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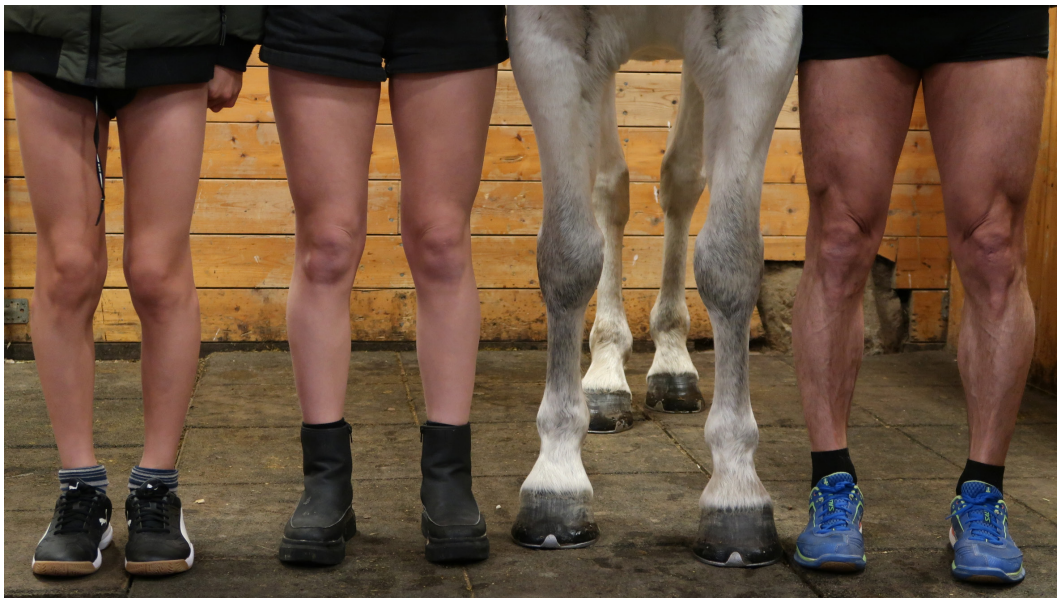
Nerve Growth Factor and its association to osteoarthritis in the horse

Anna Kendall



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Cover: Joints belonging to the Kendall-Isaksson family
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Abstract

Osteoarthritis is common in horses and is a cause of pain and compromised welfare. Osteoarthritis-associated pain in horses is currently diagnosed by subjective and objective measurements of joint pain; however, there is a need to develop methods for evaluation of the systemic osteoarthritic pain that has been recognised in other species. Nerve growth factor (NGF) is associated with pain and osteoarthritis in humans, dogs, cats and lab animals but little is known about NGF in the horse.

The aim of this thesis was to determine the presence of NGF in the equine joint and circulation, and to investigate its connection to osteoarthritis-associated lameness. Serum and synovial fluid from lame horses with osteoarthritis were analysed by ELISA, and NGF levels were compared to those in sound horses. Cartilage and synovial membranes from osteoarthritic and healthy joints were analysed by immunohistochemistry for NGF and for the NGF receptors (TrkA and $p75^{NTR}$). Chondrocyte lysates obtained from healthy and osteoarthritic cartilage were analysed by western blot and capillary simple western.

On a group level, serum NGF was higher in horses with osteoarthritis-associated lameness than in sound horses. Joints with acute or chronic inflammation had higher mean synovial fluid NGF than healthy joints and the expression of NGF and NGF receptors was increased in osteoarthritic articular cartilage compared to healthy articular cartilage. NGF receptor expression in osteoarthritic synovial membranes was also increased with marked individual variation in the relative TrkA/$p75^{NTR}$ expression. Two NGF precursor forms, 40 and 45 kDa, could be identified in equine articular chondrocytes and these forms did not appear to change with inflammation.

The results show that NGF is associated with osteoarthritis in the horse, in line with the findings in other species. The correlation between NGF and pain, individual variations in NGF receptor expression and the role of different precursor forms of NGF warrants further investigation.

Keywords: Nerve Growth Factor, osteoarthritis, pain, horse

Nerve Growth Factor hos hästar med osteoartrit

Abstract

Osteoartrit är en vanlig orsak till smärta hos häst, och kan leda till försämrad välfärd. Sjukdomen diagnostiseras vanligen med fokus på ledsmärtan och det finns ett behov av att utveckla metoder för att också kunna utvärdera systemisk osteoartritsmärta, då denna har konstaterats vara en viktig del av sjukdomen hos människa och andra djurslag. Nerve growth factor (NGF) har associerats med smärta och osteoartrit hos flera species, men vi vet idag mycket lite om NGF hos häst.

Målet med avhandlingen var att undersöka närvaron av NGF lokalt i leden och systemiskt i cirkulationen hos häst, samt att utforska eventuell association mellan NGF och hälta orsakad av osteoartrit.

Serum och ledvätska från halta hästar med osteoartrit samt från ohalta hästar analyserades med ELISA. Brosk och synovialmembran från friska leder och leder med osteoartrit analyserades med hjälp av immunohistokemi för NGF och dess receptorer (TrkA och $p75^{NTR}$). Chondrocytlysat erhållna från friskt och sjukt ledbrosk analyserades med western blot och kapillär simple western.

NGF var högre i serum från gruppen halta hästar med osteoartrit än i serum från gruppen ohalta hästar. NGF var också högre i ledvätska från grupperna med akut eller kroniskt inflammerade leder jämfört med gruppen friska leder. Uttrycket av NGF, TrkA och $p75^{NTR}$ var högre i osteoartritbrosk än i friskt ledbrosk. Uttrycket av NGF receptorer var också högre i synovialmembran från osteoartritleder än från friska leder, med anmärkningsvärd individuell variation i förhållandet mellan TrkA och $p75^{NTR}$. Två proNGF former, 40 och 45 kDa, kunde identifieras i chondrocytlysaten, och ingen ändring i dessa former kunde ses efter inflammatoriskt stimuli.

Resultaten visar att NGF är associerat med osteoartrit hos häst, överensstämmande med fynd hos andra djurslag. Sambandet mellan NGF och smärta, individuella variationer i receptoruttrycket och de olika proformernas betydelse bör undersökas vidare.

Keywords: Nerve Growth Factor, osteoartrit, smärta, häst

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Kendall A*, Nyström S, Ekman S, Mattson-Hultén L, Lindahl A, Hansson E, Skiöldebrand E (2021). Nerve growth factor in the equine joint. *The Veterinary Journal*, 267 (Jan), 105579.

II. Kendall A*, Ekman S, Skiöldebrand E (2022). Nerve growth factor receptors in equine synovial membranes vary with osteoarthritic disease severity. *Journal of Orthopaedic Research*, Epub ahead of print. doi:10.1002/jor.25382

III. Nyström S, Kendall A*, Adepu S, Lindahl A, Skiöldebrand, E (2022). The expression of Nerve Growth Factor in healthy and inflamed equine chondrocytes analysed by capillary western immunoassay. *Research in Veterinary Science*, 151, (Dec 10), 156-163.

IV. Kendall A*, Lützelschwab C, Lundblad J, Skiöldebrand E. Serum Nerve Growth Factor in horses with osteoarthritis-associated lameness (*Submitted manuscript*).

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Abbreviations

ADAMT	A disintegrin and metalloproteinase with thrombospondin motifs (an aggrecanase)
BGN	Biglycan
COMP	Cartilage oligomeric matrix protein
CV	Coefficient of variation
ECM	Extracellular matrix
HPF	High power field
HRP	Horseradish peroxidase
IHC	Immunohistochemistry
IL	Interleukin
LPS	Lipopolysaccharide
MMP	Matrix metalloproteinase
mNGF	mature Nerve Growth Factor
NGF	Nerve Growth Factor[1]
$p75^{NTR}$	pan neurotrophin receptor
proNGF	precursor forms of NGF
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TrkA	Tyrosine kinase A

[1] When the forms of NGF are not known or not defined, or when mentioned in general terms, the abbreviation NGF is used.

1. Introduction

1.1 General background

Osteoarthritis is a chronic, systemic and progressive low-grade inflammatory disease associated with joint pain and lameness (Motta *et al*., 2022; Berenbaum, 2013). In humans, the disease is associated with severe pain, loss of function with decreased ability to participate in social activities and decreased quality of life (Desmeules *et al*., 2009). Osteoarthritis is one of the main causes for early retirement and euthanasia of sport horses (Egenvall *et al*., 2006). Once structural changes are manifest in the joint, horses may have recurring lameness and require repeated treatments and periods of rest and rehabilitation in order to be able to perform. In clinical practice, pain evaluation in equine osteoarthritis is mainly directed at diagnosing joint pain by examining for signs of lameness or reaction to flexion tests. However, by extrapolating from humans and other animals, it is a reasonable assumption that horses with osteoarthritis may also experience a more generalised, systemic pain. Early detection and intervention could slow down the disease process and have implications for animal welfare, but current methods used for diagnosing osteoarthritis in clinical practice such as lameness examination, ultrasound and radiography are not always sufficient. Considering the challenges in diagnosing osteoarthritis in equine athletes, where behaviours caused by osteoarthritis-associated pain may be mistaken for frowardness (Dyson *et al*., 2018), a biomarker would be a great advantage and many substances have been researched for this purpose (McIlwraith *et al*., 2018). Nerve growth factor (NGF) is a neurotrophin that in several species has been shown to be increased in synovial fluid and articular cartilage from osteoarthritic joints compared to healthy joints (Montagnoli *et*

al., 2017; Isola *et al*., 2011; Orita *et al*., 2011; Iannone *et al*., 2002). In humans, NGF is increased in serum of patients with osteoarthritis compared to healthy controls (Chandran *et al*., 2019; Montagnoli *et al*., 2017). If similar increases occur in horses, NGF could be a potential marker for osteoarthritis or osteoarthritis-associated pain in this species.

1.2 The normal joint

Synovial joints act as hinges, connecting parts of the skeleton and thereby making movement possible. As such, joints have to withstand forces created by motion (Merritt *et al*., 2008). This creates high demands for a strong yet flexible structure with smooth and resilient contact surfaces for easy movement with minimal friction. In order to solve these diverging needs, joints are comprised of different types of tissue: articular cartilage, subchondral bone, synovial fluid, synovial membranes, ligaments and joint capsule. These tissues are not just located together anatomically - they should be viewed as parts of a whole. The joint is an organ (van Weeren, 2016).

1.2.1 Articular cartilage

The articular cartilage is a connective tissue consisting of chondrocytes and extracellular matrix (ECM) containing collagen and non-collagenous proteins such as proteoglycans and glycoproteins (Heinegård *et al*., 2002; Bruckner & van der Rest, 1994). The chondrocytes are derived from the mesoderm and it is by these metabolically active cells the ECM is produced (Archer & Francis-West, 2003). The tissue is arranged in different zones. The surface is acellular and contains collagen arranged in parallel to the surface. The presence of flattened chondrocytes defines the upper/superficial zone. Subjacent is the middle zone, with more rounded chondrocytes present singly or in small groups (chondrones). In the deep zone chondrocytes align in columns perpendicular to the surface of the joint and beneath this is the tidemark, the border to the calcified cartilage which in turn leads down to the subchondral bone (Vanden Berg-Foels *et al*., 2012; Pritzker & Aigner, 2010). The arrangement of collagen fibrils also somewhat follow these layers, with fibrils aligning parallel to the surface in the upper zone, have a varied orientation in the middle layers and are arranged perpendicular to the surface in the deep zone. (van Weeren, 2016; Vanden Berg-Foels *et al*., 2012). The resultant arcade-like structure of the collagen fibrils contribute to

reinforcing the articular cartilage, providing resilience against both loading and superficial sheering forces.

As the mature articular cartilage is avascular and aneural, chondrocytes depend on diffusion for the transport of nutrients and metabolites. Following compression due to weight bearing the limb is unloaded and fluid influx to the cartilage is enhanced by negatively charged proteoglycans in the ECM. The high water content also enables the articular cartilage to undergo reversible deformation and sustain high compressive loading (Cohen *et al*., 1998).

Cartilage has a poor regenerative capacity and collagen turnover is minimal after maturity (Heinemeier *et al*., 2016).

1.2.2 Subchondral bone

There are three distinct layers of mineralised tissue in the joint. The calcified cartilage layer starts at the histologically visible border to the uncalcified cartilage, the tidemark line. Under the calcified cartilage lies the subchondral bone plate, which resembles a layer of cortical bone. Deep to this layer is the inhomogeneous trabecular subchondral bone, which is well vascularised and innervated (Burr, 2004). The layers have different mechanical properties such as the elastic modulus (Choi *et al*., 1990) hence they will have different impact on distribution and transfer of loads to and from the joint surface. The subchondral bone has an important role in providing nutrients to the articular cartilage (Malinin & Ouellette, 2000) and the uncalcified articular cartilage has prolongations protruding into the calcified cartilage layer (Lyons *et al*., 2006), facilitating the connection.

1.2.3 Synovial membrane

The synovial membrane has a distinct intimal lining consisting of one to a few rows of synoviocytes. Two celltypes are represented; Type A is a macrophage type cell and type B, which is the most abundant, is a fibroblast type. The synovial membrane lacks a basal membrane and the subintimal tissue is well vascularised and innervated. It is the synovial membrane that produces synovial fluid and via this supplies nutrients to the chondrocytes (Smith, 2011).

1.2.4 Synovial fluid

Synovial fluid is an ultrafiltrate of blood and has non-Newtonian properties, i.e. the viscosity decreases with increased shear stress (Smith *et al*., 2014). Lubricin (a glycoprotein produced by chondrocytes and synoviocytes) and hyaluronan (a glycosaminoglycan produced by synoviocytes) synergistically protect the cartilage surface by preventing the adhesion of proteins and cells (Ye *et al*., 2019), decreasing friction and contributing to the high viscosity of synovial fluid (Momberger *et al*., 2005; Rhee *et al*., 2005).

Normal equine synovial fluid has a protein concentration of less than 26 g/l and a leukocyte count of <500 cells/µl with <10% neutrophils. (Caron, 2010).

1.2.5 Ligaments

Stability to the joint is provided by a fibrous joint capsule and surrounding ligaments. Except for the distal joints, muscles also provide stability and support. Some joints contain intra-articular ligaments (Whitton *et al*., 1997; Prades *et al*., 1989). The ligaments are dense bands of collagenous (mainly type I collagen), glycosaminoglycan-containing connective tissue that anchor onto bone. Among the collagenous fibres, fibroblast cells are scattered. Surrounding the ligament is the epiligament, a thin, vascularised layer that fuses with the periosteum. The cruciate ligament is sheathed within synovial membrane (Stamenov *et al*., 2019; Amiel *et al*., 1984).

1.3 Osteoarthritis

There is increasing evidence that osteoarthritis is a systemic disease, resulting in various co-morbidities (Motta *et al*., 2022; Swain *et al*., 2020; Robinson *et al*., 2016). As the systemic aspect of the inflammation is not within the scope of this thesis, this section will focus on local processes in the joint.

Osteoarthritis is a chronic, progressive and low-grade inflammatory disease involving the whole joint (Robinson *et al*., 2016). Two very different pathways can lead to the same end result with destruction of the articular cartilage, osteophyte formation, and chronic pain; abnormal loading of normal cartilage (Kamekura *et al*., 2005) and normal loading of abnormal cartilage (Knowlton *et al*., 1990). In sport horses, the disease is likely to be most often associated with repetitive mechanical loading (or overloading) of

the joint during training (Murray *et al*., 1999; Bramlage *et al*., 1988), but secondary OA due to other causes e.g. osteochondrosis, joint instability or septic arthritis also occurs (Tahami *et al*., 2020; Al-Hizab *et al*., 2002; Carlson *et al*., 1995; Attenburrow & Goss, 1994). Ultimately, the disease leads to progressive loss of articular cartilage, new bone formation (osteophytes and subchondral bone sclerosis) and synovial proliferation. It is not clear where in the joint the disease first starts and this may vary.

1.3.1 Articular cartilage

The inflammatory events in the articular cartilage are initiated by chondrocyte expression of inflammatory mediators. Cytokines such as IL-1β and TNFα trigger the destructive inflammation that is a hallmark of the disease (Zhao *et al*., 2019). Initially, chondrocytes are stimulated to increase the production of extracellular matrix components, e.g. collagen II (Dreier, 2010; Sandell *et al*, 2007), as an attempt to heal. Simultaneously, the IL-1β and TNFα stimulate chondrocytes to produce other inflammatory mediators such as MMPs and ADAMTs, which degrade the extracellular matrix components (Robinson *et al*., 2016; Xue *et al*., 2013). This leads to articular cartilage fibrillation and chondrocyte death. The surviving chondrocytes proliferate and hypertrophy and form clusters, chondrones (Sandell *et al*, 2007; Tetlow *et al*., 2001). The chondrocyte production of extracellular matrix constituents such as proteoglycans and collagen II is decreased and there is an increased production of collagen X (von der Mark *et al*., 1992) and proteases (Tetlow *et al*., 2001; Shlopov *et al*., 1997), leading to further destruction of the extracellular matrix integrity and cartilage breakdown. Subsequently, chondrocytes undergo apoptosis and the diseased cartilage is frailed and mineralised (Dreier, 2010), eventually leading to painful exposure of the subchondral bone (Moisio *et al*., 2009).

1.3.2 Subchondral bone

In the subchondral bone, the balance between osteoblasts and osteoclasts is disrupted. Bone remodelling increases, with resultant trabecular thickening, sclerosis and stiffening of the subchondral plate and widening of the tidemark (Zhao *et al*., 2019; Bonde *et al*., 2005). Angiogenesis is increased with vascular breaching of the tidemark, which may contribute to the clinical signs of pain (Walsh *et al*., 2007). In clinical studies, bone marrow lesions detected by magnetic resonance imaging (MRI) are associated with cartilage

damage (Muratovic *et al*., 2019). These areas represent structural changes with increased subchondral bone tubular thickness and decreased mineral density, indicating an imbalance in bone resorption and synthesis (Hunter *et al*., 2009). There are several studies on the extensive bidirectional crosstalk between articular cartilage and subchondral bone in osteoarthritis (Hu *et al*., 2021). As an example of this, active Transforming growth factor (TGF-β) is increased in the subchondral bone in osteoarthritis, and inhibition of TGF-β in subchondral bone attenuated cartilage degeneration in a murine model of osteoarthritis (Zhen *et al*., 2013).

1.3.3 Synovial membrane

Early in the osteoarthritic disease process, matrix fragments from cartilage breakdown can stimulate synoviocytes into entering a proinflammatory state (Frevert *et al*., 2018). Hypertrophy of the synovial lining, influx of inflammatory cells and increased vascularisation can be seen in the osteoarthritic synovial membrane (McIlwraith *et al*., 2010). TNFα and IL-1β expression in the synovium stimulates the release of prostaglandin (PGE_2) from synoviocytes (Benito *et al*., 2005). Macrophages play an important role in osteoarthritis progression, releasing several inflammatory mediators including TGFβ which has been shown to increase fibrosis and osteophyte formation (Zhang *et al*., 2020).

1.3.4 Synovial fluid

The viscosity in synovial fluid from osteoarthritic joints is decreased (Jebens & Monk-Jones, 1959) and the fluid contains several inflammatory mediators such as IL-1β, IL-6, TNFα and MMP 13 (Li *et al*., 2020). Also, the hyaluronic acid molecules are heavily degraded in osteoarthritic joints (Tulamo *et al*., 1996; Hilbert *et al*., 1984). Lubricin is increased in synovial fluid from equine osteoarthritic joints, but changes in glycosylation patterns may cause alterations in function (Reesink *et al*., 2017; Svala *et al*., 2017).

1.3.5 Ligaments

Rupture or tears of articular ligaments create joint instability and severing the cruciate ligaments is a way to induce osteoarthritis in experimental osteoarthritis models (McCoy, 2015). However, degradation of the cruciate ligaments is a common finding in human osteoarthritic joints and it is likely

that these changes occur as a part of the joint inflammation, in parallel with the osteoarthritic process in cartilage (Hasegawa *et al*., 2013). In horses, cruciate ligament injuries associated with avulsion fractures and intercarpal ligament tears associated with chip fractures may indicate a traumatic pathogenesis. However, there are also cases of desmitis and osteoarthritis with no or partial cruciate tears, and intercarpal ligament fraying without chip fractures have been described (McIlwraith, 1992; Prades *et al*., 1989), in line with the theory that ligament injuries may occur as part of an osteoarthritic process and not just as a causative factor.

1.4 Pain in osteoarthritis

Osteoarthritis-associated pain in humans is described in two parts as both an acute, intense and sharp pain that may be unpredictable in onset, and as a more subtle but constant "background" pain. (Hawker *et al*., 2008). Osteoarthritic patients also experience hyperalgesia (an increased amplitude of the pain signal) and allodynia (stimuli that would normally cause signals of "mechanical" nature, such as touch, now elicit pain signals) (Arendt-Nielsen *et al*., 2010; Imamura *et al*., 2008). In humans, the chronic pain state associated with osteoarthritis causes a substantial decrease in quality of life (Hawker *et al*., 2008) and chronic pain is now considered a disease in itself (The national pain strategy report, accessed on Dec 4th, 2022: https://www.iprcc.nih.gov/sites/default/files/documents/NationalPainStrategy_508C.pdf).

