA, indicating an ongoing inflammation of the joint [4, 5]. In horses with chronic or structural OA, the articular cartilage have been degraded and inflamed for a longer period of time and the specific neoepitope might have been degraded further [4].
This COMP neoepitope has the theoretical molecular weight of 52 kDa or 66 kDa, if a second further cleavage have occurred or not, based on the obtained amino acid sequence. In vivo, the molecular weight of proteins is influenced by post-translational modifications such as glycosylation, phosphorylation and further proteolysis [16]. The specific COMP neoepitope has been detected around 58 kDa in children's urine (un-published data) and serum and synovial fluid from horses (un-published data) with Simple Western Wes™(ProteinSimple).

2.4 Saliva as medium for detection of biomarkers

Serum, synovial fluid and urine are bodily fluids which has been used as medium for diagnostic monitoring of different diseases. In general, the collection of these bodily fluids is rather complex. Instead saliva, which have been shown to reflect the physiological state of the body and be an equivalent to serum, has been regarded as a substitute for diagnostic monitoring and detection of different diseases [6, 7]. The use of saliva as a medium has several advantages, including relatively easy and non-stressful collection procedure for both animals and humans [6, 7, 8].
Saliva is not a homogeneous fluid, but rather a mixture of secretion fluids from the salivary glands and its composition can vary between individuals, which makes standardisation of saliva collection, storage and analysis extremely important where the consistency is more important than the chosen method itself [6, 17]. Saliva is mainly composed of water together with other compounds such as phosphorus, glucose, fatty acids, enzymes, hormones, where its composition can be affected by different disorders [6, 8, 18].
Saliva contains a large number of different peptides and proteins, some of which only exists within saliva, while other proteins have their origin from synovial fluid or blood [6, 7, 8]. The clinical value of saliva as a diagnostic tool can be evaluated when a correlation of the biomarker concentration can be established between different bodily fluids, and provide reproducible measurements [7].
Biomarkers related to pain, such as nerve growth factor (NGF), substance P, glutamate and calcitonin-gene related peptide (CGRP), have been found and quantified in saliva from humans [6]. The proteins albumin and Immunoglobulin G (IgG) are two proteins which exist in both saliva and other bodily fluids, but in lower amount in saliva compared to blood plasma [7]. Albumin is highly abundant and can interfere with the analysis or conceal proteins which can be used as biomarkers [19]. The concentrations of some

biomarkers are usually low in saliva compared to the levels in other body fluid, therefore the analysis method may have to be adjusted prior analysis of saliva [17].
In horses, saliva is secreted as a result from the mechanical effect of chewing and the salivary flow stopped shortly after discontinued mastication [20]. The presence of food in saliva may interfere with the chosen analysis method and should therefore ideally be avoided [21]. Usually horses eat around 16-20h/ day, the presence of food in saliva is hard to avoid, even if the hour of feeding is easier to control in the modern equestrian industry [21, 22]. The knowledge regarding circadian and circannual impact on salivary biomarkers is lacking, together with the influence from physiological factors such as breed, gender, age, body condition score, or fitness [23]. Daily and seasonal variations of salivary biomarkers have been observed, however COMP neoepitope was not included in this study [23].

2.5 Capillary western blot analysis Simple Western

The Simple Western system Wes™(ProteinSimple) is a capillary western blot technology allowing protein detection without usage of gels [24]. Wes™is a modern, modified and automatised version of the common traditional technique western blot, which is used to detect specific proteins from a complex mixture e.g. in body fluids. Traditional western blot has several well-known disadvantages. It is a time consuming analysis, based on several manual steps, reduced sensitivity and has insufficient reproducibility [25].
In Figure 2.3 the detection of a target protein with Wes™can be viewed as a schematic. Wes™has automatised all steps such as protein loading, separation, immunoprobing, washing and detection, which minimise the negative impact manual factors may have on the analysis and results are received within 5 hours [24, 25, 26].
The separated target protein is immobilised on the capillary wall before being immunoprobed with the primary antibody, which is visualised by a horseradish peroxidase-conjugated secondary antibody, referred as secondary conjugate. The sample together with antibodies and necessary reagents are added to the prefilled assay-plate and inserted into Wes™, where the target protein is automatically separated based on size. Biomarkers in human saliva have preciously been detected and analysed with Wes™and results suggests that this type of analysis is likely to positively impact the discovery of biomarkers [27]. The analysis requires a small volume of antibodies and samples, has a high specificity and a high number of samples can be assessed in a short period of time [25, 27].

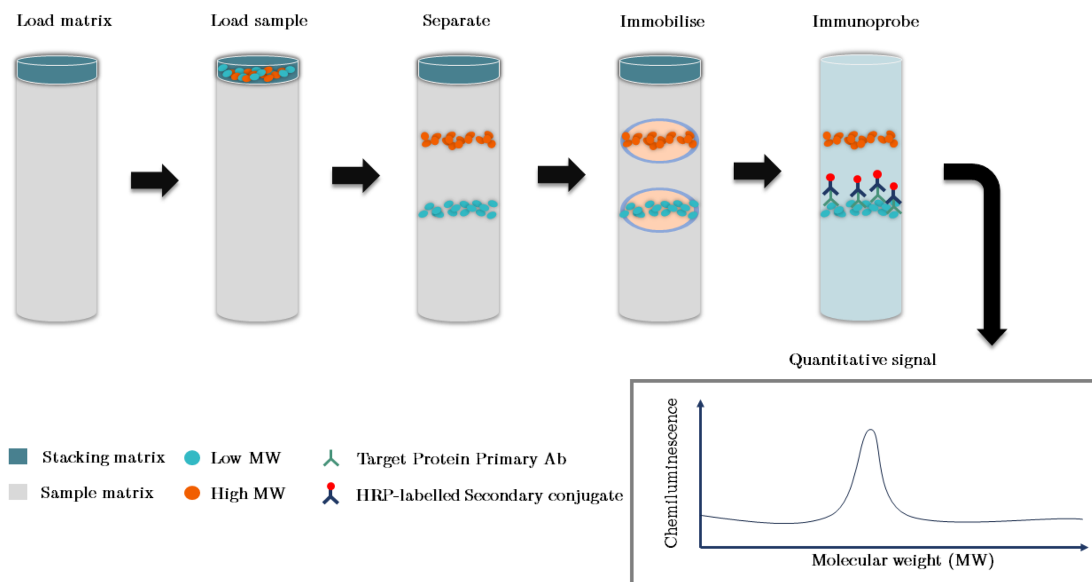

Figure 2.3: The automated procedure of Wes™. The stacking matrix and sample matrix is loaded into the capillaries followed by the sample proteins. The sample proteins are separated by charge or size before immobilisation by UV light on the capillary walls. The stacking- and separation matrix is extruded from the capillary and the target proteins are probed with the primary antibody followed by the secondary conjugate. Chemiluminescence substrate is added and the obtained signal is quantified. Illustration made by Moa Lord.

3 Materials and Methods

In this chapter, the following sections provides information regarding the horse material, salivary collection with two different techniques, determination of total protein concentration of saliva samples, detection of COMP neoepitope with Wes™and quantification of COMP neoepitope with inhibition ELISA (polyclonal neoepitope ELISA).

3.1 Horse material

Saliva was collected from both healthy horses and horses diagnosed with OA. The healthy horses were considered sound according to the owners assessment. The anamnesis, injury history, of interest, age, gender, breed and discipline were noted for each horse. The information regarding clinical diagnosis of OA was provided by the horse owner. OA was diagnosed by a veterinarian with no connection to this thesis. The severity of OA or radiology of the joint was not provided and therefore not included in this project. No lameness examination was performed prior to collection of saliva of horses. Saliva was collected with two different sampling techniques. Sampling technique 1, where saliva was collected after massaging the buccinator muscle for 20 minutes. Sampling technique 2, where saliva was collected with salivette ®.

3.2 Salivary sampling after massaging the Buccinator muscle

Salivary sampling after massaging the Buccinator muscle is referred to as sampling technique 1. Sampling of saliva with sampling technique 1 occurred between May 2020 and October 2020. A bit was placed in the mouth of the horse and was softly wiggled together with massaging and scratching the buccinator muscle, seen in Figure 3.1, for 20 minutes in order for the horse to masticate and produce saliva.

Figure 3.1: The a) buccinator muscle, b) placing of the bit and c) the lips where the saliva were sampled with sampling technique 1 are shown. Picture from Moa Lord private collection.

