



Sveriges lantbruksuniversitet  
Swedish University of Agricultural Sciences

**Faculty of Veterinary Medicine  
and Animal Science**

Department of Clinical Sciences

# **Histological Investigation of the Testicular Nerves at the Castration Site in Geldings**

*Emma Angelina Bengtsdotter*

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# Histological Investigation of the Testicular Nerves at the Castration Site in Geldings

## Histologisk undersökning av testiklarnas nerver vid kastrationsstället hos valacker

*Emma Angelina Bengtsdotter*

**Supervisor:** Pia Haubro Andersen, Department of Clinical Sciences

**Assistant Supervisor:** Stina Ekman, Department of Biomedical Sciences and Veterinary Public Health

**Assistant Supervisor:** Maria Löfgren, Department of Biomedical Sciences and Veterinary Public Health

**Examiner:** Fredrik Södersten, Department of Biomedical Sciences and Veterinary Public Health

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## SUMMARY

Castration is one of the most common surgical procedures in equine medicine and associated with a number of postoperative complications such as swelling, infection, haemorrhage, peritonitis, funiculitis, omental herniation and eventration. But what happens with the testicular nerves after crushing and severing during the procedure? An injured nerve can respond with degeneration and/or regeneration. If the distal segment of the nerve is missing, such as after castration, the newly regenerating nerve fibres can be tangled in scar tissue, forming bundles of nerve fibres, creating a neuroma. In human medicine neuromas are well known a cause of pain sensations after different types of nerve injuries, for example in relation to amputations.

Studies of animals have documented the existence of neuroma formation after somatic nerve injuries, and in a few cases signs of pain originating from such a neuroma have been described. Neuroma formation on the testicular nerves at the castration site in geldings has never been investigated before. Can neuroma develop as response to injury of the testicular nerves? If so could this cause pain in geldings? What could this pain lead to and which consequence could this have for the horse? As a first step to answer these questions, the aim of this pilot study was to investigate if neuromas can form at the castration site on the testicular nerves in geldings.

Stumps of twelve spermatic cords were collected from six geldings (between 6-25 years of age and all of different breeds) admitted from the University Animal Hospital in Uppsala, Sweden for post mortem examination after euthanasia for unrelated reasons. Owners consent for examining the spermatic cords were obtained. The tissue samples were fixed in formalin and the spermatic cord was sectioned in a distal, mid and proximal segment. These were dehydrated and embedded in paraffin before sectioned in 4 µm longitudinal sections for histological investigation. The slides were stained with Haematoxylin and Eosin (HE) and Masson's Trichrome (MT). After examination in light microscope twelve slides with presumptive neuroma formation were selected for further investigation. Expression of nerve specific enolase (NSE) for visualisation of nerve fibres and S-100 protein for detection of Schwann cells were examined in slides after immuno-histochemical labelling. The expression of nerve growth factor (NGF) by immuno-histochemical labelling was investigated as a possible pain marker.

Neuromas were found in six spermatic cords belonging to four geldings. The neuromas consisted of an irregular and abnormal distribution of nerve fibres dispersed in and surrounded by connective tissue. Most of them were classified as delimited and containing Schwann cells. One neuroma was classified as dispersed, containing nerve fibres dispersed in a large area of connective tissue without Schwann cells. All neuromas expressed mild immunostaining of NGF, but no conclusions regarding clinical relevance from this could be drawn.

This pilot study has proved that neuromas of the testicular nerves indeed may form at the castration site in geldings. Though, further studies are required to establish the extent of neuromas in this location. The influence of castration methods and the possible consequences for the individual horse need to be investigated to answer important questions on the possible welfare implications of this elective surgery. Possible clinical implications include documentation of presence of inguinal pain, unexplained hind limb lameness or movements, back pain or unexplained behavioural problems in geldings.

## SAMMANFATTNING

Kastrering är en av de vanligaste kirurgiska åtgärderna inom hästmedicin. Väl kända komplikationer är svullnad, infektion, blödning, peritonit, funikulit, nätframfall och evisceration. Men vad händer egentligen med testikelns nerver som både krossas och skärs av under ingreppet? En skadad nerv kan svara med degeneration och/eller regeneration. Om nervens distala segment saknas, så som vid kastration, kan de nyregenererade nervfibrerna trassla in sig i ärrvävnad och bilda ett neurom. Neurom har inom humanmedicinen länge varit känt för att kunna orsaka smärtupplevelser efter olika typer av nervskador, exempelvis efter amputation.