The pain signal starts with activation of free nerve endings, nociceptors. As the cartilage lacks innervation, pain in the osteoarthritic joint arises from nociceptors in the synovium and subchondral bone. These nociceptors can be either polymodal, responding to various stimuli such as thermal, mechanical or chemical input, or more specialised, e.g. reacting to either potentially harmful mechanical stimulation or noxious temperatures. The pain signal is transmitted to the dorsal horn through Aδ (myelinated) or C (unmyelinated) nerve fibres. (Dubin & Patapoutian, 2010). In the dorsal horn, the signal is transferred via synapses to neurons ascending to the brain. In the sensory cortex, the thalamus and the amygdala, the nociceptive signal is interpreted into perceived pain. Nociception can occur without subsequent awareness of pain (for example in the case of general anaesthesia, where the signal from nociceptors is not interpreted by the brain), but pain can also be

continuously present without a measurable underlying noxious stimulus. When the nociceptive signalling input is prolonged, several adaptions take place in the spinal cord. This leads to an increase in synaptic excitatory transmitters such as glutamate and substance P, postsynaptic upregulation of receptors for these transmitters, and a decrease in inhibitory substances such as GABA. There is an increased "synaptic efficacy" and neurogenic, maladaptive pain (Latremoliere & Woolf, 2009). Osteoarthritis-associated pain is a combination of local processes in the joint (nociceptive pain) and a chronic, centralized (neurogenic) pain state (Clauw & Hassett, 2017). Interestingly, evidence suggests some dissociation between joint destruction and pain perception (Zhu *et al*., 2019) and structural evidence of osteoarthritis without signs of pain is common (Horga *et al*., 2020).

Pain is a personal, subjective experience that is not only influenced by the intensity of the stimulus. Stress, mood and expectation are all examples of emotions that can have effect on our cognition of pain (Reicherts *et al*., 2016).

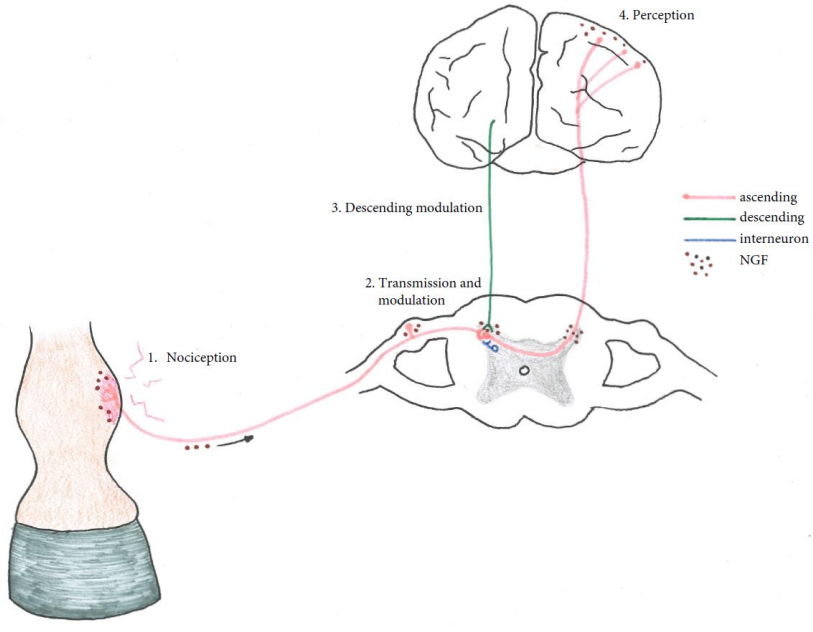

Figure 1. Pain pathway. 1. Painful signals from the joint are sent from the nociceptors via nerve fibres to the dorsal horn of the spinal cord. With chronic signalling in osteoarthritis there can be sprouting of nerve endings and ingrowth of nerves into previously aneural cartilage, leading to more nociceptors. Inflammatory mediators in the joint can cause sensitisation of these nociceptors and lower the threshold for when a pain signal is generated. Nerve growth factor (NGF) is present in the joint and in the central nervous system, and has been shown to increase in the osteoarthritic joint and in the dorsal root ganglia in chronic, osteoarthritis-associated pain. 2. In the spinal cord the signal can be modulated. As an example, touching an injured area sends signals through mechanoreceptors. In the spinal cord these signals can be transferred through connecting interneurons and dampen the ascending pain signal. On the contrary, in chronic pain inhibiting substances are downregulated and pain promoting substances are upregulated, exacerbating the ascending pain signal. 3. Further modulation occurs via signals from the thalamus and amygdala, depending on the situation. If there is perceived imminent danger, e.g. if the painful joint is caused by an attacking predator, descending pathways may downregulate the pain signal as to facilitate escape. With chronic pain, negative feelings can cause amplification of the signal. 4. After synapsing in the spinal cord, the neuron crosses over to the contralateral side and ascends to higher centres (the brain) for conscious perception of the pain.

Measuring pain in the horse (or any non-verbal individual) is a difficult task. Research in equine pain evaluation have mainly focused on acute pain (Broome *et al*., 2022; Ask *et al*., 2020; van Loon & Van Dierendonck, 2018; de Grauw & van Loon, 2016), but the more centralised, chronic pain states are equally important to consider and initial research on chronic pain in horses has been performed (van Loon & Macri, 2021). As equine osteoarthritis can be challenging to diagnose (especially in case of mild osteoarthritic changes or lesions in proximal joints where a complete radiographic evaluation can be difficult), combining behavioural parameters with biomarkers associated with osteoarthritis may be an appropriate way forward. Owner assessment as a tool of evaluating chronic osteoarthritis-associated pain in dogs and cats is based on behavioural parameters (Zamprogno *et al*., 2010; Hielm-Bjorkman *et al*., 2003). As horses are normally not a part of everyday family life the way cats and dogs are, defining and obtaining relevant data poses some challenges. Moreover, despite being a herd animal not all horses are kept in groups, which complicates evaluation of "normal" interaction with herd mates. The gold standard for measuring pain in humans is based on visual analogue scales (VAS) and other subjective measurements including questions about mobility, sleep and mood (e.g. the WOMAC scale) (Woolacott *et al*., 2012; Yarnitsky *et al*., 1996), necessitating the patient to be able to verbally communicate their experience of pain. At present, attempts to find measurements of chronic pain in the horse must be performed without a gold standard. "Triangulation" is therefore required (Thurmond, 2001), where different ways of assessing pain (e.g. extrapolating knowledge from other species, using "classic" methods such as flexion tests or pain face evaluations, exploration of potential serum biomarkers and evaluation of behavioural changes) are used in combination in order to approach the actual, elusive and subjective parameter of interest – the experience of pain.

1.5 Nerve growth factor

Nerve growth factor (NGF) was discovered in the 1950's when it was noted that sarcoma cells implanted in chick embryos stimulated heavy ingrowth of sympathetic and sensory nerves into the tumour mass (Levi-Montalcini & Hamburger, 1951). Since then, NGF has been implicated in a wide range of physiological and pathological processes ranging from nerve development,

growth and survival to pain signalling, apoptosis and inflammation (Masoudi *et al*., 2009; Nilsson *et al*., 1997; Indo *et al*., 1996). Mutations in the NGF pathway result in congenital insensitivity to pain (Sung *et al*., 2018; Shaikh *et al*., 2017; Minde *et al*., 2004; Indo *et al*., 1996), providing evidence that NGF is a signalling molecule for nociception.

1.5.1 NGF in osteoarthritis-associated pain

NGF is produced by chondrocytes, synovial fibroblasts, osteocytes and macrophages (Ohashi *et al*., 2021; Stapledon *et al*., 2019; Takano *et al*., 2017; Pecchi *et al*., 2014). Articular cartilage and synovial membranes show increased NGF in osteoarthritis (Iannone *et al*., 2002; Wu *et al*., 2000), and NGF is also increased in synovial fluid from osteoarthritic joints in several species, compared to synovial fluid from healthy joints (Montagnoli *et al*., 2017; Isola *et al*., 2011). Moreover, in an immunohistochemistry study of subchondral bone, study subjects with joint pain had more staining for NGF than subjects with subclinical (non-painful) osteoarthritis, despite equivalent structural changes in the joint (Aso *et al*., 2019). NGF can induce angiogenesis (Nico *et al*., 2008) and was found to stimulate intra-articular nerve sprouting in a murine arthritis model (Ghilardi *et al*., 2012). In a rat osteoarthritis model, NGF-inhibition decreased the signs of both acute and more chronic osteoarthritis-associated pain (Xu *et al*., 2016). In another rat model, NGF transcription increased in the dorsal horn eight weeks after destabilisation of the medial meniscus (Miller *et al*., 2020). These findings indicate that NGF plays a role in both acute and more chronic/systemic osteoarthritis-associated pain states. Monoclonal anti-NGF antibody therapy has been shown to attenuate osteoarthritis-associated pain in humans, dogs, cats and lab animals but does not stop disease progression (Corral *et al*., 2021; Gruen *et al*., 2021; Dakin *et al*., 2019; Xu *et al*., 2016). In fact, due to severe side effects with rapidly progressing osteoarthritis in some patients, monoclonal NGF-antibody therapy has not yet been approved for human use despite many years of trialling. The reason for the adverse reaction is still unknown (Hochberg, 2015). Whatever the reason, these adverse events indicate that even if NGF plays a role in osteoarthritis-associated pain, is not a primary driver or a causative factor for the structural disease.

1.5.2 NGF in inflammation

Many cells of the immune system express NGF receptors (Labouyrie *et al*., 1997; Ehrhard *et al*., 1993; Kannan *et al*., 1991) and several inflammatory diseases are associated with enhanced NGF production systemically or in the inflamed tissues (di Mola *et al*., 2000; Bonini *et al*., 1996). LPS-stimulation increases NGF synthesis and secretion in astrocytes and peripheral blood mononuclear cells (Caroleo *et al*., 2001; Galve-Roperh *et al*., 1997) and the downstream cytokines involved in the inflammatory response, such as IL-1β and TNFα can promote NGF release from chondrocytes and synovial fibroblasts (Pecchi *et al*., 2014; Manni *et al*., 2003). Histamine has also been shown to induce the production of NGF in some cell types (Kanda & Watanabe, 2003; Lipnik-Stanglj & Carman-Krzan, 2000). In an in vitro study on murine neutrophils, NGF enhanced both cell survival and phagocytosis (Kannan *et al*., 1991). NGF can also cause degranulation of mast cells (Mazurek *et al*., 1986) and trigger the respiratory burst in monocytes (Ehrhard *et al*., 1993). The induction of NGF release by inflammatory mediators and the effects on several different immune cells indicate that NGF is involved in inflammatory responses and may be a link between neural and inflammatory processes.

1.5.3 Mature NGF and precursor forms of NGF

Many different forms of NGF have been described in the literature, with molecular weights ranging from >100 kDa down to 13 kDa (Soligo *et al*., 2019; Lobos *et al*., 2005; Lee *et al*., 2001; Reinshagen *et al*., 2000). Equine full-length proNGF has 294 amino acids and the protein weighs 32 kDa (https://www.uniprot.org/uniprotkb/F6SVV7/entry; https://www.bioinformatics.org/sms/prot_mw.html accessed on November 2nd, 2022). This means that any larger precursor forms will likely be glycosylated variants (Fahnestock *et al*., 2004). Previously, research interest was mainly directed at the mature 13 kDa NGF (mNGF) and the larger forms (proNGF) were thought to be inactive precursors (Edwards *et al*., 1988). It is now known that several precursor forms are also active molecules. The significance of the different variants described is still largely unknown but relative mNGF/proNGF expression has been shown to shift toward proNGF in several inflammatory and neurodegenerative diseases (Minnone *et al*., 2017; Mysona *et al*., 2015). Many different cell types secrete NGF, including structural- (e.g. epithelial cells and fibroblasts), accessory- (e.g. glial cells

and astrocytes) and immune cells (e.g. macrophages and mast cells) (Farina *et al*., 2022; Takano *et al*., 2017; Othumpangat *et al*., 2009; Yu & Fahnestock, 2002; Leon *et al*., 1994). It is likely that different cell types produce different proNGF forms, depending on what arsenal of post-translational and proteolytic cleavage enzymes are available (Bruno & Cuello, 2006; Yu & Fahnestock, 2002; Seidah *et al*., 1996). It has been shown that mNGF and proNGF have different effects on macrophages although the significance of these differences is not entirely clear. Overall, mNGF promoted growth factor secretions while proNGF induced neurotoxin production (Williams *et al*., 2015).

Commercial NGF-ELISA kits and antibodies are often directed at mNGF, and many studies on NGF have used antibodies directed at the mature sequence. As proNGF also contains this sequence, the antibodies may recognise a mixture of mNGF and all or some precursor forms. Also, if proNGF is present it can interfere with the readouts in ELISA studies in unpredictable ways, which further complicates interpretation and comparison of quantitative data (Malerba *et al*., 2016; Soligo *et al*., 2015; Lobos *et al*., 2005).

1.5.4 NGF receptors

NGF binds to two receptors, Tyrosine kinase receptor A (TrkA) which is selective for NGF, and a pan-neurotrophin receptor ($p75^{NTR}$) which also binds to many other neurotrophins (Denk *et al*., 2017; Vilar, 2017). Both mature NGF and proNGF can bind to TrkA (Ioannou & Fahnestock, 2017). The receptor homodimerises and the internal part is phosphorylated, setting off intracellular pathways that mainly trigger survival and growth (Soligo *et al*., 2019). TrkA bound to NGF can also be internalised and transported to the cell nucleus with impact on a translational level (Ure & Campenot, 1997; Ehlers *et al*., 1995). The TrkA receptor is important for neurite development and growth (Soligo *et al*., 2019; Shaikh *et al*., 2017; Indo *et al*., 1996), and the NGF affinity to TrkA and the rate of internalisation after binding is further enhanced if the receptor heterodimerises with the $p75^{NTR}$ receptor (Mahadeo *et al*., 1994). On the other hand, the effects of binding to the $p75^{NTR}$ receptor is more unpredictable as the combination with various co-receptors will set off varied downstream signalling pathways and events, e.g. the proNGF-$p75^{NTR}$-sortillin combination will result in apoptosis, while the proNGF-$p75^{NTR}$-TrkA receptor combination will result in cell survival

(Ioannou & Fahnestock, 2017; Nykjaer *et al*., 2004). Due to this diversity, not only the dominating forms of NGF but also the balance of receptor expression (TrkA vs $p75^{NTR}$) will influence what the resultant effects of NGF (or inhibition of NGF) will be.

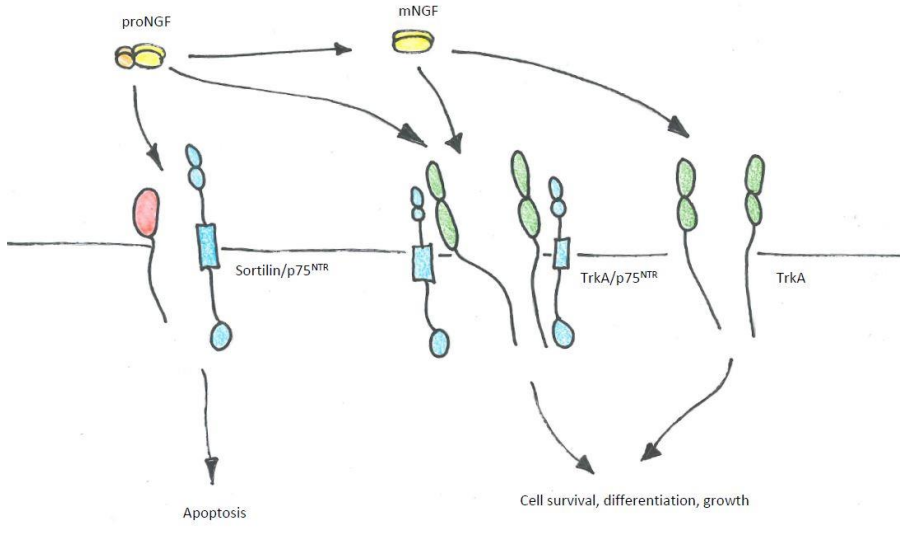

Figure 2. ProNGF and mNGF can bind to various receptor combinations in the cell membrane, leading to several possibilities in pathways and resultant action. ProNGF has the potential to induce either apoptosis or growth/differentiation depending on the balance of receptor expression in the target cell.

1.5.5 NGF in horses

There are only a few published studies on NGF in horses and no studies regarding the association with osteoarthritis in this species. Several of the studies were performed using incubation techniques, looking at induced nerve growth (Ellero *et al*., 2022b; Druart *et al*., 2013; Kawamoto *et al*., 1996; Matsuda *et al*., 1991; Banks *et al*., 1973). These methods provide information on neurotrophin activity, but several neurotrophins can cause nerve growth (Tucker *et al*., 2001), hence the method does not provide specific, quantitative information. A recent study in neonatal foal- and post

parturient mare plasma and amnion used an equine specific NGF ELISA (Ellero *et al*., 2022a; Ellero *et al*., 2022b). However, this ELISA has been noted in our lab to have unacceptably high CV values (unpublished data) and the study does not provide any information on quality control, sample duplicates etc., limiting the reader´s ability to assess the quality of the data. There is a high degree of amino acid homology between the human and equine NGF protein (Amagai *et al*., 2016) which increases the likelihood of cross-reaction and opens up the possibility to use ELISA kits developed for detection of human NGF.

2. Aims

The overall aim was to explore the association between NGF and osteoarthritis-associated lameness in the horse, and to investigate if NGF can be an appropriate candidate biomarker for the diagnosis of osteoarthritis-associated pain in this species.

The specific aims were:

- To identify NGF in horses, locally in the joint and systemically in serum, and to compare the levels of NGF in healthy (sound) horses and in horses with osteoarthritis-associated lameness.

- To explore the presence of NGF receptors (TrkA and $p75^{NTR}$) in synovial membranes and articular cartilage from healthy and osteoarthritic equine joints, and to examine if the receptor expression varies in different disease stages.

- To investigate synovial fluid NGF in acute lameness (septic) and chronic lameness (osteoarthritis) in vivo.

- To investigate NGF expression in cultured equine chondrocytes harvested from healthy joints and osteoarthritic joints. The aim was also to examine the response in NGF expression to LPS stimulation, representing acute inflammation or exacerbation of a chronic inflammatory state.

- To evaluate the impact of short-term stress on serum NGF in the horse.

3. Materials and Methods

This section contains a summary of the methods used in the four studies included in this thesis. A more detailed description is provided in each of the manuscripts.

3.1 Horses and samples

All sampling was approved by the Animal Ethics Committee in Uppsala and the experiments followed national and institutional guidelines for care and use of animals in research.

3.1.1 Study I

The synovial fluid samples were collected from painful joints with osteoarthritis or septic arthritis in horses presented to referral hospitals, and from healthy joints in horses sampled either at their home training facility or post mortem.

The horses in the osteoarthritis group were lame at a trot on a straight line, either initially or after flexion tests. The lameness or reaction to flexion could be abolished or ameliorated by intra-articular anaesthesia and all horses had radiographic changes consistent with osteoarthritis (i.e. osteophytes) in the sampled joint. Horses diagnosed with carpal chip fractures by radiographic examination as the first diagnostic procedure did not receive intra-articular anaesthesia in order to confirm that the sampled joint was the cause of the lameness, and were included without findings of osteophyte formation.

The horses in the septic arthritis group were >1 year old and admitted to a referral hospital with a wound communicating with the sampled joint. The synovial fluid was macroscopically abnormal with >30 g/L of protein and >80% neutrophils.

The healthy synovial fluid samples were collected from biobanks stored at the Swedish University of Agricultural Sciences. Horses had no history of lameness from the sampled joint. The synovial fluid had a normal macroscopic appearance and total protein showed values <25 g/l. In addition, joints were either radiographed or visually inspected by post mortem arthrotomy to exclude visible structural changes.

All synovial fluid samples were centrifuged and aliquoted. Healthy and osteoarthritic group samples were stored at -80 °C and septic group samples were stored at -20 °C until analysis.

Articular cartilage was sampled from healthy and mildly osteoarthritic joints post mortem, based on macroscopic and histological findings. Severely osteoarthritic articular cartilages were sampled from horses undergoing arthroscopic surgery for osteochondral carpal chip fractures.