An eppendorf® tube was dragged gently along the lips, saliva was collected on both sides of the horse's head. The collection was continued until a volume of at least 500 µL saliva was collected. The sample was temporarily refrigerated and transported to Sahlgrenska University hospital where the samples

were freezed at arrival. The samples were thawed and centrifuged at 4 °C 15000g for 5min where the supernatants were collected and stored at -20°C until analysis.

3.3 Salivary sampling with Salivette®

Salivary sampling with Salivette® is referred to as sampling technique 2. Collection of saliva with sampling technique 2 occurred in February 2021. Saliva was collected by introducing the small cotton swab from Salivette® inside of the cheek on the horse's tongue before the premolar teeth for 30 s, allowing the swab to be soaked with saliva, as seen in Figure 3.2. The cotton swab was then placed in the collection device (Salivette®). Three different cotton swabs were used for each horse, one placed on the tongue, one placed on the left side inside of the cheek and one placed on the right side inside of the cheek. The samples were refrigerated immediately after collection and transported to Sahlgrenska University hospital where the samples were centrifuged at 3000g for 10 min, where 400-1500 µL of saliva could be obtained. The centrifuged saliva was transferred to eppendorf® tubes and stored at -20°C until analysis. Salivette have previously been used for saliva collection in horses [8, 28].

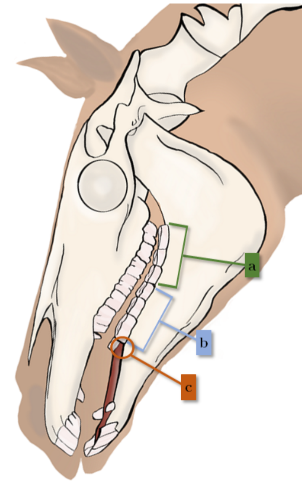

Figure 3.2: Showing the position of a) molar teeth, b) premolar teeth and position where c) the cotton swab was held inside the cheek on both sides when saliva were sampled with sampling technique 2. Illustration by Moa Lord.

3.3.1 Salivary sampling with Salivette® throughout the day

Saliva was sampled from one horse with salivette ® throughout the day where the point of time of collection were noted together with information if the horse had been fed prior to collection. The samples were collected in the morning 08:30 AM, midday 11:30 AM and evening 07:50 PM. The horse had no food intake within one hour prior to collection in the morning and evening, but had fed within one hour prior to collection in the midday. The horse was considered sound according to the owner assessment. The anamnesis of interest, age, gender, breed and training/competition discipline were noted.

3.4 Determination of total protein concentration in horse saliva

The total protein concentration of collected saliva samples were determined with Pierce™BCA Protein Assay Kit. This kit was purchased from Thermo Scientific™and kit instructions

were followed and bovine serum albumin was used as a standard. Absorbance was measured at 560 nm by the absorbance microplate reader Infinite® F50 with Magellan™tracker software from Tecan.

3.5 Detection of COMP neoepitope with Wes™

Equine saliva samples were diluted to a specific concentration in 0.1x sample buffer (ProteinSimple) together with appropriate amount of Fluorescent Master Mix (FMM) (ProteinSimple) to obtain the final concentrations of 1.49 mg/mL, 1.88 mg/mL, 2.0 mg/mL or the specific dilution series required for analysis. FMM is used as a control for protein migration within each capillary, which can be seen in Figure 3.3.

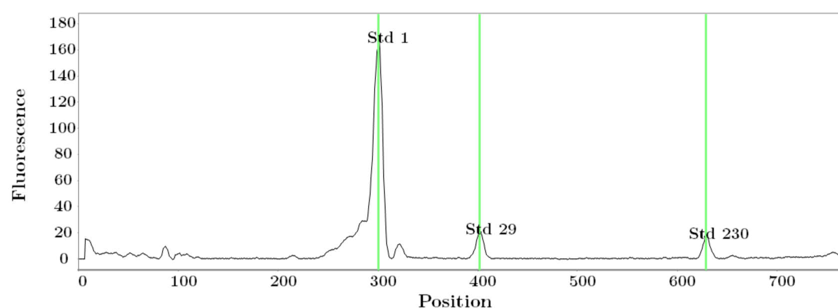

Figure 3.3: Fluorescent labeled proteins used as control for protein migration within capillaries displayed in an electropherogram with the registered fluorescence signals as a function of the positions of each internal standard.

The sample dilutions and the biotinylated 12-230 kDa ladder (ProteinSimple), seen in Figure 3.4, were vortexed and heated at 95 °C for 5 min. After incubation the sample dilutions and biotinylated ladder were vortexed, centrifuged and vortexed again, and stored on ice until loading into the Wes™prefilled assay-plate. The samples were run with 12-230 kDa assay capillaries, using default parameters with increased stacking matrix load time (21 s) and sample load time (12.6 s).

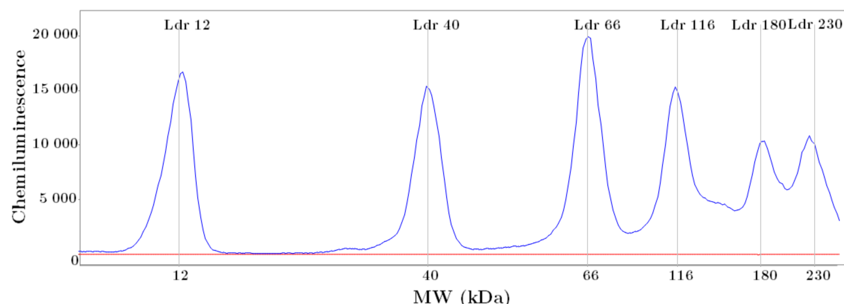

Figure 3.4: The biotinylated ladder 12-230kDa (ProteinSimple), displayed in graph view, included in each Wes™-analysis. The electropherogram display the chemiluminescence units, the molecular weight (MW) and the shape with assigned reference peaks.

The primary antibodies used were monoclonal anti-COMP neoepitope antibody, referred to as COMP neoepitope mAb, diluted 1:25 in antibody diluent (ProteinSimple) and

polyclonal anti-Albumin antibody (ABIN2776970, antibodies-online), referred to as Anti-albumin pAb, diluted 1:2000 in antibody diluent (ProteinSimple) [29]. Anti-albumin pAb was used as a control for the position of albumin related to COMP neoepitope. The theoretical molecular weight of albumin detected with this antibody is 67 kDa [29]. The primary antibody was visualised using the anti-rabbit horseradish peroxidase-conjugated secondary antibody (ProteinSimple), referred to as secondary conjugate and chemiluminescent substrate, luminol-peroxide mixture, (ProteinSimple) is added multiple times. A charge-coupled device (CCD) camera captures the emission of light, the chemiluminescence signal is quantified and viewed in an electropherogram generated by Compass for Simple Western Software v.5.0.1 (ProteinSimple), showing the amount of target protein bound to primary antibody as a function of their apparent molecular weight. The antibodies bind to an internal marker protein in Wes™-system, resulting in a non-specific peak at $\sim$ 230 kDa. This peak has been detected in other studies, visualised in presented electropherograms as internal marker (I.M) [27]. The peak area can be used for quantification of signal related to the target protein, if the measurements have been performed within the linear range of the assay. The primary antibody dilution has to be optimised to ensure that changes in peak area are proportional to changes in protein concentration [26]. For possible quantification, the primary antibody should be used at saturated concentration [26].

3.6 Quantification COMP neoepitope with polyclonal neoepitope ELISA

A custom made inhibition ELISA was used to measure the concentration of the COMP neoepitope in saliva. The specific inhibition ELISA has previously been developed and used for quantification of COMP neoepitope in synovial fluid and serum [4, 5].
A NUNC plate was coated with 4.0 µg/mL peptide (sequence SGPTHEGVC) diluted in 100 mM carbonate buffer (pH 9.6), and incubated at 4 °C overnight. A serial dilution of 5.0 µg/mL peptide (sequence SGPTHEGVGMA) in 10 mM phosphate buffered saline (PBS) with 0.6% BSA and 0.8% SDS was used as a calibration curve (range=0.078-5 µg/mL). The saliva samples were analysed as single sample except for horse 4 which had two samples included, this due to low sample volume. The samples were analysed in different dilutions were the samples were diluted 1:2 in PBS with 1.6 % SDS ; diluted 1:4, 1:8 and 1:16 in PBS with 0.8 % SDS and to undiluted samples were added 5 µL 20 % SDS to 115 µL original sample. Duplicates of standard and single samples of saliva were incubated in 96-well Sterilin plate at 25°C overnight. On the second day, primary polyclonal anti-COMP neoepitope antibody (diluted in PBS with 1% BSA and 4% Triton-X-100) was added to the plates and incubated 1 h 20 min at 25 °C on a shaker at 600 rpm. The NUNC plate was washed and blocked with PBS with 0.05 % Tween (pH 7.4) and 1 % BSA for 1 h at

25 °C. A total of 100 µL was transferred from the Sterlin plate to the NUNC plate and incubated for 1 h at 25 °C on a shaker at 600 rpm. After incubation, the NUNC plate was washed and the secondary antibody was added (Goat Anti-Rabbit IgG H&L [HRP] [ab97051]), diluted 1:20 000 in PBS with 1% BSA and 0.1 % Tween was added. The plate was incubated for 1 h at 25 °C on a shaker at 600 rpm and then washed 6 times and incubated with substrate (Substrate Reagent Pack (DY999), R&D systems) for 10 min at 25°C protected from light. Stop solution (1 M H_2SO_4) was added and the absorbance was measured at 450 nm. The specificity of the assay and primary polyclonal antibody for the COMP neoepitope has previously been evaluated through spiked synovial fluid sample with different concentrations of the overlapping peptide, where the antibody was tested using a serial dilution of the overlapping peptide or neoepitope peptide, together with coating the ELISA plate with peptide overlapping the neoepitope (sequence PPGYSGPTHEGVGMC) [4].