Hos djur har man undersökt och påvisat förekomst av neurom vid vissa nervskador och i några fall har man även kunnat visa tecken som tyder på att neuromen orsakar en smärtupplevelse hos djuret. Neurom på testikelns nerver efter kastration hos häst har aldrig tidigare undersökts. Bildas det neurom på testikelns nerver efter kastration? Skulle dessa neurom kunna orsaka smärta hos valacker? Vad skulle denna smärta kunna leda till och vilken betydelse kan den ha för hästen? Denna pilotstudie avsåg som ett första steg att svara på frågan om neurom kan bildas på testikelns nerver vid kastrationsstället hos valacker.

Stumpen av tolv funiklar insamlades från sex valacker som skickats för obduktion från Hästkliniken vid Universitetsdjursjukhuset i Uppsala, efter ägarnas medgivande. Funikelstumparna fixerades i formalin och sädessträngen där nerverna löper skars sedan ut i ett distalt-, mitt-, och proximalt segment som efter dehydrering och inbäddning i paraffin snittades i 4 µm longitudinella snitt för histologisk undersökning. Snitten färgades med Haematoxylin och eosin (HE) samt Masson's trichrome (MT). Efter undersökning i ljusmikroskop valdes tolv snitt med misstanke om neurom ut för vidare undersökning. Med hjälp av immunohistokemi undersöktes uttryck av nerv specifikt enolase (NSE) för visualisering av nervfibrer och S-100 protein för påvisande av Schwannska celler. Uttryck av nerve growth factor (NGF) undersöktes med immunohistokemi som en möjlig smärtmarkör.

Totalt sex sädessträngar hos fyra valacker innehöll neurom. Neuromen bestod av en oregelbunden och onormal utbredning av nervfibrer, utspridd i och omgiven av bindväv. De flesta var avgränsade och innehöll Schwannska celler. Ett av neuromen klassificerades som utspritt och bestod av nervfiber utspridda i ett större område av bindväv, utan förekomst av Schwannska celler. Neuromen uttryckte mild immunofärgning av NGF, men någon slutsats gick inte att bestämma utifrån detta.

Denna pilotstudie har påvisat förekomst av neurom i vävnad vid kastrationsstället hos valacker. Men ytterligare studier behövs för att kunna svara på hur vanligt förekommande det är, samt om det skiljer sig mellan olika kastrationsmetoder. Men främst vilken betydelse det har för den enskilda hästen. Kan ett neurom på testikelns nerver orsaka obehag eller en smärtupplevelse hos hästen? Skulle detta kunna vara orsaken till inguinal smärta, en oförklarlig hälta, ovilja att utföra en viss rörelse, beteendeproblem eller en dålig bakbensaktivitet hos vissa valacker?

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## **LIST OF ABBREVIATIONS**

HE – Haematoxylin and Eosin

MT – Masson's Trichrome

NGF – Nerve Growth Factor

NSE – Nerve Specific Enolase

PBS – Phosphate Buffered Saline



## **INTRODUCTION**

Castration is one of the most common surgical procedures in equine medicine which is often associated with a number of complications such as swelling, haemorrhage, infection, funiculitis, peritonitis, omental herniation and sometimes even eventration that may be fatal. But what about the testicular nerves? All castration techniques are associated with severe crushing of the spermatic cord including the testicular nerves. What happens with the testicular nerves after crushing and severing?

A severed nerve regenerates with sprouting fibres; if the proximal end is separated from its distal end these sprouting fibres can form bundles of fibres in connective tissue, which creates a neuroma. In humans neuroma can be a complication to amputation of limbs and other nerve injuries such as trauma or surgical procedures. In animals tail docking in pigs (Simonsen *et al.*, 1991; Herskin *et al.*, 2015; Sandercock *et al.*, 2016), dogs (Gross & Carr, 1990), and lambs (French & Morgan, 1992), shoulder ulcerations in sows (Dahl-Pedersen *et al.*, 2013), beak trimming in poultry (Beward & Gentle, 1985), amputation models in rats (Dorsi *et al.*, 2008) and rabbits (Kim *et al.*, 2010; Ko *et al.*, 2011), and neurectomy in horses (Said *et al.*, 1984; Gutierrez-Nibeyro *et al.*, 2015) have shown neuroma formation.

Neuromas can cause chronic pain and/or hyperalgesia. In animals hyperalgesia linked to neuromas have been demonstrated in tail-docked dogs (Gross & Carr, 1990), and in sows with shoulder ulcerations; where they found signs of pain or discomfort even in cases where the ulcer was healed (Dahl-Pedersen *et al.*, 2015). In many studies of animals investigating the presence or absence of neuromas in injured nerves, it has not been investigated if these caused pain for the animal. Maybe due to the complexity and difficulty of proving if the animal experiences pain or not.