3.1.2 Study II

Synovial membranes were mainly obtained from biobank specimens, with a few additions of freshly collected samples. Synovial membranes from healthy joints were sampled from horses euthanized for reasons unrelated to the musculoskeletal system. Joints had macroscopically normal cartilage on post mortem arthrotomy. Synovial membranes and full-thickness cartilage samples from the radial facet of the third carpal bone (C3) were obtained within one hour of euthanasia and placed in 10% neutral buffered formalin prior to paraffin embedding. Prior to inclusion of healthy joints, cartilage samples were examined by light microscopy to rule out changes consistent with early osteoarthritis. Osteoarthritic carpal joints were sampled from lame horses during arthroscopic surgery. Three groups of horses were included: horses with synovitis but no gross cartilage changes, horses with synovitis and macroscopically visible cartilage damage and horses with synovitis and intra-carpal fractures.

3.1.3 Study III

Six age-matched horses were included; three horses had mild macroscopic osteoarthritic cartilage changes of the dorsal radial facet and three were without visible joint lesions. Following aseptic preparation, the joint was incised and the articular cartilage was inspected macroscopically. Cartilage on the dorsal aspect of the radial facet of the third carpal bone was incised with a scalpel down to the bone and full-thickness cartilage samples were

collected. The chondrocytes were isolated and expanded to passage 1 and then seeded in chondrogenic medium to maintain the phenotype. On day 4, cells were stimulated with LPS (10 ng/ml) or kept untreated (controls) for 24 h. Cells were grown to confluence and harvested on day 5 and immediately frozen and stored at –80 °C until further analyses. These chondrocyte lysates were obtained from the pathology unit biobank at SLU.

3.1.4 Study IV

Four groups of horses were included in the first part of the study: 1. The advanced osteoarthritis group were lame horses with radiographic changes consistent with osteoarthritis (i.e. osteophytes) in one or more joints of the lame limb. 2. The mild osteoarthritis group were lame horses or horses presented for poor performance with a lameness or a reaction to flexion and an asymmetry on objective motion analysis that was decreased or abolished after intra-articular anaesthesia. These horses had no radiographic changes in the anaesthetised joint. In the absence of structural changes, synovial fluid analysis was performed to confirm the presence of cartilage breakdown, consistent with early osteoarthritis. Biglycan neo-epitope (BGN^{262}) and cartilage oligomeric matrix protein neo-epitope ($COMP^{156}$) were analysed by ELISA. 3. The healthy group consisted of young horses that were sampled prior to commencing training. The horses were examined by a veterinarian and no lameness was detected on dynamic evaluation and flexion tests prior to inclusion. 4. In addition, horses presented to two referral hospitals for acute fractures were included. No history regarding previous lameness was obtained for this group.

The second part of the study, the stress experiment, was performed on horses owned by the Department of Clinical Sciences, SLU. The transportation of these horses was in order to get them to their summer pasture, hence they were not subjected to any stress solely for the purpose of research.

3.2 Analytical methods

3.2.1 ELISA (study I and IV)

Two different commercial sandwich ELISA kits were used, Horse NGF ELISA (MBS040618, MyBioSource) for study I and Human beta-NGF

DuoSet ELISA (DY256, R&D Systems) for study IV. ELISA wells are coated with a capture antibody and sample is then added. The antigen of interest attaches to the capture antibody and excess (unbound) sample is washed away. Thereafter, a secondary antibody, directed at a different epitope on the antigen of interest, is added and attaches to the antigen in the wells. The secondary antibody is labelled with biotin. When streptavidin-HRP is added it attaches to the biotin. A substrate solution containing tetramethylebenzidine (TMB) is used to create an enzymatic reaction with the streptavidin-HRP, directly correlated to the amount of antigen present in the sample and the resultant colour absorbance is measured. A standard curve is prepared by analysing several dilutions with known amounts of the antigen of interest, and measuring the absorbance of these dilutions. The sample absorbance can then be compared to the standard curve and the antigen concentration determined.

3.2.2 Immunohistochemistry (Study I and II)

Immunohistochemistry (IHC) experiments were performed on paraffin embedded cartilage and synovial membranes. The paraffin was removed and antigen retrieval was performed in citrate buffer in a heated water bath at relatively low temperatures (60 °C) in order to decrease the risk of dislodging cartilage from the slides. This temperature was kept the same for synovial membranes as well. After trials to determine optimal protocols for antibody dilutions and staining, efforts were made to keep all samples that were to be compared with each other within the same IHC batch, as batch-to-batch variation in staining intensity can vary considerably (Grube, 2004). Non-specific binding was blocked with goat serum. Primary antibodies were then added to the sections with rabbit IgG run in parallel on all sections as an isotype control, using identical protein concentrations as for the primary antibodies. The sections were incubated at 4 °C overnight, then incubated with secondary antibody and stained with 3,3'-diaminobenzidine tetrahydrochloride. Nuclei were stained with Mayer´s hematoxylin.

For study I, estimation of the amount of positive chondrocytes in each articular cartilage sample slide was performed by evaluating staining patterns of a total of 200 chondrocytes.

For study II, 100 synovial cells in each of 10 randomly selected high power fields (HPF, x60 magnification) were evaluated for positive intranuclear immunohistochemistry staining in each sample slide. In addition, in order to

evaluate total staining (cytoplasmic and nuclear), slides were analysed with Image J software (Fiji Downloads, ImageJ). Ten HPF (x40 magnification) were photographed from each slide. Relevant areas containing synoviocytes were manually annotated, avoiding areas of vessels or subsynovial fibrous tissue. The mean percentage (%) stained area was then calculated.

3.2.3 Western Blot (Study III)

Sample protein was loaded on a pre-cast Mini-protean TGX stain-free gel and run at 300 V for 15 minutes. The blot was transferred to a low fluorescence PVDF membrane. After blocking for 30 minutes in EveryBlot buffer, the membrane was incubated with primary antibody at 4 °C overnight. Thereafter, the membrane was washed and incubated with secondary antibody at room temperature for one hour. The membrane was once again washed and immediately imaged with the Chemidoc Touch Imaging System. Trials were also performed to determine optimal antibody dilutions and controls were run with recombinant NGF to confirm if there was appropriate binding, and with albumin and equine IgG to identify potential unspecific binding to these proteins.

3.2.4 Capillary western, Wes (Study III)

The method is based on capillary protein separation by molecular size. In an automated system samples are loaded onto capillaries, separated and incubated with primary and secondary (HRP-conjugated) antibodies. Chemiluminescence is produced after addition of luminol-peroxidase and the system software creates either curves with peaks or images with the more "traditional" western blot bands. The system is sensitive and uses very small amounts of antibodies and protein. However, the system does not work well for more complex matrices such as equine serum and synovial fluid as there is substantial unspecific binding to albumin and IgG. Due to this, IgG and albumin antibodies were always included as controls in each analysis. By doing so, the location of albumin (around 65 kDa) and IgG (around 32 and 54 kDa for the light and heavy chains and 100 kDa for the non-denatured) could be determined. Peaks in these areas were excluded as they could have been caused by unspecific binding.

3.2.5 Mass Spectrometry

Mass spectrometry was attempted twice (data not included in the studies). In the first attempt, synovial fluid from healthy, osteoarthritic and septic joints and serum from two osteoarthritic horses were sent for analysis to the Proteomics Core Facility at Sahlgrenska Academy, Gothenburg University. Samples were size separated on a gel, and bands were cut out for analysis at 15, 20, 25, 50 and 65 kDa. The second time, samples were sent to the Mass Spectrometry Facility at Uppsala University. Serum from two horses with acute painful conditions were analysed. The serum samples were separated on a gel and areas of the gel were cut out around 25, 50, 75 and 100 kDa. After mass spectrometry, data analysis was performed by data base search against the Equus (horse) proteome downloaded from Uniprot (www.uniprot.org).

4. Results and Discussion

As the aim of this thesis was to explore the association between NGF and osteoarthritis in the horse, study subjects were mainly clinical patients with naturally occurring disease. Despite the challenges with obtaining samples from well-defined and standardised groups, there are considerable advantages with studying naturally occurring disease in live animals. There is likely to be extensive crosstalk between the different tissues involved in the osteoarthritis disease process (Jiang *et al*., 2021; Hugle & Geurts, 2017; Pearson *et al*., 2017). This is something that cannot be evaluated well using in vitro models of osteoarthritis where either single cell types or tissue samples are studied in isolation. Moreover, experimental models of induced osteoarthritis may provide skewed data as different ways of inducing disease (e.g. injection of substances causing inflammation or surgically creating instability) in a previously healthy joint may not provide appropriate representation of naturally occurring disease that develops gradually over time (Kim *et al*., 2013; Mapp *et al*., 2008).

4.1 NGF in synovial fluid and serum (Study I and IV)

NGF levels were higher in synovial fluid from the group of acutely inflamed joints with septic arthritis and the group of joints with chronic osteoarthritis, compared to synovial fluid from the group of healthy joints. This increase likely represents NGF derived from several cell types and tissues in the joint, i.e. synovial membranes, subchondral bone and articular cartilage (Ohashi *et al*., 2021; Stapledon *et al*., 2019; Pecchi *et al*., 2014). NGF was also increased in serum from the two groups of lame horses, compared to the group of sound horses. Furthermore, the group of horses with structural (radiographic) changes in the painful joint had higher serum NGF than the

group of lame horses that had no abnormal findings on radiographs, indicating either a stage-related or chronicity-related difference in serum NGF (figure 3). The horses without radiographic evidence of OA had high levels of BGN[262] and COMP[156] in synovial fluid. These data are unpublished results to be included in another study and therefore could not be shared in the present thesis. However, the results support the classification of the horses as having mild osteoarthritis in the painful joint (Adepu *et al*., 2022; Skiöldebrand *et al*., 2017). There was a wide range of serum NGF concentrations in the group of horses with radiographic changes, possibly reflecting different disease stages or phenotypes. Individuals with signs of more "generalised" pain (lameness reaction to flexion test in all four limbs) tended to have considerably higher serum NGF than those with reaction to flexion test in one limb only (figure 4). This difference did not reach statistical significance ($p=0.12$) which could be due to poor power as the sample numbers were low. Serum samples obtained from horses before and after transportation did not show changes in NGF, and serum samples from nine horses with acute fractures did not show high NGF levels. This indicates that the increased serum NGF in osteoarthritic horses was not caused by external factors such as short-term stress or acute pain. The marked differences in serum NGF between healthy horses and horses with osteoarthritis-associated lameness and structural lesions make NGF an interesting candidate biomarker for chronic osteoarthritis-associated pain.

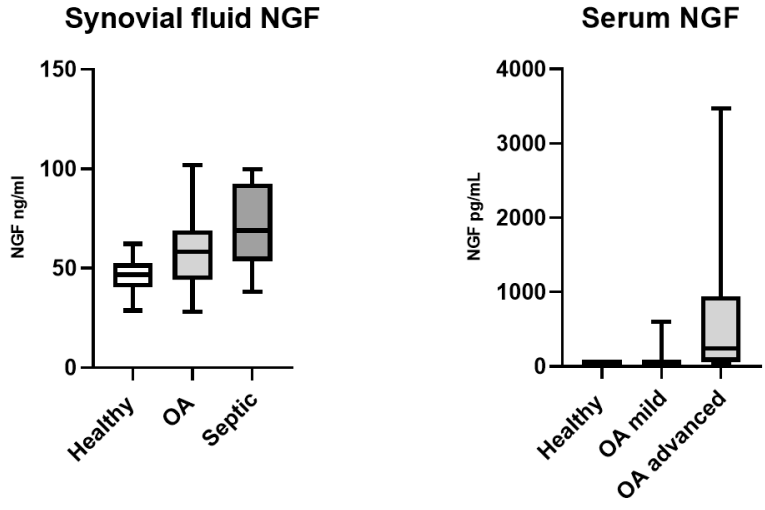

Figure 3. Left: Synovial fluid NGF in healthy equine joints (n=16), in joints with chronic osteoarthritis (OA, n=27) and with acute septic inflammation (Septic, n=9). There was a statistically significant difference between healthy and diseased joints. Right: Serum NGF in healthy horses (n=20), lame horses with no radiographic changes in the painful joint (OA mild, n=20) and lame horses with radiographic changes (OA advanced, n=20). All groups were significantly different ($p<0.05$).

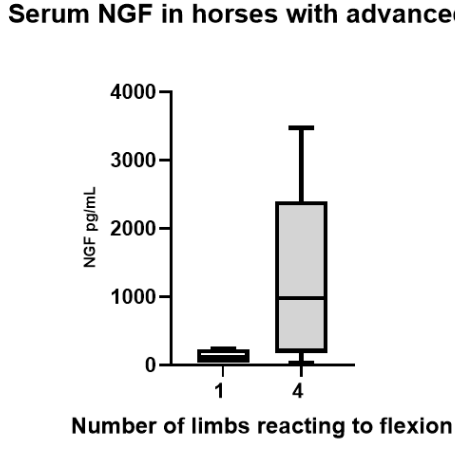

Figure 4. Serum NGF in some of the lame horses with radiographic changes consistent with osteoarthritis. Horses were grouped into those who had lameness after flexion test in either one (n=4) or all four (n=6) limbs.

In study I, an equine specific ELISA (MBS040618, MyBioSource) was used for analysis of synovial fluid. In each plate, the same aliquoted serum sample was repeatedly run in duplicate in order to provide an additional quality control. Initially, for the plates included in study I, CV values were good at <15%. However, during the course of our experiments, it became clear that the inter- and intra-plate CV values were increasing. Despite repeated contacts with the manufacturers and the use of several additional ELISA plates, the issue could not be resolved. One other equine-specific ELISA could be found on the market (Nori® Equine NGF-B ELISA, Genorise) but unfortunately this ELISA did not perform well, again with too high intra-plate CV values (data not shown). Due to this, we trialled a human NGF ELISA (DY256, R&D Systems) and added recombinant equine NGF from the Genorise kit as an additional control during the validation of the kit for equine serum samples. The ELISA proved to be robust with CV values ≤13%. Equine recombinant NGF was detected with excellent recovery (99%). This kit was used in study IV. Queries to the manufacturer regarding antibody epitopes were not answered as this was considered proprietary information. The company would not supply the ELISA antibodies for testing, and our attempts to map the epitopes using the antibodies provided in the kits have so far been unsuccessful. The antibodies provided in the ELISA kit were in low concentration, likely too low for use in western blot. Interestingly, attempts to run synovial fluid previously analysed on the equine ELISA in study I did not show measurable amounts of NGF when analysed on the human ELISA plate. It is possible that this is caused by the expression of different forms of NGF in synovial fluid and serum. One horse in the acute fracture group was a 2-year old colt that sustained a femur fracture during transportation into the equine hospital for a routine castration. The horse was in severe pain with high heart rate and respiratory rate, displaying an obvious pain face and non-weight bearing on the fractured limb. In addition, the pain was acute onset with a duration of approximately one hour. This horse did not show detectable NGF on serum ELISA (DY256, R&D Systems), however, western blot experiments repeatedly showed an obvious band at approximately 26 kDa (figure 5), consistent with previously described proNGF (Soligo *et al.*, 2019). This band has until now not been detected in other horses, but the finding underscores the importance of further research into what proNGF forms are expressed in different disease stages.

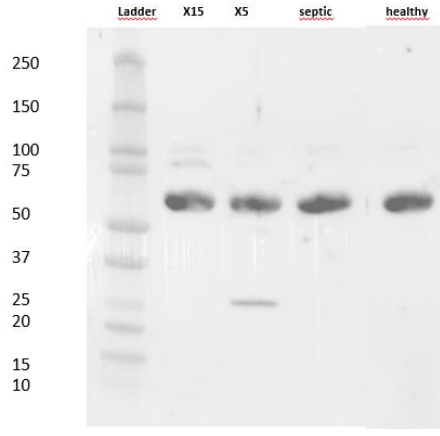

Figure 5. Example of western blot. Serum samples were diluted 1:40 and a polyclonal NGF antibody directed at the mature NGF sequence was used. From left to right: Ladder, X15: a horse with a fracture-associated lameness of a few days duration, X5: a young colt with an acute fracture since approximately one hour, septic: a horse with a septic joint, healthy: a horse with no abnormal findings on pre-purchase examination. Serum from horse X5 showed a clear band just above 25 kDa that was consistent in multiple experiments. The faint bands visible at 100 and 75 kDa were not consistently present and were considered unspecific binding. The intense band at approximately 65 kDa is unspecific binding to albumin. Serum from horses with radiographically visible osteoarthritis did not show a band around 25 kDa (data not shown)

In order to further identify NGF, mass spectrometry was attempted twice (data not shown). Neither of the two attempts were successful. The reason for this has not been further investigated within the scope of this thesis. However, poor sensitivity due to interference of other abundant proteins is likely a part of the problem and further mass spectrometry studies with the addition of techniques for amplification, such as immunoaffinity or 2D gel separation may have been advantageous.

At present, it is unknown what forms of NGF is detected by ELISA, and if the mNGF/proNGF ratio is altered in equine osteoarthritis.

4.2 NGF in articular cartilage (Study I and III)

Healthy articular cartilage and articular cartilage with mild osteoarthritic changes had similar staining patterns. NGF staining was mainly intracytoplasmic in all samples. In the superficial layer, nearly all

chondrocytes had specific immunostaining. In the middle and deep layers, approximately 50% of the cells stained positive. In articular cartilage samples with severe osteoarthritic lesions the middle and deep layers had abundant staining. These samples did not have an intact superficial layer; hence, this could not be evaluated for staining characteristics. These results indicate that equine chondrocyte expression of NGF increases with increasing osteoarthritic disease severity, in line with studies in other species (Aso *et al*., 2022; Iannone *et al*., 2002).

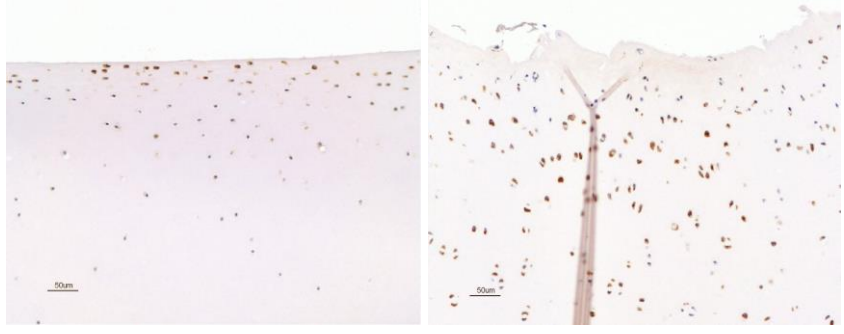

Figure 6. Left: NGF staining in healthy articular cartilage. Staining is intracytoplasmic and most intense in superficial chondrocytes Right: NGF staining in osteoarthritic cartilage. Staining is cytoplasmic and is increased in the middle and deep layers. (x20)

The NGF antibody used for immunostaining was directed at the mature NGF sequence and therefore it was not possible to determine if different forms of NGF were expressed in health or disease. In order to investigate this further we analysed NGF expression in chondrocytes with capillary simple western. Cultured healthy and osteoarthritic equine chondrocytes expressed two forms of proNGF with molecular weights of approximately 40 and 45 kDa (figure 7). Mature, 13 kDa, NGF was not expressed. These findings are in line with previous research in human patients with rheumatoid arthritis (Minnone *et al*., 2017), where proNGF and not mNGF was predominant in synovial fluid and synovial fibroblasts. Different cells show differences in regards to both the mNGF/proNGF expression and the molecular weights of the expressed proNGF. This is likely related to what arsenal of intracellular and extracellular cleavage enzymes are available (Bruno & Cuello, 2006; Seidah *et al*., 1996). Results from studies on one cell type can therefore not be extrapolated to all cells. The same forms of proNGF were found in equine

chondrocytes from both healthy and osteoarthritic cartilage. Acute inflammatory stimulation with LPS did not alter the expressed forms. In addition, preliminary results from equine chondrocyte stimulation with IL-1β did not show changes in what proforms were expressed (unpublished data, not shown).

The capillary simple western system provided a quick and sample sparing alternative to traditional western blot. The method distinguishes between different forms of NGF by size separation, however, the method was not quantitative as saturation of antibodies could not be achieved. Experiments were performed to investigate if the proNGF forms detected in chondrocytes could be detected in synovial fluid and serum; however, there was substantial unspecific binding to IgG and albumin with all tested antibodies. Attempts were made to deplete albumin and IgG from the samples (Poltep *et al*., 2018) but despite a clear reduction, too much protein remained and this caused problems in analysing serum and synovial fluid, with unspecific binding in both capillary simple western and traditional western blot (figures 8 and 9). This underscores the importance to verify peaks with positive controls and to identify the location of the peaks for IgG chains and albumin in the relevant sample protein concentrations, in order to avoid incorrect interpretation of results.

In summary, NGF increased in osteoarthritic chondrocytes and this increase appears to include two forms of proNGF.