3.7 Data analysis

There was a limited number of horses included in the study and the saliva samples obtained were unevenly distributed between horses. The soundness of horses were provided and determined by their owner. The horses with OA had been diagnosed by a veterinarian of the owner's choice, where the information about the diagnosis was provided from the owner. Information regarding the severity of OA was not provided and thereby not taken into consideration. Because of the limited sample group no statistical analysis could be made. However, age of the horses and values regarding concentrations obtained by protein determination or by inhibition ELISA, are presented as means and standard deviations (SD) in descriptive purposes. Mean-values and SD, were generated in Microsoft Excel v.2104. The electropherograms were processed and analysed using high dynamic range 4.0. in Compass for Simple Western software v.5.0.1 (ProteinSimple). The obtained peak with apparent molecular weight around 58 kDa recognised with COMP neoepitope mAb and the peak with apparent molecular weight around 64 kDa recognised with anti-albumin pAb, were analysed further, if their internal standards were in accordance over capillaries. These peaks were analysed in terms of their apparent molecular weight, position of the peak recognised with Anti-albumin pAb, peak area, signal-to-noise (S/N)-ratio >10 and background noise visualised as baseline as a percentage of peak height and that the baseline-height (B/H)- ratio is less than 20%. The B/H-ratio is used for optimisation of the assay, a too high baseline can reduce the dynamic range, therefore peaks with B/H-ratio above 20 % are used with caution. For optimisation of assay, the obtained peak area was shown as a function of the concentration of sample or dilution of antibody in terms of linearity of assay or saturation of antibodies. These graphs were processed in Compass for Simple Western software v.5.0.1 (ProteinSimple).

4 Results

In this chapter, the results from the thesis mainly in form of tables and figures, as well as the information required to interpret them are presented. The results are divided into sections, separated for further understanding. The interpretations and discussions of the presented information will be addressed in the following chapter.

4.1 Horse material

Saliva was collected from seven healthy horses with a mean age 10 $\pm$ 9.93 years and from three horses with OA with a mean age 13.67 $\pm$ 5.03 years. In Table 4.1 the collected information about the horses have been summarised. The breed of horses differed, but 4 out of 10 horses included were Swedish warmblood horses.

Table 4.1: The age, gender, breed, discipline and diagnosis of the horses included for detection and quantification of COMP neoepitope. The horse was either considered healthy according to their owner, or had confirmed OA-diagnosis from veterinarian of owners choice. Allround includes both easy level of dressage and showjumping, mostly ridden by children. Unbroken are horses not ridden due to young age.

Horse	Age (years)	Gender	Breed	Discipline	Diagnosis: **O**wner or **V**eterinarian
1	13	M	Oldenburger	Jumping	Healthy : O
2	9	M	Swedish Warmblood	Dressage	OA : V
3	19	G	Latvian Warmblood	Dressage	OA : V
4	4	G	New forest pony	Allround	Healthy : O
5	2	M	Swedish Warmblood	Unbroken	Healthy : O
6	1	M	Swedish Warmblood	Unbroken	Healthy : O
7	13	G	Oldenburger	Dressage/Jumping	OA : V
8	30	G	Swedish Warmblood	Eventing (retired)	Healthy : O
9	11	G	Dutch Warmblood	Jumping	Healthy : O
10	9	G	Dutch Warmblood	Jumping	Healthy : O

A total of seven horses were sampled with sampling technique 1 and five horses were sampled with sampling technique 2. Horse 1 and horse 4, were sampled with both techniques. Samples throughout the day were collected from horse 1.

Additional information about the horses with OA was provided from the horse owner. Horse 2 was not diagnosed with OA when saliva was collected, but obtained a diagnosis a few months later. Horse 3 was lame when the samples were taken, but the horse had other diagnoses, including decalcified navicular bone in both front hoofs. This horse was later euthanised. Horse 7 was not lame when the saliva was collected however it has previous history of repeating joint inflammations and intra-articular treatments with corticosteroids.

4.2 Determination of total protein concentration in horse saliva

The total protein concentration of samples used in further analysis were determined and the concentration of each sample can be viewed in Table 4.2. The concentration range for all samples were 4.38 ± 2.56 mg/mL. The total protein concentration was used for determining the sample dilution for Wes™analysis for detection of COMP neoepitope.

Table 4.2: Sample annotation and determined total protein concentration (mg/mL), for samples collected from the different horses presented in Table 4.1. Horse 1, horse 2, horse 3, horse 4 and horse 6 have more than one sample included.

Sample	Concentration (mg/mL)	Sample	Concentration (mg/mL)	Sample	Concentration (mg/mL)
Horse 1.T.8:30AM	3.73	Horse 1.1	3.17	Horse 5	3.78
Horse 1.R.8:30AM	2.85	Horse 1.2	2.65	Horse 6.1	4.20
Horse 1.L.8:30AM	4.70	Horse 1.3	4.17	Horse 6.2	5.22
Horse 1.T.11:30AM	2.34	Horse 2.1	9.74	Horse 7	11.83
Horse 1.R.11:30AM	3.86	Horse 2.2	6.46	Horse 8	9.62
Horse 1.L.11:30AM	1.86	Horse 3.1	3.93	Horse 9	2.40
Horse 1.T.7:50PM	2.77	Horse 3.2	2.35	Horse 10	3.64
Horse 1.R.7:50PM	2.36	Horse 4.1	2.44		
Horse 1.L.7:50PM	3.59	Horse 4.2	5.87		

Horses which had only one sample included was annotated as horse x, where x is the horse number. Horse 1, horse 2, horse 3, horse 4 and horse 6 have more than one sample included. These were annotated by an additional description after the horses were noted e.g. horse 1.1 is sample 1 from horse 1. Samples annotated horse x.T.time, is the sample sampled from horse x with salivette throughout the day, on the tongue (T), left side inside of the cheek (L) or right side inside of the cheek (R) and the time when the sample was taken.

4.3 Simple Western Controls

The COMP neoepitope mAb have not previously been used for detection of COMP neoepitope in equine saliva with Wes™. Therefore, three different controls were performed where either sample, primary antibody or secondary conjugate was excluded from the analysis. Omitted sample shows unspecific binding of primary antibody and secondary conjugate. Omitted primary antibody shows unspecific binding of protein and secondary conjugate. Omitted secondary conjugate shows unspecific binding of protein and primary antibody. The detected chemiluminescence signal is shown as a function of the apparent molecular weight in the electropherograms viewed in Figure 4.1 and obtained data from analysis is summarised in Table 4.3.

As seen in Figure 4.1, no signal was observed when the protein (sample) or secondary conjugate were excluded form the analysis. An exclusion of the primary antibody resulted in observed signal with peaks positioned at the apparent molecular weight of 32, 61 and

Table 4.3: Obtained data for control where the primary antibody was excluded. Sample annotation, concentration of sample analysed, apparent molecular weight (MW), peak area, S/N-ratio and B/H-ratio are shown. Values obtained from the electropherogram generated in Compass for Simple western software.