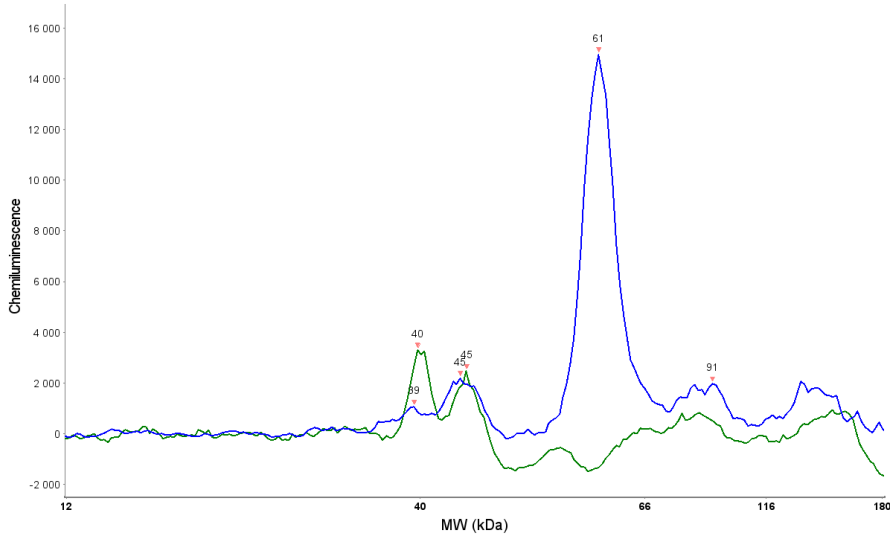

Figure 7. Capillary simple western analysis of a chondrocyte lysate from an osteoarthritic joint. Blue curve: Polyclonal antibody directed at the mature NGF sequence. The antibody detects NGF peaks at 39 and 45 kDa. The peak at 61 kDa is unspecific binding to albumin. No mature NGF is detected. Green curve: Monoclonal antibody directed at proNGF. The antibody detects peaks at 40 and 45 kDa and there is no unspecific binding to albumin.

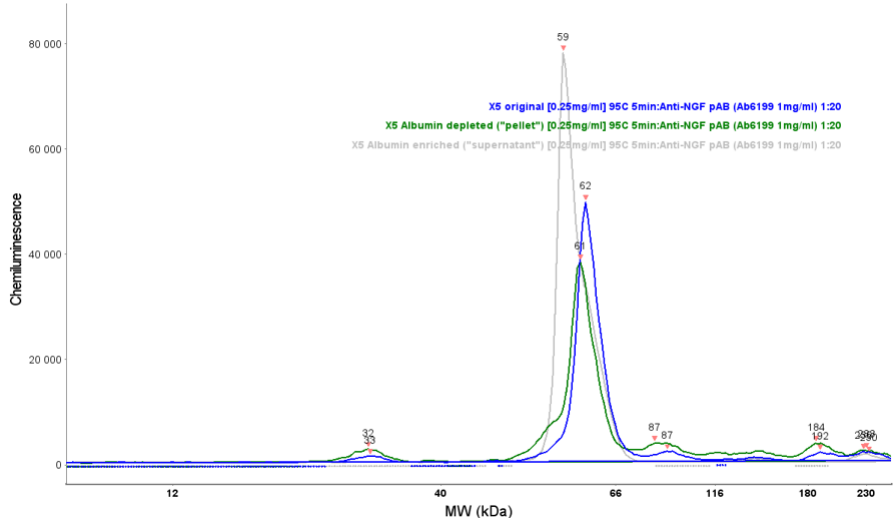

Figure 8. Serum analysis by capillary simple western of a horse with an acute fracture of approximately one hour duration (X5). There is unspecific binding to albumin (peak at approximately 60 kDa). Blue curve: original serum sample. Green curve: albumin depleted serum. Grey curve: albumin enriched fraction. No specific NGF peaks are detected. Experiments with antibodies directed at albumin show a peak at approximately 60 kDa depending on protein concentration. Experiments with an antibody directed at equine IgG showed that the peak at 32 kDa is either consistent with or overlapping the IgG light chain (data not shown).

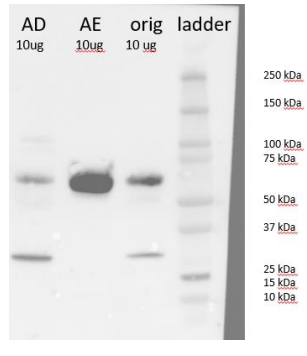

Figure 9. Western blot experiment with the same horse as in figure 8 (X5). 10 µg protein was loaded in each sample well. From left to right: AD: Albumin depleted sample. AE: The extracted albumin fraction. Orig: Untreated sample. Despite the protein depletion, there is still suspected unspecific binding to albumin, creating a band at 65 kDa. The suspected NGF band just above 25 kDa is not present in the albumin fraction, indicating that the protein of interest is not lost in the depletion process.

4.3 TrkA and $p75^{NTR}$ in articular cartilage and synovial membranes (Study I and II)

In the superficial layer, staining patterns for TrkA were similar to NGF with most superficial cells in healthy and mildly diseased articular cartilages showing positive cytoplasmic immunostaining. In the healthy cartilages, only occasional cells in the middle and deep layers stained, whereas the mild and the severe osteoarthritis cartilages had considerably more cells staining positive. For $p75^{NTR}$ the staining patterns were more inconsistent within groups, and staining was not consistently increased with increasing disease severity. Again, most cells showed cytoplasmic staining, but there were also occasional chondrocytes with intranuclear staining. As the TrkA receptor is selective for NGF, the increase in expression of TrkA in articular chondrocytes with increasing disease severity indicates that the NGF signalling will be enhanced in osteoarthritic cartilage. The other receptor, $p75^{NTR}$, can bind to several neurotrophins hence the resultant effects depend to some extent on what ligands are present. The $p75^{NTR}$ receptor also functions as a co-receptor for TrkA and is involved in pain signalling. Clinical signs of lameness had not been recorded for all horses prior to sampling, but it is possible that the difference in $p75^{NTR}$ receptor expression represents differences in phenotypes other than the structural lesions used for grouping, such as painful vs subclinical osteoarthritis, or progressive disease vs dormant.

In synovial membranes, TrkA staining was increased in early stages of osteoarthritis (represented by synovitis without gross articular cartilage changes), whereas increases in $p75^{NTR}$ were most prominent in later stages with articular cartilage damage and synovial fibrosis. These results are consistent with previous findings that expression of inflammatory markers in synovial membranes differ between early and late osteoarthritis (Benito *et al.*, 2005). However, in synovial membranes there were also individual variation in staining patterns of TrkA and $p75^{NTR}$ within the groups. Not only the number of cells that were positive for staining, but also the intracytoplasmic vs the intranuclear staining location varied. This could have implications for analgesic treatments based on inhibition of NGF. If NGF is inhibited in an individual with a dominance of TrkA expression in the joint, this may disrupt pathways of growth and differentiation, while NGF inhibition in an individual with $p75^{NTR}$ receptor dominance may result in a very different result such as disruption of apoptosis (Ioannou & Fahnestock,

2017; Masoudi *et al*., 2009). Failure to acknowledge the complexity of NGF receptor signalling may create future problems with the use of NGF-antibody therapy in a clinical setting.

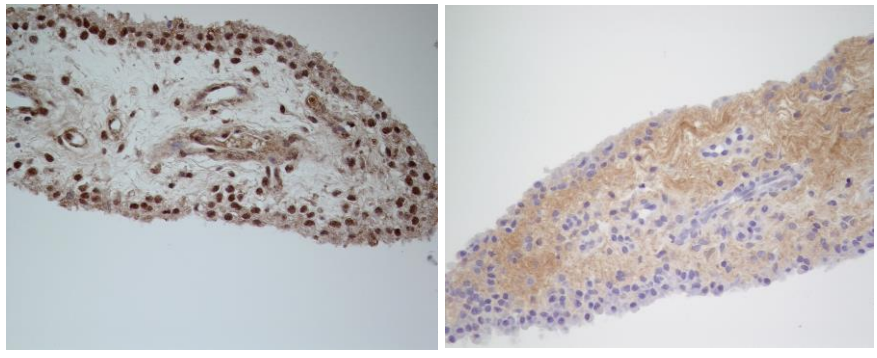

Figure 10. TrkA staining of carpal joint synovial membranes from two lame horses with synovitis and intracarpal fracture (x40). There is considerably more intranuclear staining in the sample to the left while the sample to the right shows mainly intracytoplasmic staining.

5. Concluding remarks

This thesis work has explored NGF and its association to osteoarthritis in the equine species. The following specific conclusions can be drawn:

- NGF is present in the equine joint. On a group level, NGF is increased in synovial fluid and chondrocytes of joints with osteoarthritic changes compared to structurally normal joints. NGF is also increased in synovial fluid of acutely inflamed, septic joints.

- NGF is increased in serum of horses with osteoarthritis-associated lameness, compared to sound horses on a group level. There is also a difference between lame horses with radiographic evidence of osteoarthritis compared to lame horses with no structural lesions in the painful joint, indicating a stage-related difference in NGF expression.

- Short-term stress did not cause an increase in serum NGF.

- Chondrocytes in different inflammatory stages expressed two forms of proNGF, 40 and 45 kDa, in vitro. No mature NGF could be detected. The expressed forms were not altered by osteoarthritis (chronic inflammation) or LPS stimulation (acute inflammation).

- NGF receptors (TrkA and $p75^{NTR}$) are present in synovial membranes and articular cartilage, and the expression is increased in osteoarthritis. There are individual differences in receptor expression in horses with comparable structural lesions and clinical

signs, which is likely to have implications for signalling pathways and resultant effects of NGF.

6. Future perspectives

Chronic osteoarthritis-associated pain in horses can be challenging to diagnose and clinically applicable biomarkers for this type of pain would be a great advantage when investigating horses for poor performance. However, when it comes to low-grade, chronic pain we presently lack gold standards for identifying painful and pain free horses. Moreover, despite the fact that valid pain-related outcome measures are important for ensuring reliable findings in clinical trials, we do not have the tools to objectively identify longitudinal changes in horses with chronic, low-grade pain. In order to be able to evaluate putative pain biomarkers, there is a need to develop behavioural assessment tools for chronic pain evaluation in horses. Preferably, these should be easy to perform in a stable setting without expensive equipment. Behavioural assessment tools have been developed to identify osteoarthritis-associated pain in dogs and cats (Zamprogno *et al*., 2010; Brown *et al*., 2007). Some initial work has been done on behavioural validation of osteoarthritis-associated pain in horses (van Loon & Macri, 2021; Dyson *et al*., 2018), but more work is needed.

Normal behaviour, as well as pain-related behaviours and pain-related *lack* of normal behaviour should be identified (Carbone, 2020). This could be done by field studies and focus group interviews with horse owners, trainers and veterinarians. Identified parameters should be trialled in test cohorts with "known" disease states. In addition, these horses should receive analgesic treatment and be followed longitudinally in order to further identify if the behavioural parameters change with a potential decrease in pain. The parameters that are most promising in separating painful from non-painful horses should be validated in cohorts of horses with unknown disease states that are subsequently subjected to full clinical work-ups in order to

identify horses with suspected osteoarthritis-associated pain and to evaluate if the assessment model is able to separate healthy and diseased horses.

When this work is done, the role of NGF in chronic pain can be further elucidated. Horses with and without osteoarthritis should be sampled, and NGF levels should be related to behavioural and clinical assessment scores. Longitudinal studies of serum NGF in horses treated with analgesia, as well as any changes in behavioural assessment scores after suppression of NGF with monoclonal antibodies would provide useful information regarding the role of NGF in osteoarthritis-associated pain. Morevover, species specific antibodies that distinguish between mNGF and proNGF should be developed (Soligo *et al*., 2015), and the presence and potential disease-related changes in prevailing forms of NGF in the horse should be explored.

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Popular science summary

Osteoarthritis is common in horses and is one of the most common reasons for early retirement or euthanasia due to lameness and joint pain. Osteoarthritis causes decreased quality of life in humans due to chronic joint pain, sleep deprivation and inability to partake in social activities. Joint pain in the horse can be subtle and difficult for owners and trainers to observe at an early stage. Meanwhile, early detection of osteoarthritis is important in order to tailor training and treatments so that disease progression can be prevented or slowed down. There is a need to find biomarkers for diagnosing pain and to follow progression of osteoarthritis.

Nerve growth factor (NGF) is a protein that is associated with pain and osteoarthritis in humans, dogs and lab animals. People with mutational defects in the gene coding for NGF or NGF receptors have decreased ability to feel pain. Human patients with osteoarthritis have higher NGF in blood and synovial fluid with more severe osteoarthritic changes. The degree of pain also plays a role. When patients with equal structural changes are compared, those with more severe pain have higher NGF levels. Knowledge about NGF and its association to osteoarthritis in the horse is lacking.

The aim of this thesis was to investigate the presence of NGF in healthy equine joints and in equine joints with osteoarthritis. The aim was also to determine if NGF can be analysed in blood. The impact of acute stress on NGF levels was investigated by sampling horses before and after transportation. In order to examine how NGF is influenced by acute pain, horses with acute fractures or acute septic arthritis were sampled.

The NGF protein is complex as it exists in several forms and binds to two different receptors (TrkA and $p75^{NTR}$). NGF signalling have different resultant effects in the cell depending on the present form of NGF and what receptor is activated. Due to this, the aim was also to investigate if different

forms of NGF could be detected in the horse and if differences in receptor expression occurs in different disease stages. The experiments were performed using different immunological methods (ELISA, immunohistochemistry, traditional western blot and capillary western blot) based on antibodies directed at the NGF protein.

NGF is present in the equine joint and blood, and on a group level the NGF is higher in horses with osteoarthritis-associated lameness compared to sound horses. Other factors, such as acute stress or acute pain, did not cause high NGF levels in blood, indicating that the increase is associated with the osteoarthritis. The receptors (TrkA and $p75^{NTR}$) increased in osteoarthritic joints, however, there were marked individual variations in receptor expression. Two forms of NGF with molecular weights of 40 and 45 kDa could be detected in cultured chondrocytes (cartilage cells).

In summary, the studies show that NGF is present in the horse, and that the protein is associated with osteoarthritic joint pain. This is in line with findings in other species. NGF suppression is used as a treatment for osteoarthritic pain in dogs and cats. The results of this thesis indicate that NGF suppression could be a possible alternative for use in horses and that NGF should be further explored as it is an interesting candidate biomarker for osteoarthritis-associated pain.

Populärvetenskaplig sammanfattning

Osteoartrit (även kallat artros) är en vanligt förekommande sjukdom hos häst och en av de vanligaste orsakerna till att sport- och fritidshästar pensioneras eller avlivas i förtid på grund av hälta. Osteoartrit ger hos människa försämrad livskvalité på grund av kronisk ledsmärta, sömnstörningar och svårighet att delta i sociala aktiviteter. Ledsmärta hos häst kan vara subtil och svår för ägare och tränare att upptäcka på ett tidigt stadium. Samtidigt är tidig identifiering av osteoartrit viktigt för att kunna anpassa hästens träning och behandling så att sjukdomsförloppet kan stoppas eller fördröjas. För att underlätta för hästägare, tränare och veterinärer finns ett behov av att hitta biomarkörer för att diagnostisera smärta och följa sjukdomsförloppet vid osteoartrit.

Nerve growth factor (NGF) är ett protein som associerats med smärta och osteoartrit hos människa, hund och försöksdjur. Människor med defekter i genen som kodar för NGF eller dess receptorer har nedsatt förmåga att känna smärta. Humanpatienter med osteoartrit har högre halter NGF i blod och ledvätska ju gravare osteoartritförändringarna är, men också smärtgraden spelar roll. En kraftigare smärta ger högre halter NGF när grupper med liknande grad av röntgenförändringar i knäleden jämförs. Kunskap om NGF och dess betydelse för smärta vid osteoartrtit hos häst saknas.

Huvudsyftet med denna avhandling var att undersöka förekomsten av NGF i leden hos friska hästar och hos hästar med osteoartrit. Syftet var också att undersöka om NGF kan analyseras i blodprov. Inverkan av akut stress på NGF-halten i blodet undersöktes genom att hästar provtogs före och efter transportering. För att undersöka hur NGF påverkas av akuta smärttillstånd provtogs även hästar med akuta frakturer och akuta ledinfektioner.

NGF proteinet är komplext då det förekommer i flera olika former, samt binder till två olika receptorer (TrkA och $p75^{NTR}$). NGF-signaleringen ger

upphov till olika slutresultat i celler beroende på vilken form av NGF som dominerar samt vilken receptortyp proteinet binder till. Därför var syftet även att undersöka om olika former av NGF kunde hittas hos häst, samt om närvaron av receptorer i leden ändrades med sjukdomsgraden. Undersökningarna gjordes med olika immunologiska metoder (ELISA, immunohistokemi, traditionell western blot och kapillär western blot), vilka bygger på antikroppar som riktar sig mot NGF.

Studierna visar att NGF förekommer i leden och i blodet hos häst, samt att mängden NGF är högre hos hästar med ledsmärta jämfört med ohalta hästar vid jämförelse av grupper. Andra faktorer, såsom akut smärta och stress, påverkade inte halten av NGF i blodet. Detta betyder att ökningarna som sågs hos halta hästar kan vara kopplade till osteoartritsjukdomen. Även receptorerna (TrkA och $p75^{NTR}$) ökade i leder med osteoartrit, men det fanns stora individuella variationer i närvaron av receptorer och i förhållandet mellan de två olika receptorerna. I odlade broskceller påvisades två former av NGF, med specifika molekylvikter 40 och 45 kDa.

Sammanfattningsvis visar studierna att NGF förekommer hos häst, och att proteinet liksom hos andra djurslag är associerat till ledsmärta vid osteoartrit. För hund och katt med osteoartritsmärta finns en behandling som hämmar NGF. Resultatet av denna avhandling indikerar att ett liknande läkemedel skulle kunna användas för behandling av kronisk ledsmärta även för häst, och att NGF är en intressant kandidat att undersöka vidare i jakten på en biomarkör för smärta vid osteoartrit.

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I am and always will be grateful for your support.

Thank you.

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Nerve growth factor in the equine joint

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ABSTRACT

Nerve growth factor (NGF) is a neurotrophin with many functions. In humans, it is involved in inflammation, nerve growth, apoptosis and pain signalling. Increased concentrations of NGF in synovial fluid has been shown in humans and dogs with osteoarthritis. Despite osteoarthritis being a common problem in horses, no studies have previously been published on NGF in the equine joint. The aim of this study was to quantify NGF in equine synovial fluid from healthy joints, acutely inflamed septic joints and joints with structural changes associated with osteoarthritis. A secondary aim was to identify the localisation of NGF and its two receptors, TrkA and $p75^{NTR}$, in healthy and osteoarthritic articular cartilage. NGF concentrations in synovial fluid from osteoarthritic joints (n = 27), septic joints (n = 9) and healthy joints (n = 16) were determined by ELISA. In addition, articular cartilage from osteoarthritic and healthy joints was examined for NGF, TrkA and $p75^{NTR}$ using immunohistochemistry staining.

NGF was present in equine synovial fluid and articular cartilage. Compared to synovial fluid from healthy joints, NGF concentration was higher in synovial fluid from joints with structural osteoarthritic changes (P = 0.032) or acute septic inflammation (P = 0.006). In articular cartilage with severe osteoarthritic changes, there was more abundant positive immunohistochemistry staining for NGF and its receptors than in normal articular cartilage. Further studies should focus on identifying precursor forms of NGF, and on receptor expression and downstream signalling of TrkA and $P75^{NTR}$ in health and disease.

Introduction

Nerve growth factor (NGF) is a crucial molecule for nerve cell maturation and survival and has been extensively studied in humans and laboratory animals (Denk et al., 2017; Minnone et al., 2017). It binds to two membrane bound receptors, tyrosine kinase A (TrkA) and the pan-neurotrophin receptor $p75^{NTR}$. Interaction of NGF or precursor forms of NGF (proNGF) with these receptors can cause a range of effects from cell survival and neurite outgrowth to cell death through apoptosis (reviewed in Denk et al., 2017). NGF expression increases in many inflammatory diseases (reviewed in Minnone et al., 2017) and has important functions in the nervous system for the production of pain signals. In humans, mutational defects in NGF or its receptors cause severe loss of deep pain sensation with undiagnosed, non-painful, fractures as well as disabling joint disease as a consequence (Einarsdottir et al., 2004; Shaikh et al., 2017).

Human patients with osteoarthritis (OA) of the knee have been shown to have increasing synovial fluid concentrations of NGF with increased disease severity (Montagnoli et al., 2017). Osteoarthritis is a major cause for veterinary care and early retirement of sport horses (Egenvall et al., 2005, 2006) and a cause of chronic pain and compromised animal welfare. In order to advance treatment and rehabilitation of equine athletes there is a need to identify biomarkers of inflammation that can help the clinician diagnose and prognosticate stages of OA (McIlwraith et al., 2018). Nerve Growth Factor has shown promise as a marker of OA and a candidate for therapeutic intervention in other species

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(Isola et al., 2011; Stoppiello et al., 2014; Lascelles et al., 2015; Chen et al., 2017; Montagnoli et al., 2017) but the involvement of NGF in equine OA is currently unknown.

The aim of this study was to confirm the presence and compare the concentrations of NGF in equine synovial fluid from joints with structural changes associated with OA to acutely inflamed septic joints and healthy joints. A secondary aim was to identify the localisation of NGF and its receptors TrkA and $p75^{NTR}$ in healthy articular cartilage and articular cartilage with OA.

Materials and methods

Sample collection was approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (Approval number, 5.8.18-02896/2018; Approval date, 6 April, 2018). Written owner informed consent was obtained as required.

Horses

Synovial fluid was sampled from lame horses with OA or septic arthritis and from healthy controls. The horses in the OA group were admitted for lameness with an initial lameness or flexion test reaction of ≥2/5 (AAEP)[1] where the lameness or reaction to flexion could be abolished or ameliorated by ≥ 70% by intra-articular mepivacaine anaesthesia of the sampled joint. Synovial fluid was collected prior to the administration of local anaesthetic. All horses underwent radiographic examination of the sampled joint. Inclusion criteria for the OA group were radiographic findings of intra-articular osteophytes as evaluated by the attending veterinarian. Horses diagnosed with carpal chip fractures by radiographic examination as the first diagnostic procedure did not receive intra-articular anaesthesia in order to confirm that the sampled joint was the cause of the lameness, and were included without findings of osteophyte formation. Horses were excluded if they had received any intra-articular treatment or if they had been treated with systemic corticosteroids within three months prior to sampling.

Synovial fluid samples from horses with OA were centrifuged at 5700 g for 15 min and supernatant aliquots were frozen within hours of sampling. Some samples were initially frozen at −20 °C before moving to the −80 °C freezer, but no samples were thawed prior to the time of analysis.

Horses with septic arthritis were included if they were >1 year old and admitted to a referral hospital with a wound communicating with the sampled joint. The synovial fluid was macroscopically abnormal with >30 g/L of protein and >80% neutrophils (Cousty et al., 2017). The precise duration of the joint infections was not known but was estimated from the history to vary between a few hours to a few days. Synovial fluid from the septic group was often collected out of hours, and these samples were refrigerated overnight and frozen the following day. The septic synovial fluid samples were all stored at −20 °C until analysis.

Synovial fluid samples from healthy joints were collected from biobanks stored at the Swedish University of Agricultural Sciences. Horses had no history of lameness from the sampled joint and OA inflammatory changes had been excluded by synovial fluid analysis as well as by radiographic imaging (lateromedial, dorsopalmar, dorsolateral-palmaromedial oblique, palmarolateral-dorsomedial oblique and dorsoproximal-dorsodistal oblique at a 35° angle views; n = 6; Skiöldebrand et al., 2006), or by post mortem arthrotomy and visual inspection of the joint (n = 10). All synovial fluid samples had a normal macroscopic appearance with total protein values <25 g/L in untreated samples (Caron, 2011).

For immunohistochemistry, healthy articular cartilages and articular cartilages with OA were sampled. Healthy cartilage was macroscopically normal on post mortem arthrotomy. Articular cartilage with mild to moderate OA had visible abnormalities such as wear lines or fissures visible on post mortem arthrotomy. Full-thickness cartilage samples were obtained from the lateral or medial chondylar surface of the third metacarpal bone within 1–5 h of euthanasia and placed in 10% neutral buffered formalin for 48 h prior to paraffin embedding. Articular cartilages with severe OA were sampled from horses undergoing arthroscopic surgery for osteochondral carpal chip fractures. The cartilage-covered chip was retrieved and placed in formalin as described above. No samples were decalcified prior to embedding. In addition to the macroscopic examination, all sections were stained with hematoxylin-eosin and toluidine blue and examined by light microscopy prior to inclusion to confirm that they were representative for the OA and healthy articular cartilage groups (McIlwraith et al., 2010; Schmitz et al., 2010). Chondrocyte necrosis, chondrone formation, fissuring and focal cell loss were graded (McIlwraith et al., 2010) and results are presented in Table 1.

Synovial fluid analysis

Protein analysis was performed on untreated samples as single sample analysis with the biuret test (Coba's Mira instrumentation, Hoffman-LaRoche). Microscopic examination of synovial fluid including nucleated cell differential counts were performed by trained clinical pathologists.

ELISA

The NGF ELISA assay (Horse NGF ELISA kit, MBS040618, MyBioSource) was performed according to the kit manual. The pre-determined detection range was 15.6-500 ng/mL. Briefly, 50 μL of sample or standard were added to all plate wells except for the blanks, followed by 100 μL HRP-conjugate reagent to all wells. The plate was covered and incubated at 37 °C for 60 min, followed by washing four times with buffer. 50 μL of Chromogen Solution A and 50 μL of Chromogen Solution B were added to all wells, the plate was covered, gently swirled and incubated at 37 °C for 15 min. After incubation, 50 μL of Stop solution were added to all wells, and the optical density was read within 15 min at 450 nm with a plate reader (Infinite F50, Tecan). Inter- and intra-assay coefficient of variation (CV) was tested with an aliquoted synovial control sample run in duplicate on every plate. A four parameter Marquardt standard curve and the Magellan software (Tecan) were used for calculation of concentrations.

Immunohistochemistry

Slides were deparaffinised and rehydrated. Phosphate buffered saline was used for all washes and antibody dilutions and samples were handled in room temperature. After antigen retrieval in a 60 °C water bath for 2 h, endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 min. Non-specific binding was blocked with normal goat serum (X0907, Dako) for 30 min. Primary antibodies were diluted and added to the sections; NGF: 1:500 (polyclonal, H20/SC-548, Santa Cruz Biotech), TrkA: 1:1000 (polyclonal, LS-C389392, Nordic BioSite), $p75^{NTR}$: 1:4000 (polyclonal, ABIN1917233, Antibodies Online). Rabbit IgG (X0936, Dako) was run in parallel on all sections as a negative control, using identical protein concentrations as for the primary antibodies. The sections were incubated at 4 °C overnight, incubated with secondary antibody (EnVision K4003, Dako) for 30 min and

[1] See: American Association of Equine Practitioners. www.aaep.org/horsehealth/lameness-exams-evaluating-lame-horse (Accessed 9 October, 2020).

Table 1
Histopathology scoring of articular cartilage according to McIlwraith et al., 2010[a].

Cartilage classification	Chondrocyte necrosis	Chondrone formation	Fissuring	Focal cell loss	Total score
Healthy	0	0	0	0	0
Healthy	0	0	0	0	0
Healthy	0	0	0	0	0
Mild OA	4	3	3	3	13
Moderate OA	4	3	3	3	13
Moderate OA	4	3	3	4	14
Severe OA	4	3	4	4	15
Severe OA	4	3	4	4	15
Severe OA	4	4	4	4	16

OA, osteoarthritis.
[a] The classification of severity is not clearly reflected in the total score. The scoring system was developed for an osteochondral fragment model and scoring does not include if changes are focal (as in the samples graded as mild to moderate OA) or widespread throughout the whole sample (as in the samples graded as severe OA).

stained with 3,3′-diaminobenzidine tetrahydrochloride in organic solvent for 3 min (NGF and TrkA) or 6 min ($p75^{NTR}$). Nuclei were stained with Mayer's hematoxylin. Finally, sections were dehydrated and mounted. Equine spinal cord was used as a positive control for each antibody (Appendix A: Supplementary material).

For estimation of the proportion of positive chondrocytes in each articular cartilage sample, 200 chondrocytes were evaluated for positive immunohistochemistry staining on two separate occasions. Articular cartilage sections were reviewed by light microscopy by two evaluators, one of them a board-certified veterinary pathologist.

Statistical analysis

Statistical analysis of the synovial fluid NGF concentration was performed using a commercial statistical software program, JMP Pro 14.0 (JMP Nordics). Residuals were confirmed to be normally distributed. The concentration of NGF for the OA, septic and healthy joint groups were compared using ANOVA and least square means estimates. NGF was elected as the response variable with ELISA plate and diagnosis selected as model effects. Tukey's test was used for testing pairwise differences. In addition, NGF concentration for the OA and healthy carpal joint groups and fetlock joints were analysed using ANOVA and least square means estimates. NGF was elected as the response variable with ELISA plate, joint and diagnosis selected as model effects and differences were tested using the Student's t test. Correlation between age and the concentration of synovial fluid NGF in healthy horses was tested by bivariate analysis. Data is presented as mean (± standard deviation, SD) and significance was set at $P < 0.05$.

Results

ELISA

Twenty-seven horses met the inclusion criteria for the OA group. Nine horses were included in the septic group and 16 horses in the healthy group. Table 2 contains demographic details.

Synovial fluid concentration of NGF in the OA group was 57.78 ng/mL (±17.41), in the septic group 71.34 ng/mL (±20.95) and in the healthy group 46.32 ng/mL (±9.26; Fig. 1). NGF concentrations in the OA and septic groups were significantly higher compared to the healthy group ($P = 0.032$ and $P = 0.006$, respectively). There was no significant difference in NGF concentrations between the septic and the OA group ($P = 0.72$) or between carpal and fetlock joints ($P = 0.31$). There was no correlation between age and NGF concentration in synovial fluid.

The ELISA intra-assay CV for the three plates used was 1, 4 and 12%, respectively, and the inter-assay CV was 10%.

Immunohistochemistry

Appendix A Supplementary material contains demographic details. Three horses were included in each group. The healthy cartilage samples had no abnormal findings on light microscopy of hematoxylin-eosin and toluidine blue stained sections. The articular cartilage with mild to moderate OA changes had superficial fibrillation and loss of chondrocytes with adjacent chondrocyte cluster formations (chondrones) in a localised area (one horse) and as multifocal changes (two horses). These areas had decreased colouring of the matrix on toluidine blue staining.

Table 2
Demographics of horses sampled for ELISA.

Diagnosis (*n*)	Age (mean)	Breeds (*n*)	Sex (*n*)	Sampled joint (*n*)
OA (27)	2–22 (10)	Standardbred (3) Thoroughbred (1) Pony breed (5) Quarter Horse (1) Warmblood (17)	m (13) g (13) s (1)	Fetlock (10) Carpus (9) Tarsocrural (2) Tarsometatarsal (2) Coffin (2) Stifle (2)
Septic (9)	1–23 (8)	Standardbred (3) Pony breed (2) Warmblood (4)	m (6) g (3)	Fetlock (3) Carpus (2) Tarsocrural (3) coffin (1)
Healthy (16)	1–12 (4)	Standardbred (16)	m (8) g (3) s (5)	Fetlock (4) Carpus (12)

OA, osteoarthritis; m, mare; g, gelding; s, stallion.

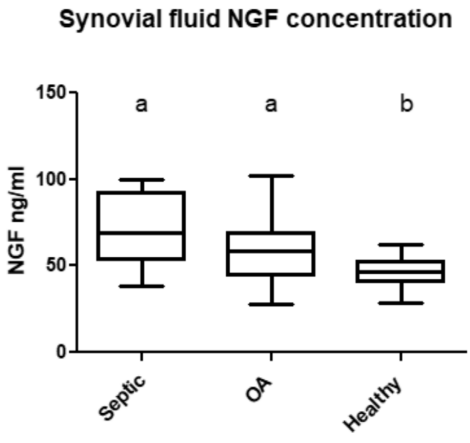

Fig. 1. Boxplot showing mean, interquartile and min-max ranges for synovial fluid nerve growth factor (NGF) concentrations in septic, osteoarthritic (OA) and healthy joints. Different letters indicate statistically significant differences in group means.

The articular cartilages with severe OA had fibrillation, areas of necrosis and of fibrous cartilage formation, as well as chondrone formation in all layers. These samples showed decreased toluidine blue staining, indicating marked matrix degeneration (Schmitz et al., 2010). Appendix A: Supplementary material contains examples of toluidine blue stained sections.

Immunohistochemistry for both NGF and TrkA showed increased number of staining chondrocytes with increasing disease severity, while the staining for $p75^{NTR}$ varied. Although $p75^{NTR}$ staining was more abundant in areas of cluster formation surrounding necrosis, there was not a consistent increase with increasing morphological disease severity.

For NGF, the healthy and the mild-moderate OA articular cartilages had similar staining patterns. In the superficial layer, nearly all chondrocytes had specific cytoplasmic immunostaining with only occasional cells without obvious staining present. In the middle and deeper layers, approximately 50% of the cells stained positive. The articular cartilages with severe OA did not have an intact superficial layer; hence, this could not be evaluated for staining characteristics. However, there were areas of fibrous cartilage formation and these cells did not stain positive for NGF. Positive staining was more abundant in the middle and deeper layers of the severe OA cartilage group compared to the healthy and mild-moderate OA cartilage groups, with more than 60% of the cells staining for NGF (Fig. 2).

For TrkA, staining patterns were similar to NGF with superficial cells in the healthy and mild-moderate OA articular cartilage groups showing positive cytoplasmic immunostaining. However, the staining of the middle and deep layers showed some variation. In the healthy cartilages, only occasional cells in the middle and deep layers stained, whereas the mild-moderate and the severe OA cartilage groups had more cells that stained positive. The increase in positively stained cells was marked in the articular cartilages with severe OA, compared to healthy cartilages. Chondrones were consistently positive for TrkA. Some articular cartilage samples also had non-specific staining of the extracellular matrix. This non-specific staining was most apparent in areas of cartilage necrosis in the severe OA articular cartilage group, but could also be seen as a very thin line in the most superficial layer of one healthy articular cartilage and one with mild OA, with no associated cartilage necrosis visible.

For $p75^{NTR}$, staining patterns were inconsistent within groups. All the healthy articular cartilages had only occasional cytoplasmic staining of cells in the middle and deep layers, but one horse had most of the superficial cells positive while two horses had positive staining of approximately 25% of the superficial cells. In the mild-moderate OA group, two samples had positive staining of more than 75% of the cells in the superficial layer and approximately 10-20% stained cells in the middle and deeper layers. In the third sample, less than 50% of the cells in the superficial layer stained positive and only occasional cells stained in the middle and deeper layers. However, there was a localised area of necrosis and cluster formation in this sample and in that area, all of the surrounding cells stained for $p75^{NTR}$. In the severe OA cartilage group, two samples had more than 60% of the cells staining positive. Most cells showed cytoplasmic staining, but there were also occasional chondrocytes with intranuclear staining. The third articular cartilage with severe OA had only a few positively stained cells.

Discussion

This study is the first to show the presence of NGF and its receptors (TrkA and $p75^{NTR}$) in equine joints. Concentrations of NGF were significantly higher in synovial fluid from joints with structural OA changes and joints with septic inflammation, compared to healthy joints. This is consistent with previous studies in dogs and humans, where joints with chronic OA had significantly higher synovial NGF concentrations than healthy control joints (Isola et al., 2011; Montagnoli et al., 2017). ELISA results were supported by immunohistochemistry; articular cartilage with severe OA showed more cells staining positive for NGF compared to normal articular cartilage. Moreover, given the role of NGF receptors in survival and apoptosis, the difference in receptor expression with more chondrocytes being positive for

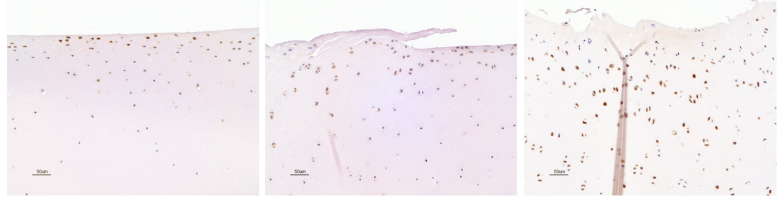

Fig. 2. Articular cartilage stained for nerve growth factor (x20). Left, normal; middle, mild osteoarthritis (OA); right, severe OA. Healthy and mild OA articular cartilage show similar staining patterns with positive chondrocyte staining of almost all cells in the superficial layer and in some cells of middle and deeper layers. In severe OA there is obvious staining of most chondrocytes in the middle and deeper layers.

TrkA in articular cartilage with severe OA, but marked variation in the expression of $p75^{NTR}$ is an interesting finding.

The NGF protein is secreted as a large molecule with several glycosylation sites, and post-translational processing involves glycosylation and splicing from the N-terminal into shorter proNGF forms and the mature NGF of 13 kDa (Fahnestock et al., 2004). The post-translational modification of NGF may differ between cell types depending on what arsenal of proteolytic enzymes are available, which means that different cell-lines are likely to have variations in what proNGF forms are expressed (Seidah et al., 1996; Reinshagen et al., 2000). Exact functions of the various forms of proNGF remain to be elucidated. It has been shown that proNGF function can change according to the relative expression of TrkA and $p75^{NTR}$. TrkA expression results in neurotrophic signalling, whereas a decrease in TrkA leads to apoptotic signalling (Ioannou and Fahnestock, 2017). The current understanding is that NGF binds preferentially to TrkA, while proNGF has been shown to have greater affinity for the $p75^{NTR}$ receptor (Lee et al., 2001; Clewes et al., 2008). Reasons for the differences in $p75^{NTR}$ expression in this study remain speculative and should be interpreted cautiously. Considering the interplay between TrkA and $p75^{NTR}$, it is possible that the lower $p75^{NTR}$ expression represents NGF signalling that stimulates healing and growth as supposed to apoptosis.

The NGF antibodies used in this study were directed at mature NGF and will not distinguish between mature and pro forms, which could potentially be a disadvantage in distinguishing the differences between NGF in synovial fluid and articular cartilage from healthy horses and those with OA, considering the different actions of proforms and mature NGF. Although the synovial fluid concentrations of NGF were different between healthy joints and those with OA, there was some overlap between the groups. Definition and quantification of proforms of NGF may have been more rewarding in terms of studying differences between these groups.

Synovial fluid from septically inflamed joints also had higher NGF concentrations than synovial fluid from healthy joints. It is not known if these horses had underlying OA of the sampled joint, as a complete lameness history and radiographs were not consistently obtained from these horses. However, it has been shown that NGF enhances neutrophil function in both murine models and humans (Kannan et al., 1991; Beigelman et al., 2009). In a carrageenan induced synovitis model in rats, NGF increased in synovial membranes within 24 h (Aloe et al., 1992). These studies indicate that NGF is not only involved in chronic OA processes but also has important functions in acute inflammation.

The horses in the healthy group included for synovial fluid analysis were younger (1–12 years) than the horses in the OA group (1–23 years), but the reverse was true for the cartilage samples, with older horses (4–12 years) in the healthy group than in the severe OA group (3–4 years). Age may have influenced the results and due to a relatively small sample size it was not possible to properly test the effect of age in the study material. However, there was no correlation between age and synovial fluid NGF concentrations in the healthy group, and it is unlikely to have affected the results of this study. Articular cartilage sections from both fetlock and carpal joints were included for immunohistochemistry as these two joints were not shown to differ statistically in synovial fluid NGF concentrations.

In the ELISA cohort, three horses with OA and three with septic joints had received systemic NSAIDs prior to sampling (data not shown). The horses with OA had not received treatment within 4 days, but the horses in the septic group had been treated the same day. The mean synovial fluid NGF concentrations (65.7 ng/mL for the NSAID-treated OA horses and 73 ng/mL for the NSAID-treated septic horses) were above the respective group means. It is possible that anti-inflammatory treatment decreased the NGF levels in the treated horses, however, it has not changed the final results of the study.

In humans, phase III studies of treatment with NGF antibody are being conducted, showing promising results in patients refractory to traditional treatments with NSAIDs and opioids (Dakin et al., 2019). Pilot studies in dogs with chronic lameness due to OA have also showed improvement after antibody treatment (Webster et al., 2014; Lascelles et al., 2015). A potential safety signal with rapidly progressing OA in human patients treated with NGF-antibody caused a temporary halt in trials in 2010. Causes for the rapid disease progression is still unknown, but combination with NSAIDs as well as neuropathic arthropathy and increased joint loading due to analgesia has been postulated (Hochberg, 2015).

NGF therapy could be an intriguing alternative to long-term NSAID treatment in horses. However, due to the varied effects of proNGF depending on the amount of TrkA receptors available (Ioannou, 2017), more research on receptor expression in different disease stages may be advisable before anti-NGF treatment in horses is considered.

Conclusions

In summary, NGF, TrkA and $p75^{NTR}$ is present in the equine joint and expression appears to be influenced by inflammation. The potential use of NGF as a biomarker or target for therapeutic intervention for OA pain should be further elucidated with emphasis on defining and quantifying the proNGF forms and the activation and downstream signalling of $p75^{NTR}$ and TrkA. Determining alterations of NGF and its proforms in other body fluids such as serum or saliva may provide a more accessible tool for future research in this area.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tvjl.2020.105579.