Sample	Concentration (mg/mL)	MW (kDa)	Peak area	S/N	B/H (%)
Horse 2	2	32	2 571.1	103.1	3.5
Horse 2	2	56	1 168.4	44.2	3.1
Horse 2	2	61	3 954.9	76.6	1.4
Horse 2	2	86	1 375.8	98.6	1.6

86 kDa. The chemiluminescence signal is below 250 chemiluminescence arbitrary units (a.u.). The peak area for registered peaks, where the primary antibody were excluded, apparent molecular weight, their S/N-ratio and baseline as percentage of peak height, can be viewed in Table 4.3. The low chemiluminescence signal follows to relatively low peak area for all detected peaks, in which all had S/N-ratio above 10. The baseline is low relative to observed peak height, the baseline is 1.4-3.5 % of the peak height.

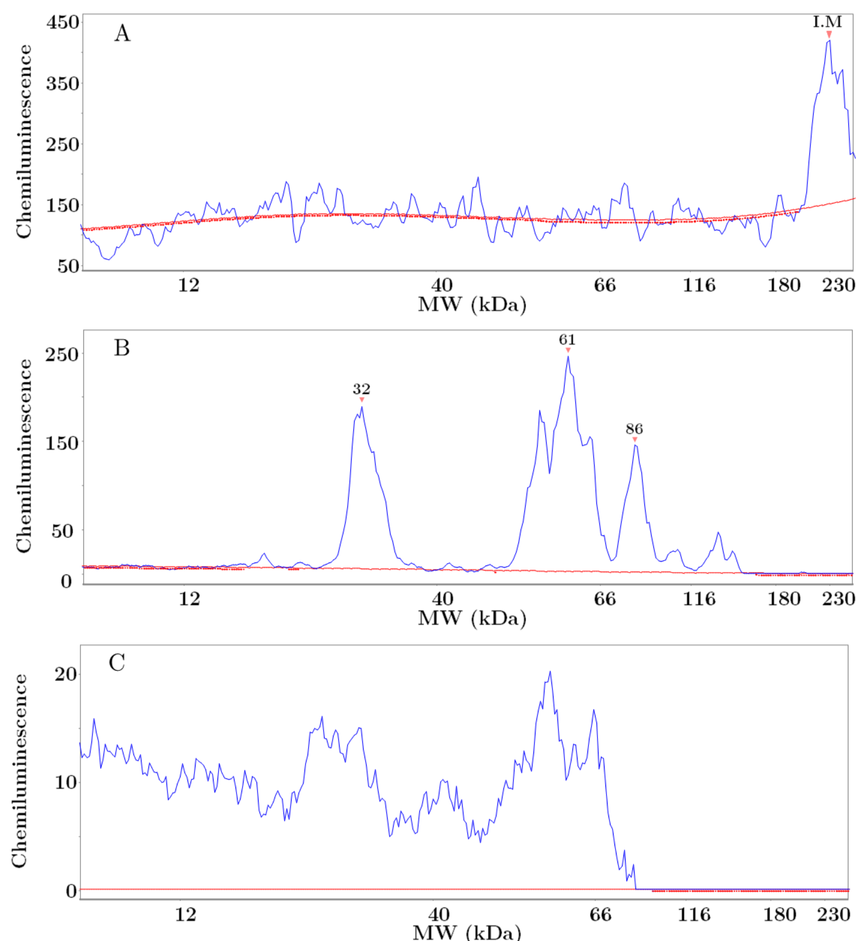

Figure 4.1: Electropherogram showing the chemiluminescence signal as a function of the apparent molecular weight (MW) for the different controls (A) excluded protein, (B) excluded primary antibody and (C) excluded secondary conjugate from analysis. The primary antibody used was COMP neoepitope mAb diluted 1:25. The sample used was collected from horse 2, analysed at a concentration of 2 mg/mL. Sample buffer was used instead of sample in (A) excluded protein. Antibody diluent was used as replacement of the excluded antibody in (B) and (C).

4.4 Optimisation of monoclonal COMP neoepitope antibody dilution and determination of linear range of analysis with Wes™

The optimal dilution of the primary antibody was evaluated through analysing the same concentration of sample with a series of dilutions in a titration of the COMP neoepitope mAb. The obtained peak area, seen in Table 4.4 together with the additional analysis data, for the registered peak for COMP neoepitope, was plotted against the dilution of antibody, viewed in Figure 4.2 A.

Table 4.4: Optimisation of COMP neoepitope mAb dilution. Showing the dilution of primary antibody (PAb) in antibody diluent (ProteinSimple), apparent molecular weight (MW), peak area, S/N-ratio and B/H-ratio. Saliva from horse 2 was analysed in 2 mg/mL and COMP neoepitope mAb was used. Values obtained from the electropherogram generated in Compass for Simple western software.

Dilution of PAb	MW (kDa)	Peak area	S/N	B/H (%)
1:5 (0.2)	59	886 314.2	313.3	4.7
1:10 (0.1)	59	376 172.4	244.4	5.9
1:20 (0.05)	59	248 041.6	424.2	2.9
1:40 (0.025)	59	73 842.3	272.5	4.9
1:80 (0.0125)	59	32 328.1	236.5	5.7
1:160 (0.00625)	59	19 977.8	224.5	3.3

As can be observed, no plateau was reached and the antibody was not saturated. The baseline was low relative to the observed peak height, the B/H-ratio was 2.9-5.9 %. However, as seen in Table 4.4, the highest S/N-ratio and lowest B/H-ratio was obtained when the antibody was diluted 1:20. The linear range of the assay was evaluated with the COMP neoepitope mAb diluted 1:25. The obtained peak area, seen in Table 4.5 together with the additional analysis data, for the registered peak for COMP neoepitope, were plotted against the concentration of sample, viewed in Figure 4.2 B.

Table 4.5: The linear range of the assay. Showing the concentration of sample analysed, apparent molecular weight (MW), peak area, S/N-ratio and B/H-ratio. Values obtained from the electropherogram generated in Compass for Simple western software. Saliva used was taken from horse 2. The COMP neoepitope mAb was diluted 1:25.

Concentration (mg/mL)	MW (kDa)	Peak area	S/N	B/H (%)
3.2	58	207 672.9	393	3.2
1.6	59	113 341.4	316.9	4.7
0.8	60	59 352.7	149.7	6.3
0.4	62	31 603.7	56.2	9.9
0.2	61	15 520.7	56.6	14.5
0.096	61	8 374.8	18.3	25.4

The obtained values for the linear assessment for the peak (58-62 kDa) representing COMP neoepitope can be viewed in Table 4.5. The B/H-ratio was 3.2-25.4 %. Based on the obtained values, linear range of the assay can be obtained within the interval of 0.2-3.2 mg/mL, with a R^2=0.998. The concentration 0.096 mg/mL were excluded since a B/H-

ratio above 20 % can reduce the high dynamic range of the assay.

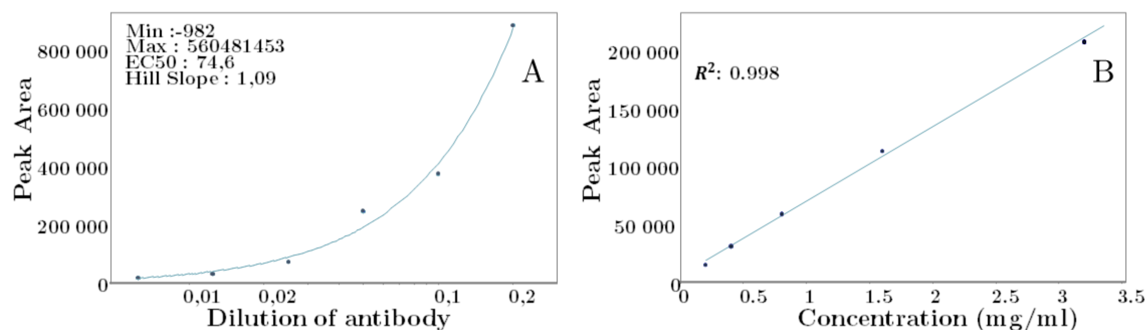

Figure 4.2: (A) Optimisation of COMP neoepitope mAb dilution (0.2, 0.1, 0.05, 0.025, 0.0125, 0.00625), peak area as function of antibody dilution. Four Parameter Logistic Regression was used. The minimum (MIN)- and maximum (MAX) -value; inflection point (EC50) and hill coefficient (Hill slope) is displayed in the graph. (B) Determination of linear range, peak area as a function of the concentration of sample (0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL) with COMP neoepitope mAb diluted 1:25. Linear regression was used. R^2 value is displayed in graph. The saliva used in (A) and (B) was taken from horse 2.