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RESEARCH ARTICLE

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Nerve growth factor receptors in equine synovial membranes vary with osteoarthritic disease severity

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Abstract

Nerve growth factor (NGF) is a neurotrophin that has been implicated in pain signaling, apoptosis, inflammation and proliferation. The resultant effects depend on interaction with two different receptors; tyrosine kinase A (TrkA) and p75^{NTR}. NGF increases in synovial fluid from osteoarthritic joints, and monoclonal antibody therapy is trialed to treat osteoarthritis (OA)-related pain. Investigation of the complex and somewhat contradictory signaling pathways of NGF is conducted in neural research, but has not followed through to orthopaedic studies. The objectives of this study were to compare the expression of NGF receptors and the downstream regulator BAX in synovial membranes from joints in various stages of OA. The horse was used as a model. Synovial membranes were harvested from five healthy horses postmortem and from clinical cases with spontaneous OA undergoing arthroscopic surgery for lameness. Four horses with synovitis without gross cartilage changes, four horses with synovitis and cartilage damage, and four horses with synovitis and intracarpal fractures were included. Samples were investigated by immunohistochemistry and results showed that nuclear staining of TrkA, p75^{NTR} and BAX increases in OA-associated synovitis. TrkA expression increased in early disease stages whereas increases in p75^{NTR} were most prominent in later disease stages with cartilage damage and fibrosis. Clinical significance: Suppression of NGF may result in varied effects depending on different stages of the osteoarthritic disease process.

KEYWORDS
immunohistochemistry, nerve growth factor, osteoarthritis, p75, TrkA

1 | INTRODUCTION

Nerve growth factor (NGF) is a neurotrophin that since its discovery in the 50s[1] has been implicated in a wide range of physiological and pathological processes such as nerve development, growth and survival, pain signaling and inflammation.[2–4] The resultant effects of NGF depend on receptor interaction with two different receptors; tyrosine kinase A (TrkA) and pan-neurotrophin receptor (p75^{NTR}). NGF is secreted in both a mature form (mNGF) and in a proform (proNGF), although many studies do not specify what forms are studied. The current understanding is that mNGF binds preferentially to TrkA, while proNGF has been shown to have greater affinity for the p75^{NTR} receptor.[5,6] TrkA binding promotes nerve growth and survival. p75^{NTR} binding to proNGF in the absence of TrkA can reduce nerve growth or

induce apoptosis via activation of Bcl-2-Associated X protein (BAX).[7–9] Both receptors impact on transmission of pain signals[10] and NGF has been shown to cause direct neuronal sensitization[11] and nerve sprouting,[12] which could cause increased nociception.

Human patients with osteoarthritis (OA) of the knee have been shown to have increasing synovial fluid concentrations of NGF with increased disease severity,[13] and serum NGF has been shown to be higher in OA patients compared to healthy controls.[14] Since OA is a whole joint disease and not only defined by articular cartilage pathology, the synovial membrane likely has an integral role in the OA disease process.[15,16] Synoviocytes have been shown to express NGF and NGF receptors and the expression was increased after stimulation with interleukin-1 and tumor necrosis factor-α, indicating an influence of inflammation.[14,17]

The horse has been suggested as an animal model for studying disease mechanisms in human OA.[18,19] Horses have a genome structure with substantial synteny with humans,[20] develop OA spontaneously without iatrogenic intervention and provide an approximation for human OA in terms of cartilage thickness and morphology.[18,21] The possibility of harvesting cartilage and synovial membrane specimens and chondrocytes from articular cartilage from euthanized horses immediately post mortem provides good access to material, both from healthy and diseased joints. Moreover, the horse has been shown to have increased concentrations of NGF in articular chondrocytes, synovial membranes and synovial fluid of OA joints compared to healthy joints,[22,23] which is in agreement with findings in humans.[13,24]

Most published studies so far use commercial antibodies that are directed at epitopes on the mature form of NGF, and these do not distinguish between mature and proNGF. In addition to this, it has been shown that proNGF function can change according to the relative expression of TrkA and $p75^{NTR}$[7] and further investigation of receptor expression is therefore important to increase understanding of the effects of NGF. Several immunohistochemistry studies have examined NGF receptor expression in rheumatoid arthritis,[17,25,26] but information on receptor expression in spontaneous OA including comparisons with a healthy control group is, to the best of the authors' knowledge, lacking.

This study aimed to compare the expression of NGF receptors and the downstream regulator BAX in synovial membranes from healthy equine joints and from equine joints in various stages of symptomatic OA determined by the morphological phenotype that can be visualized by arthroscopic surgery.

The hypothesis was that receptor expression would increase in synovial membranes with morphological changes consistent with synovitis, compared to synovial membranes harvested from healthy joints. The hypothesis was also that receptor expression would vary in different disease stages of OA and that BAX expression would increase with increased $p75^{NTR}$ [27] in more advanced OA.

2 | METHODS

Sample collection was approved by the Ethical Committee on Animal Experiments, Uppsala, Sweden (Dnr: 5.8.18-02896/2018).

2.1 | Horses

Synovial membranes from healthy and OA carpal joints were sampled for immunohistochemistry and owner consent was obtained before sampling. Healthy joints were sampled from horses euthanized for reasons unrelated to the musculoskeletal system. Joints had macroscopically normal cartilage on post mortem arthrotomy. Synovial membranes and full-thickness cartilage samples from the radial facet of the third carpal bone (C3) were obtained within 1 h of euthanasia and placed in 10% neutral buffered formalin for 2–4 days before paraffin embedding. Before inclusion of healthy joints, cartilage samples were stained with hematoxylin and eosin (H&E) and examined by light microscopy to rule out changes consistent with early OA. Osteoarthritic carpal joints were sampled from clinical cases during arthroscopic surgery. Three groups of horses were included: horses with synovitis but no gross cartilage changes, horses with synovitis and macroscopically visible cartilage damage and horses with synovitis and intra-carpal fractures. Horses were grouped according to the clinical diagnosis made by the attending surgeon.

2.2 | Synovial membrane grading

All synovial membranes were sectioned and stained with H&E. They were evaluated for degree of cellular infiltration, vascularity, intimal hyperplasia, subintimal edema and subintimal fibrosis. Grading of these parameters was performed according to a previously published method[28] by a board certified veterinary pathologist (SE) blinded to sample identity. The total score was used to divide specimens into groups based on severity of synovitis; normal (score 0–4), mild to moderate synovitis (score 5–9) and severe synovitis (score ≥10). Fibrosis was selected as an additional way of evaluating chronicity and three groups were identified; no or very slight fibrosis (score 0–1), mild fibrosis (score 2) and moderate to marked fibrosis (score 3–4).

2.3 | Immunohistochemistry

Slides (4 μm) were deparaffinized and rehydrated. Phosphate buffered saline was used for all washes. After antigen retrieval in a 60°C water bath for 2 h, endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 5 min. Nonspecific binding was blocked with Normal Goat Serum (X0907; Dako) for 30 min. Primary antibodies were diluted and added to the sections; TrkA: 1:1500 (polyclonal, LS-C389392; Nordic BioSite), $p75^{NTR}$: 1:2000 (polyclonal, ABIN1917233; Antibodies Online) and BAX: 1:800 (AF820; R&D systems). Rabbit IgG (X0903; Dako) was run in parallel on all sections as a negative isotype control, using identical protein concentrations as for the primary antibodies. The sections were incubated at 4°C overnight, thereafter incubated with secondary antibody (EnVision K4003; Dako) for 30 min and stained with

3,3′-diaminobenzidine tetrahydrochloride (DAB) in organic solvent for 2 or 6 min, respectively, for TrkA and $p75^{NTR}$/BAX. Nuclei were counter-stained with Mayer's hematoxylin. Finally, sections were dehydrated and mounted. Equine spinal cord was used as a positive control for TrkA and $p75^{NTR}$, a lymph node with malignant lymphoma was used as a positive control for BAX.[29]

All slides were stained simultaneously for each antibody, to decrease batch-to-batch variation in staining outcomes.

For estimation of the amount of positive synoviocytes in each sample, 100 cells in each of 10 randomly selected high power fields (HPF, ×60 magnification) were evaluated for positive intranuclear immunohistochemistry staining.[30,31] When there were not enough cells in one HPF to fill the quota, counting was continued in an immediately adjacent field. Slides were coded to decrease the risk of evaluator bias. In addition, to evaluate total staining (cytoplasmic and nuclear), slides were analyzed with ImageJ software (Fiji Downloads, ImageJ, National Institute of health, Bethesda, MD). Ten HPF (×40 magnification) were photographed from each slide.[31] Images were colour deconvoluted to separate the DAB positive areas. The software set the thresholds and the same settings were used for all images within the same antibody staining. Relevant areas containing synoviocytes were manually annotated, avoiding areas of vessels or subsynovial fibrous tissue. The mean percentage (%) stained area was then calculated.

2.4 | Statistical analysis

Statistical analyses were performed using a commercial statistical software program, JMP Pro 16.0 (JMP Nordics). Residuals were confirmed to be normally distributed. The total synovitis score and the percentage of stained nuclei or total area staining in the four clinical groups were compared using analysis of variance and least square means estimates. Total synovitis score or percentage of positive staining was selected as the response variable with clinical diagnosis selected as model effect. Tukey's test was used for testing pairwise differences. In addition, fibrosis scores were compared between clinical groups and total and nuclear staining in groups based on total synovitis score or fibrosis score were compared as described above. The same analysis was used to investigate the impact of sex. Correlation between age and positive staining was tested by bivariate analysis.

The results are presented as mean ± *SD* and significance level was set at $p < 0.05$.

3 | RESULTS

3.1 | Horses

Sampled horses were 2–12 years old and four different breeds were represented. Stallions, geldings and mares were sampled. A total of 17 carpal joints were included in the study; five healthy, four with synovitis and macroscopically normal articular cartilage, four with synovitis and articular cartilage damage and four with synovitis and intra-articular fractures with associated articular cartilage and subchondral bone damage. Three samples were obtained from radiocarpal joints, and the rest were sampled from intercarpal joints. All horses with synovitis were clinically lame at the time of sampling. For demographic data, see Table S1.

3.2 | Synovial membrane grading

The healthy joints had lower total synovitis scores than the OA joints ($p < 0.01$), but there were no statistical differences for mean total synovitis score between the OA groups ($p = 0.60$). Fibrosis scores were not significantly different between the synovitis groups ($p = 0.16$). Total scores and fibrosis scores for the different groups are presented in Figure 1. The complete synovitis scoring of the synovial membranes is presented in Table S2.

3.3 | Immunohistochemistry

The % positive nuclear staining for TrkA was higher in horses with synovitis only and in horses with synovitis and articular cartilage changes, compared to healthy synovial membranes. The staining in horses with intracarpal fractures was inconsistent with scarce staining in some individuals and abundant staining in others (Figures 2–4 and Figure S3).

The % positive nuclear staining for $p75^{NTR}$ was higher in horses wit more advanced disease of the cartilage; the group with synovitis and cartilage changes as well as the carpal fracture group had significantly more abundant nuclear staining compared to the healthy group (Figures 2 and 3 and Figure S3).

The % positive nuclear staining for BAX was higher in all synovitis groups compared to healthy synovial membranes (Figures 2 and 3 and Figure S3).

There were no statistically significant differences in total TrkA, $p75^{NTR}$ or BAX staining between OA groups (nuclear + cytoplasmic area stain evaluated by ImageJ analysis).

When horses were grouped according to the histologic synovitis scores instead of according to the clinical diagnosis, TrkA, $p75^{NTR}$ and BAX nuclear staining was significantly increased in synovial membranes with total synovitis scores >5 compared to those with scores <5. When groups were based on fibrosis score only, nuclear TrkA staining was increased in groups with scores ≥2, compared to the group with scores 0–1. For $p75^{NTR}$ and BAX, nuclear staining was only significantly increased in the group with the highest fibrosis scores of 3–4 (Figure 5). There were no statistical differences in total staining (nuclear + cytoplasmic area stain) between groups (data not shown). There was no correlation between age and % staining and there was no difference due to sex for any of the receptors (data not shown).

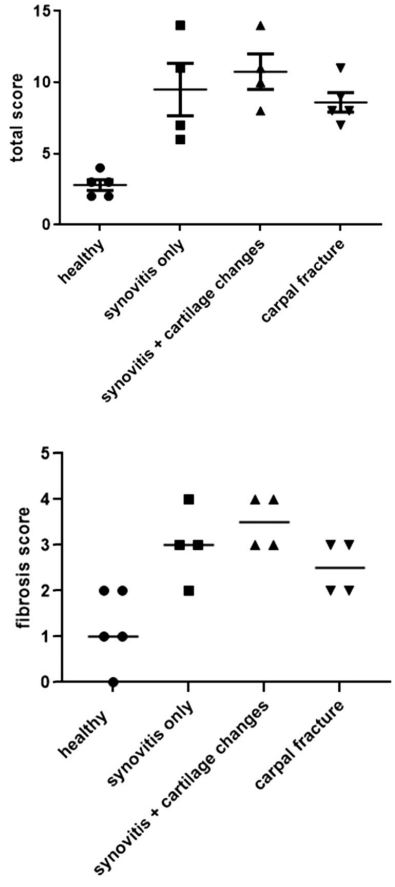

FIGURE 1 Total synovitis score and fibrosis score for the healthy and OA groups classified according to McIlwraith et al.[28] Bars show means ± *SD*. Mean total score for healthy joints was lower than means for OA joints ($p < 0.01$) but the OA group means did not differ from each other ($p = 0.50$). Mean fibrosis score was lower for healthy joints than for OA joints ($p < 0.001$) but the OA group means did not differ from each other ($p = 0.16$). OA, osteoarthritis

4 | DISCUSSION

This is the first study to show NGF receptor expression in equine synovial membranes, and to show differences in receptor expression in different stages of OA.

TrkA staining was increased in early OA disease stages represented by synovitis without gross articular cartilage changes, whereas $p75^{NTR}$ increases were most prominent in later stages with articular cartilage damage and synovial fibrosis. These results are in line with previous findings that expression of inflammatory markers in synovial membranes differ between early and late OA.[32]

Quantification of nuclear staining was utilized as a way of indicating receptor activation[33,34] and this was increased for TrkA in early OA disease stages without macroscopic cartilage lesions. The $p75^{NTR}$ receptor staining was only significantly increased in synovial membranes from joints with macroscopic articular cartilage changes, a later stage of OA.

Our results showed an increased expression of BAX in all synovitis groups, compared to healthy synovial membranes. NGF binding to $p75^{NTR}$ without involvement of TrkA can activate the c-Jun N-teminal kinase (JNK) pathway.[9] JNK causes a conformational change and polymerization of cytoplasmic BAX. These polymers form pores in the mitochondrial membranes, leading to the release of cytochrome C and subsequent activation of caspases, initiating apoptosis.[8] However, it was not the synoviocyte cytoplasm but the nucleus that showed a marked increase in BAX staining in diseased synovial membranes. The apoptotic effects of BAX have been shown to vary in different cell types[35] and regulatory functions of cell growth and differentiation have been suggested for intranuclear BAX.[36]

Immunohistochemistry provides a snapshot image of the receptor expression in the synovial membranes. Although epitopes are identified, it is not possible from the present study to discern the activation of downstream signaling. Not only membrane bound receptors, but also cleaved internal fragments can be biologically active in the complex signaling systems. $p75^{NTR}$ can bind to several neurotrophins (e.g., BDNF, NT-3, and NT-4) and interacts with a multitude of coreceptors, creating varied downstream signaling events (reviewed in[37,38]). TrkA is more specific for NGF and is activated by phosphorylation of the cytoplasmic domain after binding. The NGF-bound TrkA is also internalized via endocytosis and can be transported retrograde toward the soma in nerve cells (reviewed in Marlin & Li[39]). This can promote several downstream signaling pathways and TrkA receptor stimulation can increase cell proliferation in synoviocytes.[14] TrkA binding to NGF is enhanced by the presence of $p75^{NTR}$.[40] It is possible that intranuclear staining of $p75^{NTR}$ represents pathways with TrkA interaction, as well as with other co-receptors. The multiple possible pathways could be the reason why differences were not detected between groups when evaluated for total staining, as this will represent receptors in various stages; nuclear (active signaling), cytoplasmic (active signaling and/or endocytosed receptor fragments) and membrane bound receptors (active signaling as well as unbound, inactive receptors).

In immunohistochemistry, batch-to-batch variation in staining intensity can vary considerably.[41] As the TrkA and $p75^{NTR}$ receptor expression has not previously been quantified in different stages of OA, an effort was made to only compare within-batch stainings for a specific antibody. Due to this, the sample sizes are small and it is possible that some differences in receptor expression between healthy and inflamed synovial membranes were missed.

Receptor expression in different stages of OA warrants further research and failure to acknowledge the complexity of NGF receptor signaling may create problems in a future clinical setting.

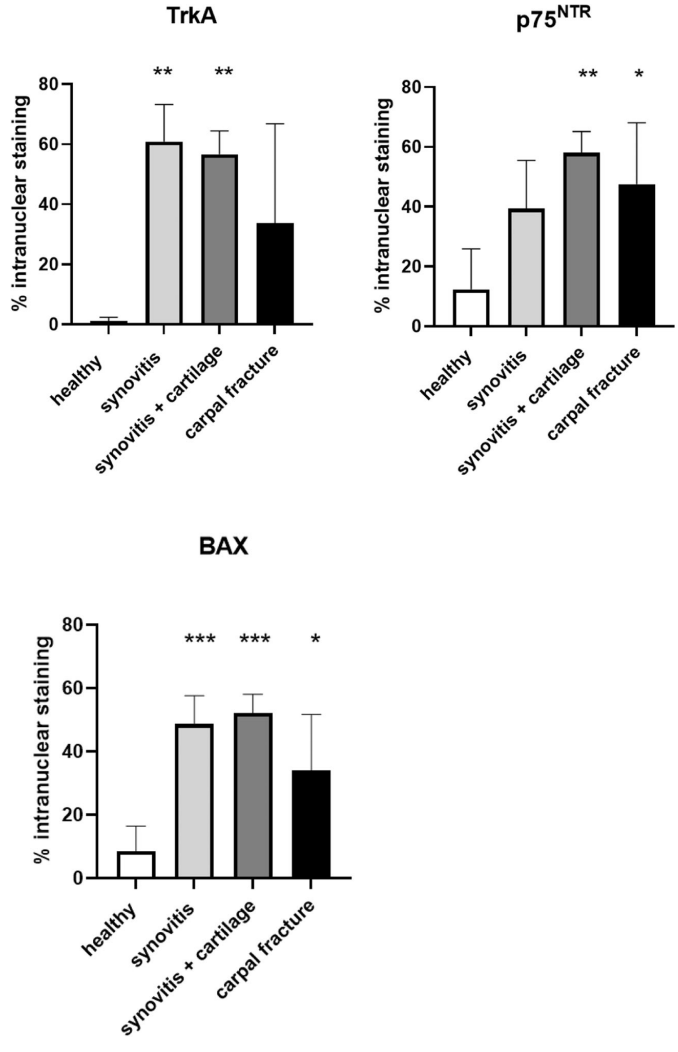

FIGURE 2 TrkA: Quantification of nuclear staining of TrkA. Staining is increased in the groups with synovitis (61% ± 12) and synovitis + articular cartilage changes (57% ± 8) compared to healthy (1% ± 1). $p75^{NTR}$: Quantification of nuclear staining of $p75^{NTR}$. Staining is increased in the synovitis + articular cartilage changes group (58% ± 7) and in the intracarpal fracture group (48% ± 21) compared to healthy (12% ± 14). BAX: Quantification of nuclear staining of BAX. Staining is increased in all synovitis groups (49% ± 9 for synovitis only, 52% ± 6 for synovitis and articular cartilage changes and 34% ± 18 for intracarpal fractures) compared to healthy (9% ± 8). There were no statistical differences in mean nuclear staining between different synovitis groups for either receptor. Bars indicate *SD* from mean. Asterisks indicate statistically significant difference from healthy; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. BAX, Bcl-2-Associated X protein; $p75^{NTR}$, pan-neurotrophin receptor; TrkA, tyrosine kinase A

Anti-NGF antibody has shown promise as a novel treatment for OA-associated pain in patients refractory to traditional analgesic treatments.[42] In 2010, trials were temporarily halted due to adverse events related to unexplained, rapidly progressing OA in some test subjects. The risk of occurrence had a dose-response relationship and also seemed to be increased with concurrent NSAID treatment.[43] Phase III trials have since been resumed with measures such as restricted concurrent NSAID use, close monitoring of OA progression and use of lower treatment doses. Despite this, 1–3% of the subjects in the treatment groups experienced rapidly progressing OA in one phase III trial with long-term follow-up.[42] The reason for this is still unclear and no drugs have so far (2022) been approved for clinical use. If the relative TrkA/$p75^{NTR}$ receptor expression in different stages of OA varies, or if individual differences in receptor expression occur, the effect of inhibiting NGF could also be varied. Anti-NGF therapy in a state of TrkA receptor dominance could inhibit signaling of growth and repair, while it could inhibit apoptosis in the presence of abundant $p75^{NTR}$ receptor expression, as has been shown in other cell types.[2]

Performing the study on clinical, client-owned cases had the disadvantage that it was not possible to fully control for factors such as prior treatment or level of training, and microscopic evaluation of articular cartilage was not possible. Using client-owned horses instead of experimental animals also limited the information on disease duration. It is very common for owners and trainers to be unaware of mild lameness in horses[44] and as this

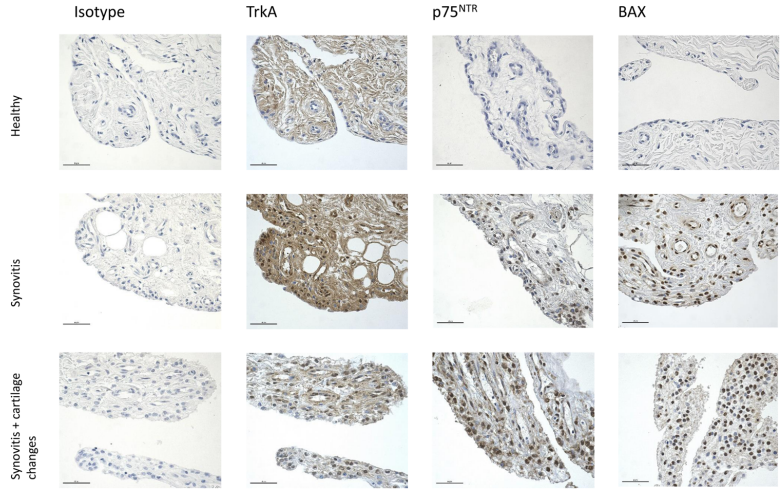

FIGURE 3 Expression of TrkA, p75^{NTR} and BAX in synovial membranes from a healthy horse, a horse with synovitis without visible articular cartilage changes, and a horse with synovitis + visible articular cartilage changes. Isotype controls are selected from the TrkA staining but are representative of isotypes from all experiments (×40, scale bar: 50 μm).BAX, Bcl-2-Associated X protein; p75^{NTR}, pan-neurotrophin receptor;TrkA, tyrosine kinase A

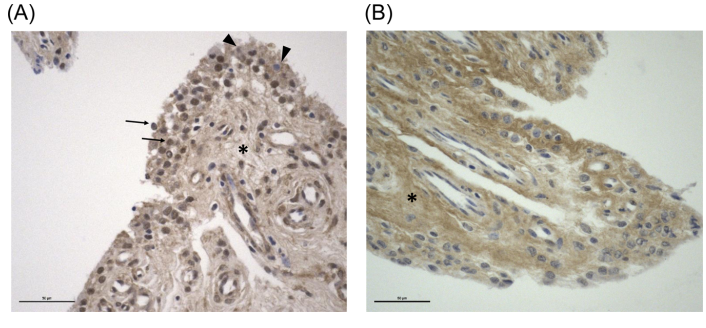

FIGURE 4 Expression of TrkA in synovial membranes from two horses with intracarpal fractures representing different nuclear staining patterns. (A) Synoviocytes with abundant intranuclear staining (arrows) and cytoplasmic staining only (arrowhead). (B) Synoviocytes with cytoplasmic staining only. In both (A) and (B) there is a marked extracellular matrix staining (asterisks). Total synovitis score for the two horses were 9 (left) and 8 (right) (×40, scale bar: 50 μm). BAX, Bcl-2-Associated X protein; p75^{NTR}, pan-neurotrophin receptor;TrkA, tyrosine kinase A

could create information bias, collection of data on disease duration was not attempted. For six horses a full treatment history could not be obtained hence it is possible that some of these individuals had received intra-articular treatments some weeks before surgery. However, all OA horses were lame at the time of sampling, and synovial membrane histology confirmed the presence of synovitis. The carpal fracture group had a high degree of intranuclear p75^{NTR}, but total p75^{NTR} stain was inconsistent (Figure S3). This could be due to individual variation or to the fact that the p75^{NTR} receptor has many different ligands that it may respond to, making expression less relatable to NGF dynamics only. However, intranuclear TrkA staining also varied markedly in this group as demonstrated in Figure 4. It is possible that, although the carpal fracture group shared a common phenotype and all had evidence of synovitis, the pathogenesis and disease duration for the fractures could be different. Horses were of different breeds and two of the horses in the carpal fracture group were used for racing whereas the two Warmbloods would have been intended for lighter work such as pleasure riding. Some horses could have had progressive OA with secondary fractures, whereas some could have been traumatic fractures caused by excessive forces during exercise.[45] The groups were considered too small

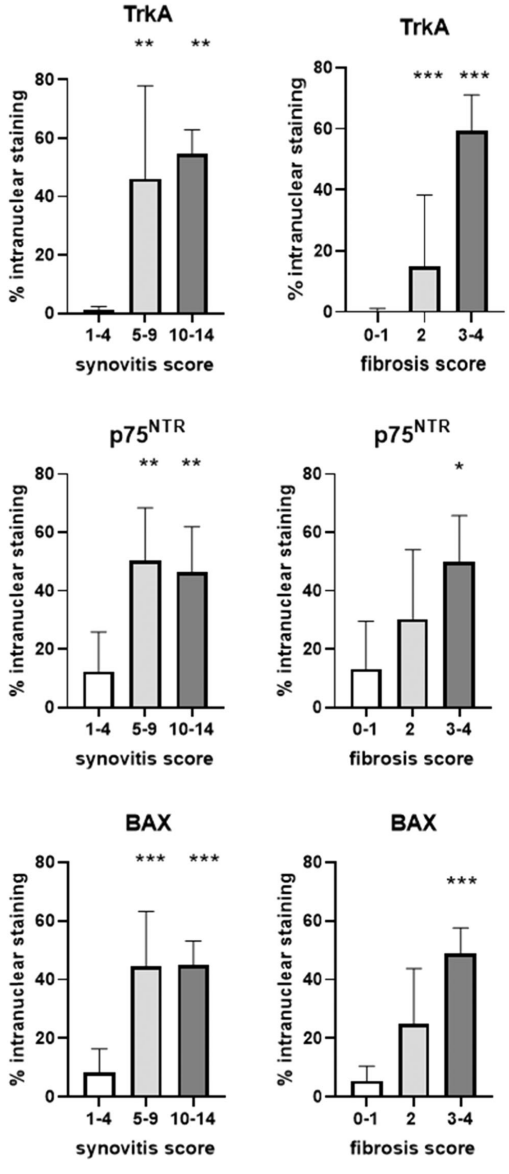

FIGURE 5 Nuclear staining for TrkA, p75^{NTR} and BAX in groups with increasing synovitis severity (synovitis score) and with increasing fibrosis (fibrosis score). Staining was significantly increased for all three in horses with synovitis compared to healthy horses. TrkA was increased in all horses with increased fibrosis, but p75^{NTR} and BAX was only significantly increased in the horses with moderate to severe fibrotic changes. Bars indicate *SD* from mean. Asterisks indicate statistically significant difference from synovitis score 1–4 or from fibrosis score 0–1; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. BAX, Bcl-2-Associated X protein; p75^{NTR}, pan-neurotrophin receptor; TrkA, tyrosine kinase A

to provide conclusive data on the impact of breed on receptor expression.

There are substantial advantages of using clinical cases as supposed to creating adjuvant inflammation for the study of receptor expression when aiming to make inferences to human OA. Creating adjuvant inflammation will produce an acute inflammatory process in a previously healthy joint. This may cause different pathological processes than those represented in naturally occurring OA disease. The same applies to models of traumatic OA, where acute destabilization and/or joint surface incongruity in a previously healthy joint is not equal to spontaneous and often slowly progressing disease involving all joint structures. Innate immunity is involved in disease progression in OA and the systemic inflammatory processes of chronic OA[46] is not present in OA models using healthy research animals. The clinical cases in the present study represent naturally occurring OA disease in varied stages and include individual variation. Also, no animals were subjected to any procedures for the sole purpose of research, in line with the 3Rs.

In summary, the results of this study show that the NGF receptor and BAX expression in equine synovial membranes is increased in synovitis, and that there may be disease stage-related differences in the relative receptor expression. This study provides information that can be used for planning of study protocols and sample size calculations for further research on TrkA and p75^{NTR} expression and signaling pathways in human OA.

AUTHOR CONTRIBUTIONS

All authors: contributed to sample collection, read and approved the final version. **Anna Kendall**: performed the laboratory work and all authors analysed the data and contributed to drafting the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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The expression of nerve growth factor in healthy and inflamed equine chondrocytes analysed by capillary western immunoassay

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ABSTRACT

Nerve Growth Factor (NGF) is a signalling molecule for pain and inflammation. NGF is increased in synovial fluid from osteoarthritic humans and animals, compared to healthy controls. Monoclonal antibody therapy directed against NGF has been approved to treat pain in osteoarthritic dogs but despite many years of trialling, therapy has not been approved for human use. One reason for this is that adverse reactions with rapidly progressing osteoarthritis has occurred in some individuals. More detailed knowledge of NGF expression in joints is needed. In this study, capillary-based Simple Western was used to analyse NGF in cultured equine chondrocytes. Chondrocytes were collected post mortem from three macroscopically healthy intercarpal joints and three intercarpal joints with mild osteoarthritic changes. The chondrocytes were expanded to passage one and seeded in chondrogenic medium to maintain the phenotype. On day four, cells were either stimulated with LPS or kept untreated in medium. All cells were harvested on day five. Wes analysis of lysates did not show mature NGF but two proforms, 40 and 45 kDa, were identified. Results were confirmed with western blot. The same proforms were expressed in chondrocytes from healthy and osteoarthritic joints. Acute inflammation induced by LPS stimulation did not change the forms of expressed NGF. Capillary Simple Western offers a sensitive and sample-sparing alternative to traditional western blot. However, confirmation of peaks is imperative in order to avoid misinterpretation of findings. In addition, in this case the method did not offer the possibility of quantification advertised by the manufacturers.

1. Introduction

Nerve Growth Factor (NGF) has since its discovery (Levi-Montalcini and Hamburger, 1951) been implicated to play a part in many inflammatory, neurodegenerative and painful signalling processes (Denk et al., 2017; Minnone et al., 2017a). Synovial fluid from osteoarthritic human, canine and equine joints have higher concentrations of NGF compared to synovial fluid from healthy joints (Isola et al., 2011; Kendall et al., 2021; Montagnoli et al., 2017) and NGF has been found to increase in human osteoarthritic chondrocytes (Iannone et al., 2002). NGF is produced by human and murine chondrocytes, synovial fibroblasts and macrophages (Ohashi et al., 2021; Pecchi et al., 2014; Takano et al., 2016; Takano et al., 2017) and immune cells such as human monocytes and mast cells express NGF receptors (Ehrhard et al., 1993; Nilsson et al., 1997). Morevover, NGF can induce angiogenesis (Nico et al., 2008) and was found to stimulate intra-articular nerve sprouting in a murine arthritis model (Ghilardi et al., 2012). These findings indicate that NGF plays a role in osteoarthritis-associated pain and inflammation. In 2021, monoclonal NGF-antibody therapies were launched to treat pain associated with osteoarthritis (OA) in dogs and cats. In humans, NGF-antibody therapy for OA related pain has been trialled for many years (Berenbaum et al., 2020) but has to date (2022) not received FDA approval. Adverse events in a small percentage of the treated patients with rapidly progressing OA caused a temporary halt in trials in 2010 (Hochberg, 2015). Trials have since been continued but he reason for these adverse reactions is still unknown.

NGF is secreted both in a mature form, mNGF (molecular weight of 13 kDa) and as a larger precursor, proNGF (Fahnestock et al., 2004).

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Several different proNGF forms have been described in laboratory animals, with molecular weights between 17 to >100 kDa reported (Lee et al., 2001; Reinshagen et al., 2000; Soligo et al., 2015). The significance of the differences in specific proforms is not known. Initially, the precursor part was thought to be biologically inactive and important only to ensure proper folding and processing into mNGF (Edwards et al., 1988). This has since been disproven, and proNGF and mNGF have been shown to have varying functions due to different receptor binding properties (Ioannou and Fahnestock, 2017; Lee et al., 2001). Mature NGF is primarily neurotrophic whereas proNGF can be either neurotrophic if it interacts with the TrkA receptor, or apoptotic if it binds to the $p75^{NTR}$ receptor in combination with the co-receptor sortillin (Ioannou and Fahnestock, 2017). Altered mNGF/proNGF balance with increases in proNGF have been found in neurodegenerative diseases such as Alzheimer (Peng et al., 2004) and diabetic retinopathy (Mysona et al., 2015), as well as in juvenile inflammatory- and rheumatoid arthritis (Minnone et al., 2017b).

Commercial NGF-ELISA kits and antibodies are often directed at mNGF and can therefore detect a mixture of mNGF and proNGF in biological samples. (Malerba et al., 2016; Soligo et al., 2015). This has implications for research in OA, where most published studies have focused on determining NGF without differentiating between mature- and proforms (Aloe et al., 1992; Montagnoli et al., 2017; Stoppiello et al., 2014). It is currently unknown what role the mNGF/proNGF balance plays in the different OA disease stages.

The ProteinSimple® capillary western immunoassay method may be appropriate for further research into the expression of different forms of NGF. The method is based on capillary protein separation by molecular size. In a fully automated system, samples are loaded onto capillaries, separated and incubated with primary and secondary (HRP-conjugated) antibodies. Chemiluminescence is produced after addition of luminol-peroxidase and is detected at multiple exposure times. Only small amounts of protein are needed for detection. As analytes are automatically quantified by calculation of the area under the curve for the detected peaks, the system provides the opportunity for both quantitative and qualitative analysis.

The aim of this study was to investigate which forms of NGF are expressed in healthy and osteoarthritic equine chondrocytes. The aim was also to determine how acute inflammation induced by lipopolysaccharide (LPS) influences NGF expression. The hypothesis was that not only mNGF, but also proNGF would be expressed and that the expression would differ in healthy, acutely inflamed and chronically inflamed chondrocytes.

2. Materials and methods

2.1. Chondrocyte isolation and culture

The chondrocytes were obtained for previous projects (Ley et al., 2011; Skiöldebrand et al., 2019) and horses were euthanized for reasons unrelated to the study.

Articular cartilage samples were collected from six age-matched horses within 48 h post mortem; three horses had mild macroscopic osteoarthritic cartilage changes of the dorsal radial facet and three were without visible lesions. (For demographic data, see Supplementary Table 1.) Following aseptic preparation, the joint was incised and the articular cartilage was inspected macroscopically. The macroscopic classification of the joints was performed by a board certified veterinary pathologist (ECVP). Cartilage on the dorsal aspect of the radial facet of the third carpal bone was incised with a scalpel down to the bone and full-thickness cartilage samples were collected.

Samples were placed in sterile saline (0.9% NaCl) solution with gentamicin sulfate (50 mg/l) and amphotericin B (250 μg/ml). The cartilage samples were transported chilled (approx. 5 °C) to the laboratory. Isolation and expansion of chondrocytes were performed as previously described (Ley et al., 2011). Briefly, the chondrocytes were expanded to passage 1 and then seeded at 20,000 cells/cm^2 in chondrogenic medium to maintain the phenotype. On day 4, cells were stimulated with LPS (10 ng/ml, *Escherichia coli* 055:B5; List Biological Laboratories, Campbell, CA, USA) or kept untreated (controls) for 24 h. Cells were grown to confluence and harvested on day 5 and immediately frozen and stored at −80 °C until further analyses.

2.2. Protein determination

A protein determination assay was performed in accordance with the manufacturer's instructions using a detergent-compatible protein assay (Bio-Rad, Hercules, CA, USA) based on Lowry's method. The standard (0–4 mg/ml BSA) and samples were mixed with the reagents and incubated for 15 min at room temperature. The absorbance was read at 750 nm with a VersaMax microplate reader and analysed using SoftMax Pro 4.8 (Molecular Devices, Sunnyvale, CA, USA).

2.3. Wes

Capillary western analyses were performed on the ProteinSimple Wes system (ProteinSimple, San Jose, CA, USA), according to the manufacturer's instructions using a 12–230 kDa Separation Module (SM-W004) and either the Anti-Rabbit Detection Module (DM-001) or the anti-rat secondary antibody (HAF-005, R&D systems, Minneapolis, MN, USA) depending on the primary antibody used.

Lysates were thawed and diluted in 0.1× Sample Buffer (ProteinSimple) to an appropriate concentration and mixed with 5× Fluorescent Master Mix (containing 5× sample buffer, 5× fluorescent standard, and 200 mM DTT) and heated at 95 °C for 5 min. Four parts of diluted sample were mixed with one part Master Mix.

The denatured samples, biotinylated ladder, antibody diluent, primary antibody, HRP-conjugated secondary antibody, chemiluminescent substrate, and wash buffer were pipetted into the assay plate according to the manufacturer's instructions, and loaded on Wes. The separation electrophoresis and immunodetection steps were then fully automated.

Instrument default settings were adjusted to maximize the protein sensitivity and protein signal: stacking gel loading time 21 s, sample loading time 12.6 s, separation at 475 V for 30–35 min, blocking reagent 5 min, primary antibody 60 min, secondary antibody 30 min, luminol-peroxide chemiluminescence detection for ~15 min (exposures of 1-2-4-8-16-32-64-128-512 s).

The resulting electropherograms were inspected and automatic peak fits/detections were manually corrected when required. In order for a peak to be considered, a signal to noise (S/N) ratio >10 was required (Beekman et al., 2018). A shift in apparent molecular weights of ±10% was accepted as representation of the same peak. The intra-run CV for area under the curve was set to ≤15% according to information from the manufacturer (data not shown).

Data analysis was performed with the Compass Software (ProteinSimple).

Results in Compass are in the form of electropherograms, where the software reports data as graphs showing chemiluminescence versus apparent molecular weight (MW). Apparent MW is determined by using the peak signals from the protein standard ladder. The fluorescent standards present in the 5× Master Mix are used to adjust for any differences in migration within the capillaries. The software calculates peak areas using the Gaussian method and these peak areas were used to compare samples.

2.3.1. Primary antibodies

Two NGF antibodies were selected: ab6199 (rabbit polyclonal, Abcam, Cambridge, UK) and MAB2562 (rat monoclonal, R&D Systems). For ab6199, the immunogen was mouse native salivary gland β-NGF hence the antibody could potentially recognise both mature- and proforms of NGF. The MAB2562 immunogen was *E. coli*-derived recombinant human proNGF, AA19–241, hence the antibody is specific for

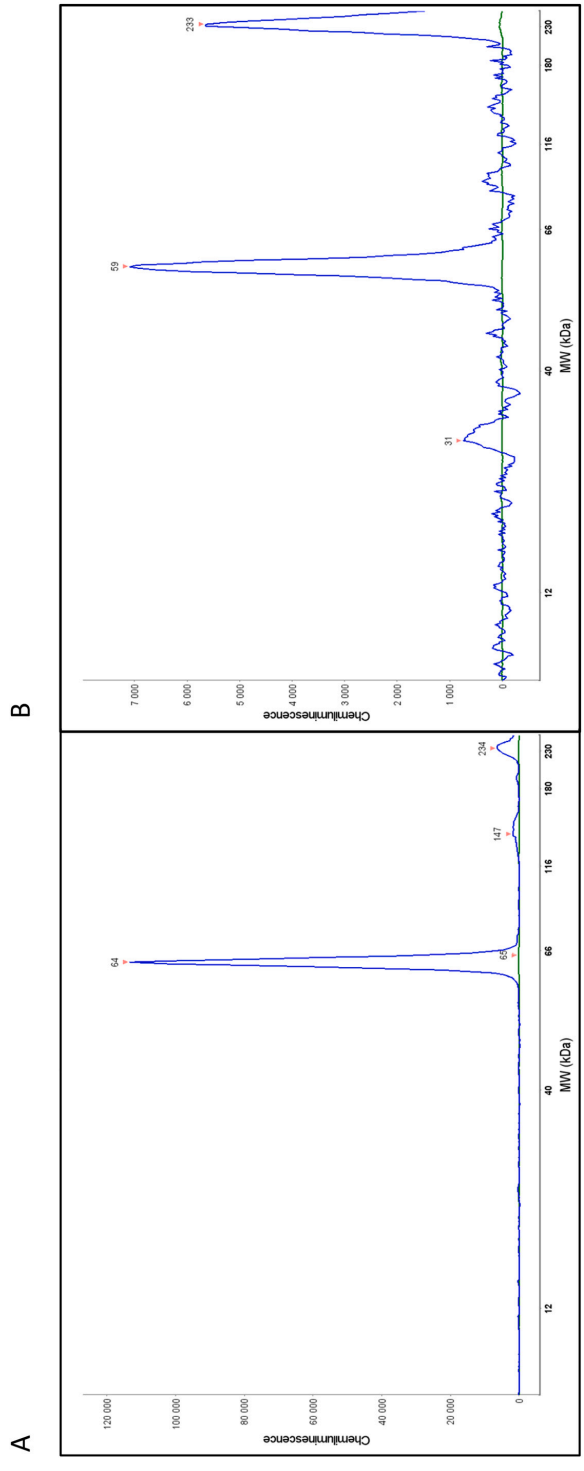

Fig. 1. A. Peaks detected by anti-NGF antibodies ab6199 (blue) and MAB2562 (green) in a solution of 0,015 mg/ml human serum albumin. There is unspecific binding to albumin with ab6199 but not with MAB2562. B. Peaks detected with the same antibodies in a solution of 0,007 mg/ml equine IgG protein. There is unspecific binding to IgG heavy and light chain with ab6199 but not with MAB2562. Peaks detected above 230 kDa are non-migrated proteins and are not considered significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proNGF only. Sequence identity between full-length human and full-length equine NGF is 94% (Uniprot BLAST). As an additional control the polyclonal antibody was compared to a no longer commercially available NGF antibody (H20, Santa Cruz Biotechnology, Heidelberg, Germany) that has previously been used in published research (Amagai et al., 2016; Bruno and Cuello, 2006; Malerba et al., 2016; Soligo et al., 2015). The two antibodies ab6199 and H20 were found to result in peaks at comparable molecular weights in the Wes system (data not shown).