4.5 Comparison of salivary sampling techniques and samples throughout the day

The two sampling techniques used were compared through analysis with Wes ™. The peaks for COMP neoepitope and albumin were analysed and the peak area, apparent molecular weight, S/N-ratio and B/H-ratio, have been summarised in Table 4.6. The COMP neoepitope could be detected in all samples except in Horse 1.L.11:30AM, where only albumin was detected. The COMP neoepitope was detected with the apparent molecular weight of 57-62 kDa and albumin with the apparent molecular weight of 58-64 kDa. The peak area and apparent molecular weight for the different time points with sampling technique 2 were: morning 44 501.33 ± 8741.46 and 57.33 ± 0.58 kDa, midday 14 867.7 ± 2857.14 and 59 kDa, evening 38 556.5 ± 11 591.58 and 59.67 ± 1.15 kDa. Saliva sampled with sampling technique 1 had a peak area of 23 911.5 ± 11 803.99 and apparent molecular weight of 60.33 ± 1.53 kDa.
In Table 4.6 it is important to observe that 6 out of 11 samples with registered peak for COMP neoepitope had a B/H-ratio above 20 %. The highest obtained peak area and S/N-ratio for COMP neoepitope peak was obtained for the sample Horse 1.T.08:30AM, sampled with sampling technique 2 in the morning on the tongue. The electropherograms for the samples collected with sampling technique 1 and saliva collected in the morning with sampling technique 2, can be viewed in Figure 4.3.

Table 4.6: Sample, primary antibody, apparent molecular weight (MW), peak area, S/N-ratio and B/H-ratio. Values obtained from the electropherogram generated in Compass for Simple western software. The samples were analysed in the concentration of 1.49 mg/mL. The COMP neoepitope mAb was diluted 1:25 and anti-albumin pAb was diluted 1:2000.

Sample	Primary antibody	MW (kDa)	Peak area	S/N	B/H (%)
Horse 1.T. 08:30AM	COMP neoepitope mAb	57	51 815.5	110.9	12.4
Horse 1.T. 08:30AM	Albumin pAb	59	124 750.7	966.8	1.2
Horse 1.L. 08:30AM	COMP neoepitope mAb	57	46 868.4	84.9	12.2
Horse 1.L. 08:30AM	Albumin pAb	58	179 749.2	1 613.2	0.9
Horse 1.R. 08:30AM	COMP neoepitope mAb	58	34 820.1	50.8	22.1
Horse 1.R. 08:30AM	Albumin pAb	61	10 5681	947.3	1.3
Horse 1.T. 11:30AM	COMP neoepitope mAb	59	16 888	12	64.8
Horse 1.T. 11:30AM	Albumin pAb	63	115 461	1 341.7	1.0
Horse 1.L. 11:30AM	COMP neoepitope mAb	-	-	-	-
Horse 1.L. 11:30AM	Albumin pAb	64	55 321.1	636.6	2.5
Horse 1.R. 11:30AM	COMP neoepitope mAb	59	12 847.4	25.4	74.2
Horse 1.R. 11:30AM	Albumin pAb	63	91 352.4	1 040.6	1.4
Horse 1.T. 07:50PM	COMP neoepitope mAb	59	39 294.4	57.3	19.3
Horse 1.T. 07:50PM	Albumin pAb	62	296 531.2	3 073.1	0.4
Horse 1.L. 07:50PM	COMP neoepitope mAb	59	49 761.5	70.8	13.6
Horse 1.L. 07:50PM	Albumin pAb	61	383 481.9	4 416.8	0.4
Horse 1.R. 07:50PM	COMP neoepitope mAb	61	26 613.6	27.9	25.7
Horse 1.R. 07:50PM	Albumin pAb	63	131 621.4	1 250.8	1.1
Horse 1.1	COMP neoepitope mAb	59	37 541.2	66.7	18.0
Horse 1.1	Albumin pAb	62	321 200.8	2 952.3	0.3
Horse 1.2	COMP neoepitope mAb	62	17 009.2	17.5	44.2
Horse 1.2	Albumin pAb	64	112 451.8	1 251.4	1.1
Horse 1.3	COMP neoepitope mAb	60	17 184.1	12.5	52.1
Horse 1.3	Albumin pAb	64	100 441.7	1 068.1	1.1

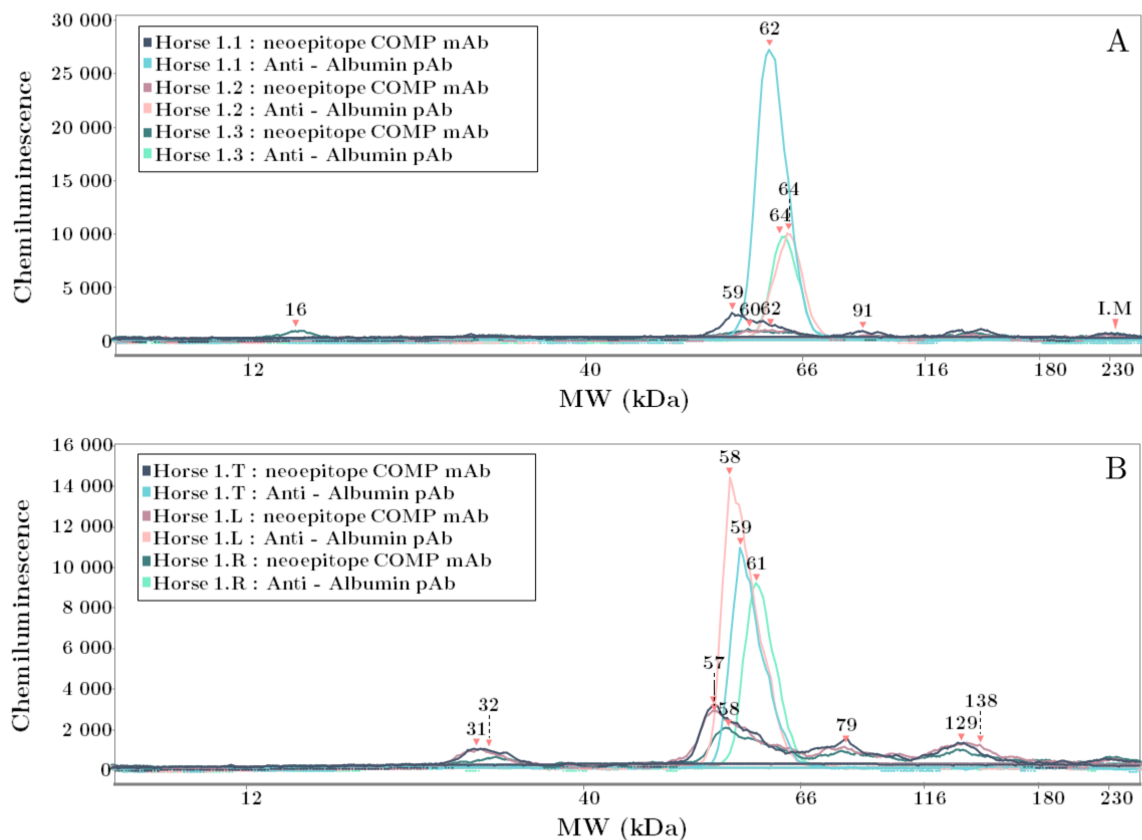

Figure 4.3: Electropherogram showing the chemiluminescence signal as a function of the apparent molecular weight (MW), for samples from horse 1 taken with two different sampling techniques. (A) These analysed samples were collected from horse 1 with sampling technique 1. (B) These analysed samples were collected morning 08:30 AM when the horse had no food intake within 1 h prior to salivary collection. These samples were collected with sampling technique 2. All samples were analysed at a concentration of 1.49 mg/mL with COMP neoepitope mAb diluted 1:25, together with anti-albumin pAb diluted 1:2000 used as a control. I.M shows the position of internal marker.

4.6 Detection of COMP neoepitope in horses with osteoarthritis

Three horses with OA mean age of 13.67 ± 5.03 years were analysed with Wes™, for detection of COMP neoepitope. A comparison was made with three healthy horses with a mean age of 5.33 ± 6.66 years. The analysis data from the electropherograms can be viewed in Table 4.7.

Table 4.7: Sample source, primary antibody, apparent molecular weight (MW), peak area, S/N-ratio and B/H-ratio. Values obtained from the electropherogram generated in Compass for Simple western software. The samples were analysed in the concentration of 1.49 mg/ml. The COMP neoepitope mAb was diluted 1:25 and anti-albumin pAb was diluted 1:2000.

Sample	Primary Antibody	MW (kDa)	Peak area	S/N	B/H (%)
Horse 2.2	COMP neoepitope mAb	57	324 205.2	245.8	6.5
Horse 2.2	Albumin pAb	61	640 147.7	4 355	0.2
Horse 3.1	COMP neoepitope mAb	58	110 807.3	59.2	20.4
Horse 3.1	Albumin pAb	62	184 200.7	2 778.6	0.4
Horse 7	COMP neoepitope mAb	60	83 036.7	41.5	21.3
Horse 7	Albumin pAb	66	189 237.4	2 644.2	0.3
Horse 1.T.08:30AM	COMP neoepitope mAb	57	51 815.5	110.9	12.4
Horse 1.T.08:30AM	Albumin pAb	59	124 750.7	966.8	1.2
Horse 5	COMP neoepitope mAb	-	-	-	-
Horse 5	Albumin pAb	64	24 678.3	918.8	0.6
Horse 6.1	COMP neoepitope mAb	57	44 002.4	25.2	59.6
Horse 6.1	Albumin pAb	63	117 067.2	2 270.7	0.5

In horse 2.2 the COMP neoepitope had the apparent molecular weight of 57 kDa, highest peak area, highest S/N-ratio and lowest B/H-ratio, compared to the other samples analysed. The COMP neoepitope could not be detected in horse 5. The average peak area and apparent molecular weight for COMP neoepitope in horses with OA were 172 683.07 ± 131 954.61 and 58.33 ± 1.53 kDa. The average peak area and apparent molecular weight for detected COMP neoepitope in healthy horses were 47 908.95 ± 5 524.70 and 57 kDa. The electropherogram for the horses with OA and horse 6, can be viewed in Figure 4.4. The apparent molecular weight of COMP neoepitope (57-60 kDa) and albumin (61-66 kDa) vary between samples. As can be observed in Figure 4.4 A the horses with OA, has more defined peaks compared to horse 6 seen in Figure 4.4 B, which was used as a healthy reference.

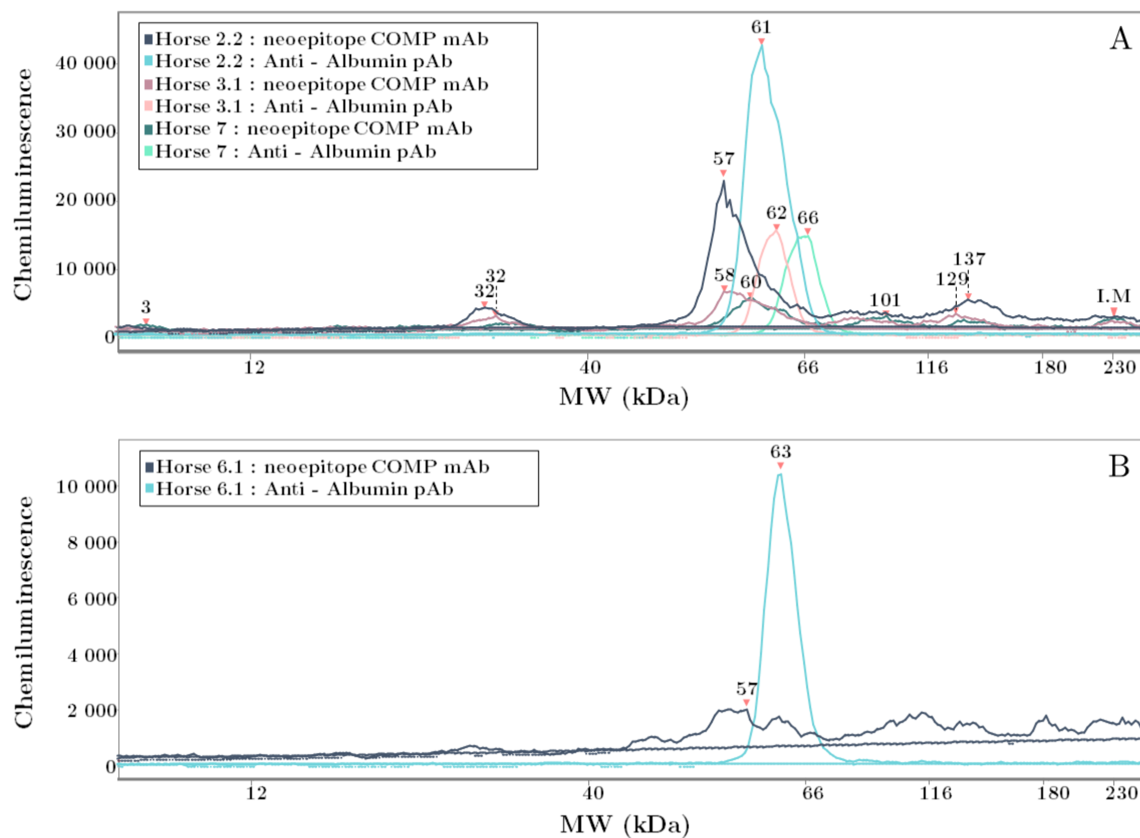

Figure 4.4: Electropherograms showing the chemiluminescence signal as a function of the apparent molecular weight (MW) for samples from (A) horse 2, horse 3, horse 7 (OA) and (B) horse 6 (healthy). All samples were analysed at a concentration of 1.49 mg/mL with COMP neoepitope mAb diluted 1:25, together with anti-albumin pAb diluted 1:2000 used as control. I.M shows the position of internal marker.

4.7 Quantification and detection of COMP neoepitope in equine saliva

A selected group of horses were analysed with Wes™and polyclonal neoepitope ELISA. A total of six healthy horses with the mean age of 9.83 ± 10.87 years and two horses with OA with the mean age of 14 ± 7.07 years, were included.

In polyclonal neoepitope ELISA, samples were analysed in a dilution series of non-diluted, 1:2, 1:4, 1:8 and 1:16-dilution. However, the samples from horse 8, horse 5 and Horse 6.2 were analysed in 1:2, 1:4 and 1:8-dilution, while horse 4.2 was only analysed in 1:2 and 1:4-dilution. The concentration of COMP neoepitope was obtained and corrected for each dilution. The corrected concentrations were used for calculating a mean value and corresponding SD, which can be viewed in Table 4.8. However, not all obtained concentrations were within the linear range of the assay and an additional average was calculated based on concentrations within the linear range. These can also be seen in Table 4.8, where n is the number of dilutions obtaining a measured concentration and n* is the number of dilutions within the linear range.

Table 4.8: The concentration of COMP neoepitope quantified with inhibition ELISA. n is the number of dilutions which obtained a concentration, presented as a mean-value and standard deviation (SD). n*, is the number of dilutions which obtained a concentration within the linear range, presented as mean* and SD*.

	Horse 1.3	Horse 2.1	Horse 3.2	Horse 4.1	Horse 4.2	Horse 5	Horse 6.2	Horse 8	Horse 9	Horse 10
n	5	5	4	5	1	3	3	3	4	5
Mean (µg/ml)	1.01	89.27	0.21	0.62	0.03	0.10	0.15	0.23	0.33	1.14
SD (µg/ml)	0.58	155.49	0.20	0.50		0.08	0.16	0.15	0.17	0.16
n*	5	3	0	3	0	0	0	0	0	4
Mean* (µg/ml)	1.01	16.86		0.70						1.15
SD* (µg/ml)	0.58	4.38		0.52						0.18

Horse 1.3, horse 2.1, horse 4.1 and horse 10 had concentrations within the linear range of the assay. As can be observed, horse 2.1 had distinctly higher concentration of COMP neoepitope compared to the other samples.
In Table 4.9 the analysis data from the electropherograms is shown. The COMP-neoepitope could be detected in all samples except in horse 4, where only albumin could be detected. Horse 2.1 had the highest obtained peak area and S/N-ratio for COMP neoepitope mAb with apparent molecular weight of 58 kDa, compared to the other samples. The sample from horse 3 did not have any prominent peak area compared to the other sample, even though it had an OA-diagnosis.
The electropherograms for horse 1.3, horse 2.1, horse 4.1 and horse 10 which had concentrations within the linear range in the polyclonal neoepitope ELISA, can be viewed in Figure 4.5. COMP neoepitope could be detected with the apparent molecular weight of 58-62 kDa and albumin with the apparent molecular weight of 62-64 kDa. In Figure 4.5 C, the sample Horse 4.1 had a detected double peak at 59 kDa and 64 kDa with COMP neoepitope mAb, where the values for the 59 kDa peak is presented in Table 4.9. The peak detected at 64 kDa is positioned on the same apparent molecular weight as albumin, preventing possible separation of COMP neoepitope from albumin for this specific peak.