2.3.2. Linearity and saturation

To ensure optimal sample protein load, a series of sample dilutions were made with a constant antibody concentration, to determine the linear range of the assay. Sample concentration for further analysis was performed within the linear range.

To determine optimal antibody concentration, our preferred sample concentration was tested against different antibody dilutions to determine the saturation point for the antibody. If saturation could not be reached, optimal antibody concentration was based on electropherogram characteristics such as baseline, S/N ratio and baseline to height ratio.

For detailed information on linearity and saturation see Supplementary table 2.

2.3.3. Controls

As many different molecular weights have been described for NGF, the NGF antibodies were run with recombinant NGF protein (256GF/CF, R&D Systems), protein load 0.00625–0.1 mg/ml, in order to better determine the specific NGF peaks.

A no lysate control (no protein control) was performed to check for primary antibody cross reactions. A no primary antibody control was also performed to check for cross reaction with the secondary antibody. In addition, all antibodies were tested against pure equine IgG (Bio-Rad), protein load 0.003–0.015 mg/ml and human serum albumin (Europa Bioproducts Ltd., Ipswich, UK), protein load 0.015 mg/ml, in order to identify any unspecific peaks.

2.4. Western blot

Analysis was carried out according to standard protocols. Protein concentrations were determined as described above. Western blot samples were prepared with 4× Laemmli sample buffer (Bio-Rad) with 10% β-mercaptoethanol, and sample in PBS. Three parts of diluted sample was mixed with one part sample buffer.

The solution was boiled at 95 °C for 5 min and samples were centrifuged for 1 min at 16,000 *g*. Nine microgram sample protein was loaded in each well of a pre-cast Mini-protean TGX stain-free gel (Bio-Rad) and run at 300 V for 15 min. The gel was activated and the blot was transferred to a low fluorescence PVDF membrane (Bio-Rad). After transfer the membrane was imaged for later protein normalisation. After blocking for 30 min in EveryBlot buffer (Bio-Rad), the membrane was incubated with primary antibody (MAB2562, 1:1000 dilution) at 4 °C overnight. Thereafter, the membrane was washed four times and incubated with secondary antibody (HAF-005, 1:1000) at room temperature for one hour. The membrane was once again washed four times and immediately imaged with the Chemidoc Touch Imaging System (Bio-Rad). Protein normalisation was performed by the Image Lab software (Bio-Rad) according to the manufacturer's instructions.

3. Results

3.1. Wes linearity and saturation

Linearity was obtained for both antibodies, however, saturation could not be obtained despite multiple experiments with increasing antibody concentrations up to 1:5 dilution, and decreasing protein concentrations until the disappearance of the specific peaks. Due to this,

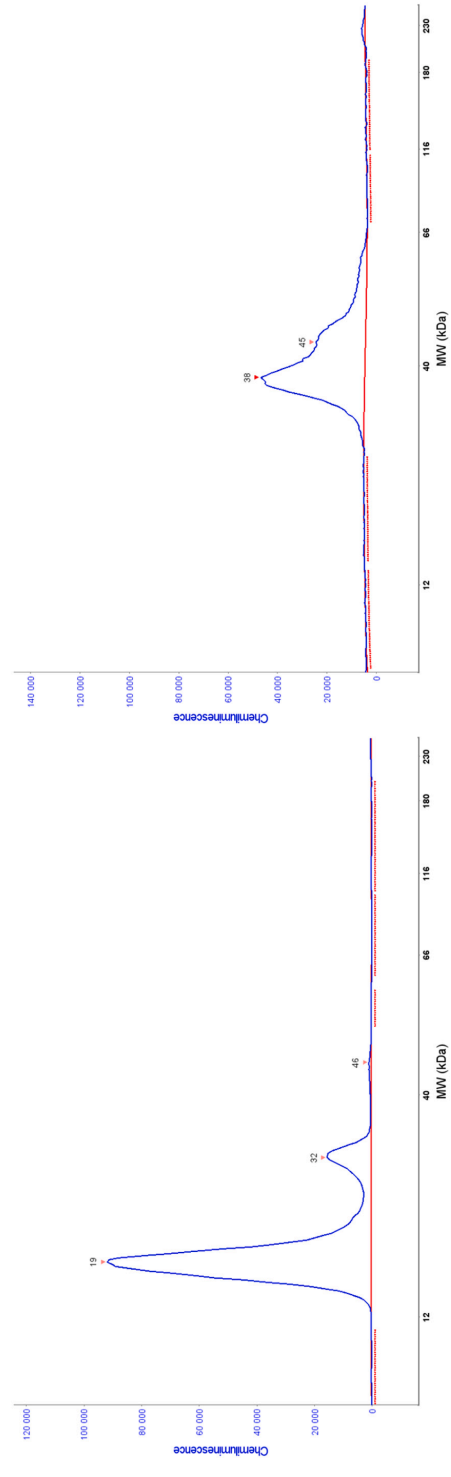

Fig. 2. Wes results for recombinant Nerve Growth Factor.
A. At 0.01 mg/ml protein load, the primary antibody ab6199 detects NGF at molecular weights 19, 32 and 46 kDa. The 19 kDa peak is interpreted as mNGF.
B. Protein load is increased to 0.1 mg/ml and primary antibody MAB2562, specific for proNGF, detects peaks at 38 and 45 kDa.

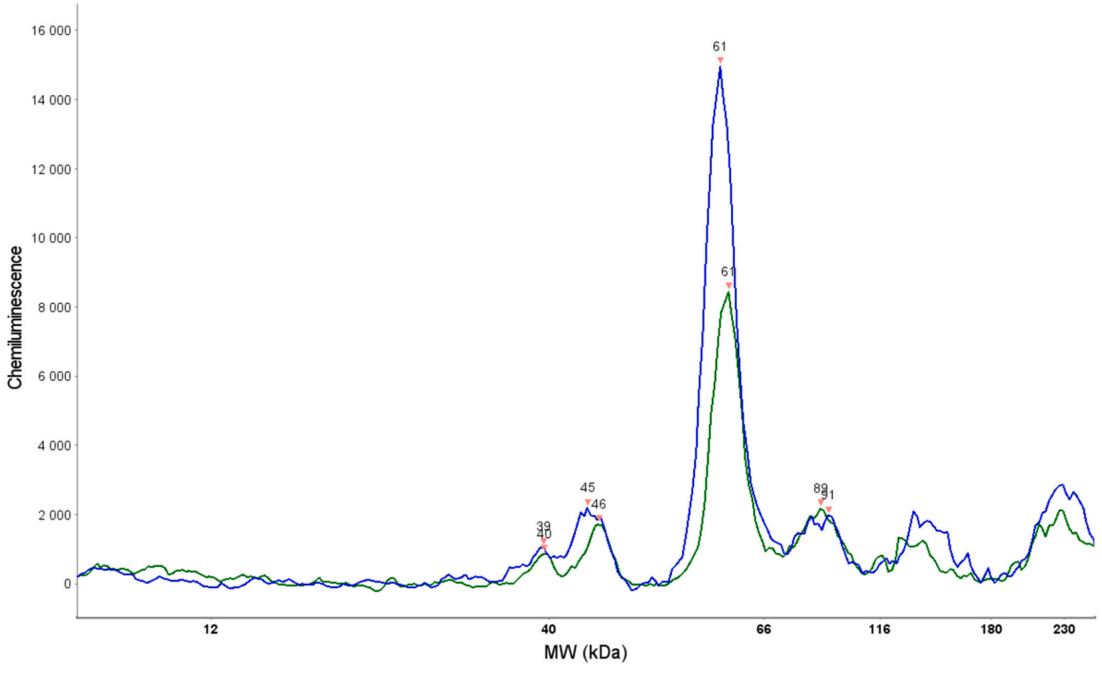

Fig. 3. 40 and 45 kDa ProNGF detected in OA chondrocytes by polyclonal antibody ab6199. The green curve shows the LPS stimulated sample and the blue curve shows the control. Peaks detected at 61 and around 90 kDa are not specific for NGF. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quantification of NGF expression could not be performed. For information on the protein- and antibody concentrations used, see Supplementary table 2.

3.2. Wes controls

No unspecific binding was detected for the secondary antibodies, however, ab6199 showed unspecific binding to equine IgG at 31 and 59 kDa, and substantial unspecific binding to albumin at 61–64 kDa. MAB2562 showed no unspecific binding to either IgG or albumin (Fig. 1).

3.3. Nerve growth factor in Wes and western blot

When run with recombinant protein, peaks were detected by the polyclonal NGF antibody (ab6199) at 19, 32 and 46 kDa. The experiment was repeated with monoclonal proNGF specific antibody (MAB2562) at a higher protein concentration, and this time peaks were detected at 38 and 45 kDa, confirming that these peaks represented proforms of NGF (Fig. 2). In lysates, both antibodies detected consistent and specific peaks at 39/40 and 45/46 kDa (Figs. 3 and 4). No mature NGF could be detected with ab6199 at the concentrations used in this experiment. Peaks detected at other molecular weights could not be confirmed as NGF peaks as the ab6199 antibody showed various degrees of unspecific binding to albumin and IgG at corresponding molecular weights (Fig. 1). The same proNGF forms were excreted in both healthy and OA chondrocytes, and this was not altered by LPS stimulation. Bearing in mind that there was not saturation, no differences above the previously reported CV of 15% were seen in peak height between control cells or LPS stimulated cells, or between healthy and OA chondrocytes. Western blot confirmed the peaks at 40 and 45 kDa (Fig. 4).

4. Discussion

This is the first study to determine what specific forms of NGF are produced by chondrocytes. The results show that both healthy and osteoarthritic equine chondrocytes express NGF in 40 and 45 kDa proforms. Acute inflammation does not alter the expressed forms of NGF. The mature form of NGF (molecular weight of 13 kDa) could not be detected in the chondrocytes. These findings are in line with previous research in human patients with rheumatoid arthritis (Minnone et al., 2017b), where proNGF and not mNGF was predominant in synovial fluid and synoval fibroblasts. ProNGF with molecular weights around 40–45 kDa has been detected in dorsal root ganliga and colon of rats (Reinshagen et al., 2000), human and rodent cerebral cortex (Bruno and Cuello, 2006; Lobos et al., 2005), and commercial cell lines (Seidah et al., 1996). Moreover, not all cells in these studies expressed detectable levels of mature NGF which is in line with the results of Wes analysis of equine chondrocyte lysates.

Different cells have different NGF expression in regards to both the mNGF/proNGF expression and the molecular weights of the expressed proNGF. This is likely related to what arsenal of intracellular and extracellular cleavage enzymes are available (Bruno and Cuello, 2006; Seidah et al., 1996). Results from studies on one cell type can therefore not be extrapolated to all cells. In vivo research has shown that human chondrocytes express NGF, and that expression increases with worsening OA (Iannone et al., 2002). Osteoarthritic human and murine chondrocytes stimulated with IL-1β or visfatin in vitro showed increased NGF production (Pecchi et al., 2014) and TGF-β, another molecule in the downstream LPS signalling cascade has been shown to increase NGF production in murine, bovine and human chondrocytes (Blaney Davidson et al., 2015). However, in these studies mNGF was not distinguished from proNGF hence it is not known what NGF form was represented in

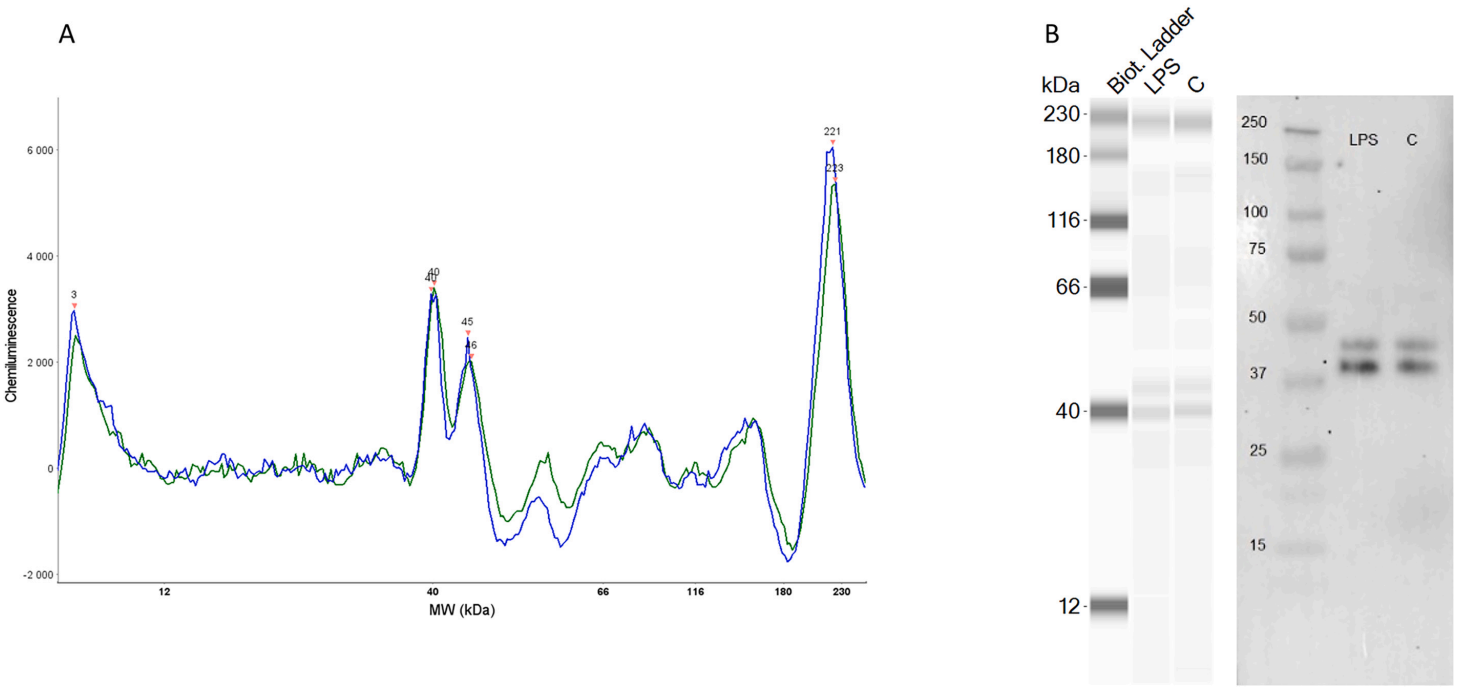

Fig. 4. ProNGF detected in OA chondrocytes by monoclonal antibody MAB2562.
A. Wes results: The green curve shows the LPS stimulated (LPS) sample and the blue curve shows the control (C). Peaks detected above 220 kDa are non-migrated proteins and are not considered significant.
B. Comparison of Wes data and western blot (far right). Both methods show proNGF at 40 and 45 kDa. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the increase. Preliminary results from equine chondrocyte stimulation with IL-1β in our lab did not show changes in what proforms were expressed (unpublished data).

The ELISA method normally offers a quantitative analysis for comparison of sample concentrations of analytes. Most studies investigating NGF concentrations by ELISA have used antibodies that do not distinguish between proNGF and mNGF, but changes in the mNGF/proNGF ratio may be a better marker of disease and disease progression than changes in total NGF concentrations (Soligo et al., 2015). As most antibodies recognising mNGF will also have affinity for proNGF, antibodies specific for proNGF could offer a better alternative for ELISA. However, many different forms of proNGF have been described (Lee et al., 2001; Reinshagen et al., 2000; Soligo et al., 2015) and if proNGF antibodies are used they should preferably recognise the potential proforms in the sample of interest. Adding to these difficulties is that the NGF forms can reciprocally interfere in ELISA analysis, causing substantial variations in assay results depending on the mNGF/proNGF ratio (Malerba et al., 2016).

The Simple Western Systems are marketed as a quick and reproducible alternative to traditional western blots, with the additional advantage of being quantitative (https://www.proteinsimple.com/simple_western_overview.html?gclid=EAIaIQobChMI56nLzefq9gIVr4xoCR05qQ9XEAAYASAAEgLLsfD_BwE reference on March 29th 2022). As molecules are size separated, proNGF can be distinguished from mNGF. The method was tested for NGF analysis in equine chondrocyte lysates in this study. In our hands, the Simple Western was indeed quick and sample sparing. However, it did not allow for quantification as saturation of antibodies could not be achieved. One interesting possibility could be to perform a calibration curve in each run and determine concentrations based on this (Fourier et al., 2019). That method would significantly decrease the number of samples that can be compared within each run and it would also require a recombinant or purified source of appropriate forms of proNGF. Moreover, several attempts were made to analyse NGF in equine serum and synovial fluid on Wes but these experiments were unsuccessful due to substantial unspecific binding to albumin and IgG with all antibodies tested. Interestingly, unspecific binding of ab6199 to IgG was found to be both linear and saturable (data not shown).

The Wes method did show that equine chondrocytes in vitro produce 40 and 45 kDa proNGF, and the types of proforms expressed were not different between healthy and OA chondrocytes. Also, the proforms did not change when cells were subject to an acute inflammatory event by LPS stimulation. Galve-Roperh et al. (1997) showed that LPS at 10 ng/ml induced NGF production in rat astrocytes, but the maximal increase was seen with an LPS dose of 2 μg/ml which is considerably more than was used in the present study. In addition, varied effects are seen with different types and purities of LPS (Parusel et al., 2017). It is possible that the 10 ng/ml LPS dose used in this study was not sufficient to induce changes in NGF production in our model, but the model has previously been shown to increase OA chondrocyte intracellular Ca^{2+} release and expression of glutamate which indicates an inflammatory response (Skiöldebrand et al., 2019).

It is also possible that mNGF would have been detectable at a much higher protein concentration. Soligo et al. (2015) showed that mNGF could be detected in rat brain tissue when the protein load was increased from 20 to 100 μg. The concentration used in Wes (1 mg/ml) was within the linear range and was chosen based on the best performance for baseline and signal to noise ratio. Further increase of protein load up to 1.64 mg/ml did not improve peaks or reveal other proforms but it increased the baseline/background interference. The 9 μg protein load for western blot was the maximum dose that could be loaded equally for all samples when considering the total protein content. The samples used in the present study were pure chondrocyte lysates, likely providing a more concentrated source of analyte than whole tissue lysates.

5. Conclusion

Equine chondrocytes produce 40 and 45 kDa proNGF. If mNGF is produced, the amount is considerably less as no mNGF was detected either in Wes or western blot at the protein concentrations analysed. The finding of only proNGF may have implications for development of future therapies for OA-related pain, as monoclonal antibody therapies may in fact be better directed against the specific proforms produced by chondrocytes. Currently available anti-NGF therapy has an undetermined specificity for the different forms of NGF. In addition to this, the NGF receptor expression (TrkA and $p75^{NTR}$) and thereby the signalling pathways may also differ between disease stages (Kendall et al., 2022). Therefore, further definitions of NGF expression (mNGF/proNGF balance) and receptor expression, as well as more detailed definition of individual osteoarthritic disease stages may provide a key to determine what patients with OA would benefit from monoclonal NGF antibody therapy and what patients would be at increased risk of detrimental side effects.

Further studies on extracellular processing of NGF and the proNGF/mNGF ratio in health and OA disease are warranted and there is a need for developing quantitative methods such as ELISA that are specific for the 40 and 45 kDa proforms.

Declaration of Competing Interest

The authors have no competing interests.

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Appendix A. Supplementary data

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Osteoarthritis-associated lameness is common in horses and there is a need to develop new methods for diagnosing osteoarthritis-associated pain. Nerve growth factor (NGF) is a signalling molecule that is associated with pain and osteoarthritis in several species but little is known about NGF in the horse. The aim of this thesis was to determine the presence of NGF in the equine joint and circulation, and to investigate its connection to osteoarthritis-associated lameness in the horse.

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