Table 4.9: Used sample, primary antibody, apparent molecular weight (MW), peak area, S/N-ratio and B/H-ratio. The samples were analysed in the concentration of 1.88 mg/mL except for 1.95 mg/mL for horse 4.1. The COMP neoepitope mAb was diluted 1:25 and anti-albumin pAb was diluted 1:2000

Sample	Primary Antibody	MW (kDa)	Peak area	S/N	B/H (%)
Horse 1.3	COMP neoepitope mAb	60	94 562.4	42.8	15.7
Horse 1.3	Albumin pAb	63	290 189	2 263	0.6
Horse 2.1	COMP neoepitope mAb	58	192 465.7	284.2	3.8
Horse 2.1	Albumin pAb	63	1 577 613.5	6 516.8	0.2
Horse 3.2	COMP neoepitope mAb	59	45 220	50.6	33.9
Horse 3.2	Albumin pAb	62	207 160.1	1 451.4	1.2
Horse 4.1	COMP neoepitope mAb	59	57 756.1	99.2	13.2
Horse 4.1	Albumin pAb	64	1 371 089.3	7 048.1	0.2
Horse 4.2	COMP neoepitope mAb	-	-	-	-
Horse 4.2	Albumin pAb	64	19 515.9	124.3	16.0
Horse 5	COMP neoepitope mAb	62	19 535.7	11.9	73.0
Horse 5	Albumin pAb	64	194 308.7	1 297.2	0.9
Horse 6.2	COMP neoepitope mAb	58	81 895.7	62.4	29.8
Horse 6.2	Albumin pAb	62	2 040 658.7	7 306.5	0.2
Horse 8	COMP neoepitope mAb	58	35 584.1	30.7	29.5
Horse 8	Albumin pAb	63	268 709.1	1 500	0.5
Horse 9	COMP neoepitope mAb	59	114 235.3	115.5	8.9
Horse 9	Albumin pAb	63	1 039 405.2	4 457.2	0.3
Horse 10	COMP neoepitope mAb	61	104 931.4	118.8	7.8
Horse 10	Albumin pAb	64	2 440 219.1	11 804.8	0.1

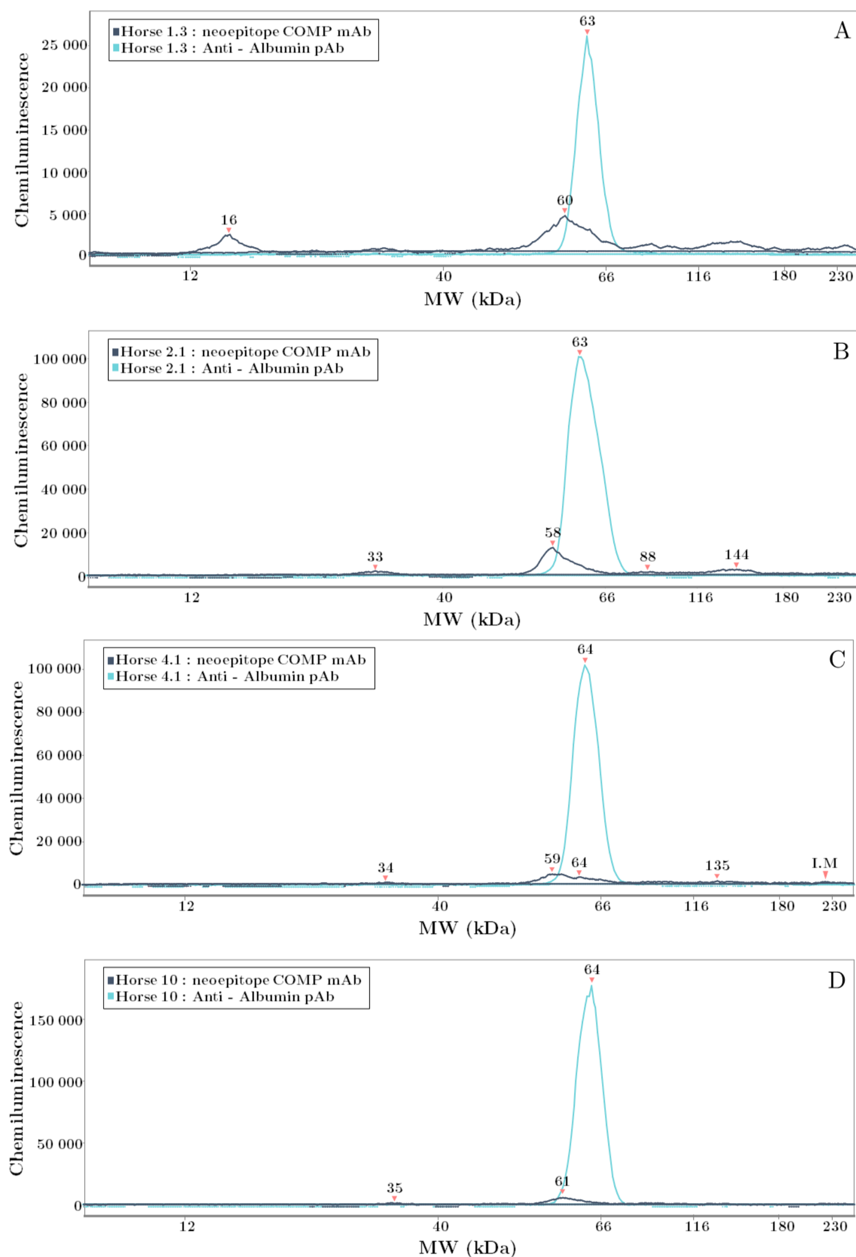

Figure 4.5: Electropherograms showing the chemiluminescence signal as function of apparent molecular weight (MW) for (A) horse 1.3, (B) horse 2.1, (C) horse 4.1 and (D) horse 10. All samples were analysed at a concentration of 1.88 mg/mL except for 1.95 mg/mL for horse 4.1. COMP neoepitope mAb was diluted 1:25, together with anti-albumin pAb diluted 1:2000 used as a control. I.M shows the position of internal marker.

5 Discussion

In previous studies, the concentration of COMP neoepitope have been shown to increase in serum and synovial fluid from horses with acute lameness [4, 5]. Different biomarkers present in synovial fluid and serum have also been detected in saliva. The aim of this thesis was to detect and quantify COMP neoepitope in saliva sampled from healthy horses and horses with OA. The results of this thesis showed that COMP neoepitope could be detected and quantified in equine saliva, and the highest concentration of COMP neoepitope could be observed in a horse with OA.

Equine saliva have not previously been used as medium for detection nor quantification of COMP neoepitope with Wes™or inhibition ELISA. It could be concluded that the COMP neoepitope could be detected but not quantified with Wes™, since the antibody used was not saturated. Instead, COMP neoepitope was quantified with an custom made developed inhibition ELISA developed for serum and synovial fluid [4, 5]. The highest concentration of COMP neoepitope detected was for horse 2, which also obtained the highest peak area and S/N-ratio in Wes™-analysis compared to six healthy horses and another horse with OA. The other horses analysed with inhibition ELISA had concentrations within the linear range, and had noticeably lower concentrations of COMP neoepitope than horse 2. Horse 3, which had an OA-diagnosis, did not have any prominent peak area compared to the healthy horses nor concentration of COMP neoepitope within the linear range. The exact reason behind this can not be determined, but several aspects could have great importance. The clarity or presence of food debris in saliva could interfere with the analysis method [21]. The severity of OA were not provided for this thesis and no lameness examinations of horses were performed. The increase of COMP neoepitope in synovial fluid and serum indicates an ongoing inflammation of the joint, resulting in acute lameness [4, 5]. If this applies to COMP neoepitope in saliva as well, the low detected peaks in horse 3 and lack of determined concentration may be due to sample quality or inactive inflammation of OA joint, with no acute lameness. It is important to note that a correlation between the concentration of COMP neoepitope in synovial fluid or serum, to the concentration present in saliva has not been determined. It is also important to note that concentrations of COMP neoepitope in saliva presented are based on one single measurement and should therefore be repeated to obtain any scientific conclusions.

When the three horses with OA were compared to a group of three healthy horses in a Wes™-analysis, it could be concluded that horse 2 had distinctly higher peak area than the other horses with OA and the healthy horses. The two other horses with OA, horse 3 and horse 7 did have higher peak area than the healthy horses, but had lower S/N-ratio than horse 1 during this analysis. The difference in registered chemiluminescence signal

and peak area between samples from the horses with OA, could have some correlation to the severity of their disease. Unfortunately, the severity of OA or radiological changes were not provided. However, according to the provided information about horses, horse 2 was not diagnosed with OA when saliva was collected, but obtained a diagnosis a few months later. Horse 3 was lame when the sample were taken, but also had other diagnoses. Horse 7 was not lame when saliva was collected, however it was known to have repeated incidences of joint inflammation. Even tough a limited number of horses were used, this indicates that COMP neoepitope in horse saliva could have some correlation to the state of cartilage degradation during early stages of OA.

A comparison of peak area is not totally representing the difference or the concentrations of COMP neoepitope in the samples analysed with Wes™, since it cannot be used for quantification of COMP neoepitope in saliva. As mentioned, quantification of specific proteins can be determined with Wes™if the analysis is performed within the linear range of the assay and primary antibody is used at saturating conditions. If this is fulfilled, the changes in chemiluminescence signal and peak area are proportional to amount of protein bound to primary antibody. The linear range of the assay was determined to be 0.2-3.2 mg/mL with COMP neoepitope mAb diluted 1:25. The optimal dilution of COMP neoepitope mAb was not determined with the sample concentration 2 mg/mL and dilution series of antibody, presented in Figure 4.2 A. The COMP neoepitope mAb could not be saturated in horse saliva, probably due to the high concentration required to obtain peaks with reasonable chemiluminescence signals and related data. One attempt to reach saturation by decreasing the sample concentration was performed (data not shown). The same dilutions of antibody were used with sample concentration 0.5 mg/mL. Unfortunately, saturation was not reached. A further decrease of sample concentration would be beneficial for saturating the antibody. However, decreased concentration of sample increases background noise, decreases S/N-ratio and peak area. This increases the risk of not detecting our target protein. Instead, the used antibody dilution for detection of COMP neoepitope was based on which dilution received low background noise and high S/N-ratio, with clearly defined peaks. Based on this, a suitable dilution was 1:20. However, the 1:25-dilution was chosen since it generally provided clear defined peaks with low background noise. In addition, the 1:25 dilution of COMP neoepitope mAb have previously been used for analysis of COMP neoepitope in equine serum with Wes™, in which saturation could be observed (unpublished data). In this thesis, a compromise had to be made between possible detection of COMP neoepitope in equine saliva, and attempts to saturate the antibody with lower sample concentration and quantification of COMP neoepitope with Wes™.

For possible detection of COMP neoepitope, the presence and apparent molecular weight of albumin has to be known, since albumin is known to interfere with several proteins present in serum, synovial fluid and saliva [19]. For this purpose, the Anti-albumin pAb was included as a control for each sample, to ensure the specificity of antibodies or possible

interference with COMP neoepitope. As observed, the position and apparent molecular weight of both COMP neoepitope and albumin have shown to shift between samples. It can also be observed that the COMP neoepitope peak has a shoulder to the right which may be an effect of albumin binding to COMP neoepitope. This shoulder can for example be seen in Figure 4.4 for horse 2.2. Meaning, obtained peak area for COMP neoepitope may have a contribution from albumin. However, for this thesis this possible contribution is not of interest since Wes™is not used for quantification of COMP neoepitope.

Prior to Wes™-analysis with a new medium and antibodies for a specific target protein, controls for non-specific antibody interactions should be performed. In this thesis, three controls were used by omitting either protein, primary antibody or secondary conjugate. As observed in Figure 4.1 B, peaks at 32 kDa, 56 kDa, 61 kDa and 86 kDa could be registered when the primary antibody was excluded from the analysis. These peaks were below 250 a.u. but are still valid peaks due to a S/N-ratio above 10. Peaks detected with both primary antibody and secondary conjugate present had higher chemiluminescence signal than 250 a.u. suggesting that the main source of signal is obtained from the primary antibody's recognition of target protein. The secondary conjugate used is purchased for ProteinSimple and is designed for sensitivity, but it is not cross-absorbed. Meaning, that the secondary conjugate can recognise other proteins e.g. heavy chain IgG from other species than rabbit. However, since the chemiluminescence signal is noticeable higher in the presence of primary antibody, we can be confident that most of the obtained signal results from the primary antibody being bound to the target protein rather than other proteins present in saliva.

The aim of this thesis included evaluation of sample collection and preparation. Saliva was sampled from horses with two different sampling techniques. One healthy horse was sampled with both techniques, including collection throughout the day with sampling technique 2. The two sampling techniques were compared by analysis with Wes™. The apparent molecular weight of COMP neoepitope and albumin were noted within each sample and peak area, S/N-ratio and B/H-ratio were compared between samples and sampling techniques. The highest average peak area for COMP neoepitope was obtained when saliva was collected in the morning with sampling technique 2, no food intake within one hour. These samples obtained lower background noise and higher S/N-ratio compared to samples collected with sampling technique 1. Saliva collected on the tongue in the morning, no food intake within one hour, obtained the highest peak area, highest S/N-ratio and relatively low B/H-ratio for COMP neoepitope compared to the other samples. It is important to note that this specific sample had albumin detected with the apparent molecular weight of 59 kDa, which is close to COMP neoepitope detected at 57 kDa. However, the large difference in obtained chemiluminescence signal, seen in Figure 4.3 B, peak area in Table 4.6, and the specificity of used antibodies indicates that both COMP neoepitope and albumin are present in this samples, even if COMP neoepitope and albumin

is detected close to each other. COMP neoepitope was not detected in saliva collected on the left side of the cheek in the midday when the horse had eaten. This indicates that this sample did not contain COMP neoepitope in this sample at that specific time. Due to this, it is of high interest to obtain saliva samples which does not contain e.g. food which may prohibit possible detection of COMP neoepitope. The practical aspects of salivary collection have to be taken into consideration when comparing the two sampling techniques. Sampling technique 1 takes a long time to obtain a relatively small volume, compared to sampling technique 2 where a larger volume of saliva was obtained within a minute. Based on Wes™-analysis and the practical aspects, it can be concluded that saliva should be sampled on the tongue, one hour after feed intake with sampling technique 2. Thus, the analysis showed lower background noise, higher chemiluminescence signal and more defined peaks compared to saliva sampled close to feed intake, when analysed with Wes™, and obtained a larger volume of saliva in a short time. This conclusion is based on results obtained from analysis and collection of saliva from one healthy horse. Even tough this provides a limitation in the comparison, the practical advantages of sampling technique 2 outweigh advantages with sampling technique 1. The long time required for saliva collection with technique 1 increases the risk of variations between sampling of horses. Therefore, saliva should be collected with salivette®,sampling technique 2, on the horse tongue with no food intake within one hour.

6 Conclusion and future plans

Saliva should be sampled with salivette®, sampling technique 2, since a larger volume of saliva could be obtained in a shorter time compared to sampling after massaging the buccinator muscle, sampling technique 1. Saliva should be collected on the horse tongue, no food intake within one hour, since it showed lower background noise, higher peak area and high S/N-ratio, with a defined COMP neoepitope peak.
COMP neoepitope could not be quantified with Wes™, since the optimal antibody dilution could not be determined. However, COMP neoepitope could be detected with Wes™at the apparent molecular weight around 58 kDa, where the molecular weight differed between samples. The apparent molecular weight of albumin was determined using a specific anti-albumin pAb, ensuring the specificity of antibodies and possible concealment of COMP neoepitope. Further analysis of apparent molecular weight, peak area, S/N-ratio, and B/H-ratio was used to provide an indication of the relative amount of COMP neoepitope present in saliva from different horses, where the analysis was performed with the same conditions. The concentration of COMP neoepitope could be determined by inhibition ELISA, where a horse diagnosed with OA showed the highest concentration. The same horse also showed a more defined peak with COMP neoepitope mAb when analysed in Wes™.
The future plans are proceeding with a well defined study for analysis of COMP neoepitope in equine saliva. The hypothesis is that the concentration of COMP neoepitope is also increasing in saliva from horses with OA diagnosed by a veterinarian compared to healthy horses, since COMP neoepitope indicates an ongoing inflammation of the joint in serum and synovial fluid from horses with acute lameness [4, 5]. The aim of this future study is to determine the concentration of COMP neoepitope in saliva from healthy horses and horses with OA diagnosed by a veterinarian, and relate the concentration to serum. Aged-matched groups of healthy horses and horses with OA will be included in the future study. The molecular weight of COMP neoepitope will be determined by analysis with Wes™and the COMP neoepitope will be quantified by inhibition ELISA. The initial results for detection and quantification of COMP neoepitope in saliva obtained during this thesis, is a step toward the future goal of developing a point of care diagnostics for the quantification of COMP neopitope in saliva, which could be an accessible tool for diagnosing and monitoring of OA, usable in everyday training of athletic horses.

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