Some equine clinicians claim that neuroma at the testicular nerves after castration could be the cause of hyperalgesia shown in the inguinal area of geldings. The presence of neuroma at this anatomical location has however never been investigated. Can a neuroma form on the testicular nerves? Could a neuroma in this location on the testicular nerves cause inguinal pain in geldings? If so could this be the reason for some horses behavioural problems, lack of hind leg activation, riding problems or even lameness? And if so, is there a milder way to castrate horses? A technique that does not cause severe damage on the nerves and therefore prevents neuroma formation?

The aim of this study is, as a first step in this frontier, to investigate the amputation site at the testicular nerves in geldings post mortem for the presence of neuroma. This study have to be considered as a pilot-study due to its small number of horses included and the usage of autopsy-material, making the information about castration technique and -age inaccessible.

## **LITERATURE REVIEW**

### **Innervation of the equine testis**

A testicular nerve plexus innervates the testicles with autonomic sympathetic nerve fibres derived from the aortic plexus to the caudal mesenteric plexus and from the splanchnic lumbar nerve (Constantinescu & Schaller, 2012). The plexus accompanies the testicular vessels in the spermatic cord via the inguinal canal, to the testicles (fig. 1). This is what we call the testicular

nerves and the nerves that we are investigating in this study. Somatic nerves, the genital branch of the genitofemoral nerve, innervates the cremaster muscle, scrotal skin and vaginal tunic (Budras *et al.*, 2011). The scrotal skin is also innervated by the somatic ilioinguinal nerve and the pudenda nerve.

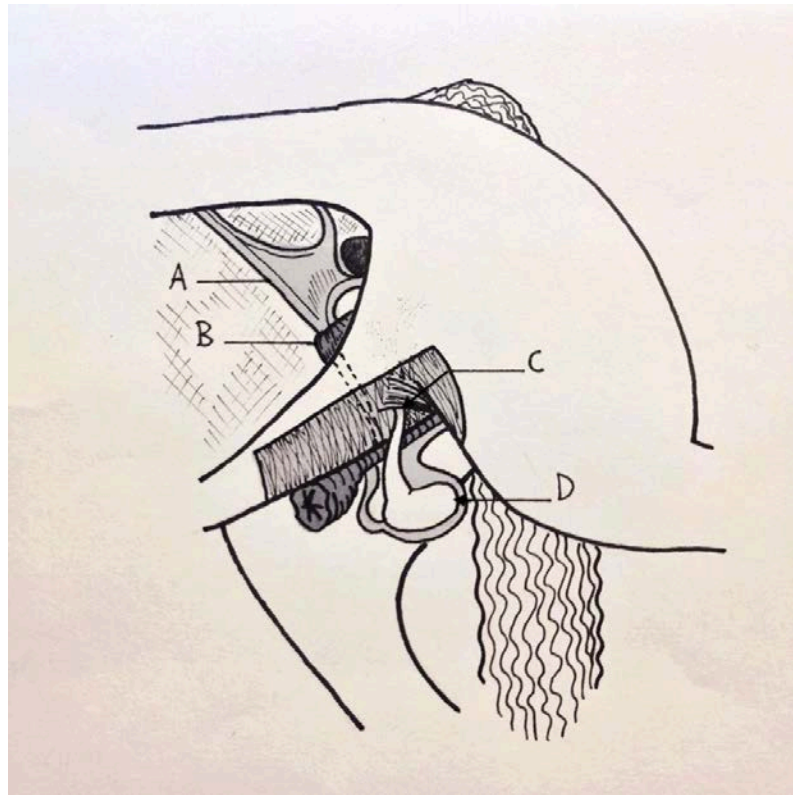


Figure 1. *Reproductive tract of a stallion. A. Right testicular artery, veins and autonomic nerves, B. Right internal inguinal (vaginal) ring, C. Left external inguinal ring, D. Left testicle in scrotum. Illustration created by Johanna Boklund based on Schumacher, 2012.*

### **Histology of the peripheral nerve**

A peripheral nerve consists of myelinated or non-myelinated axons (nerve fibres). Individual nerve fibres are together with belonging Schwann cells surrounded by connective tissue, the endoneurium (Kierszenbaum, 2007). Multiple of these nerve fibres are organised in fascicles covered by the perineurium consisting of connective tissue. A number of fascicles are enclosed by the epineurium, a connective tissue covering, creating the peripheral nerve (fig. 2). Tight junctions between fibroblasts in the perineurium form the blood-nerve barrier together with endoneurial capillaries consisting of endothelial cells with tight junctions.

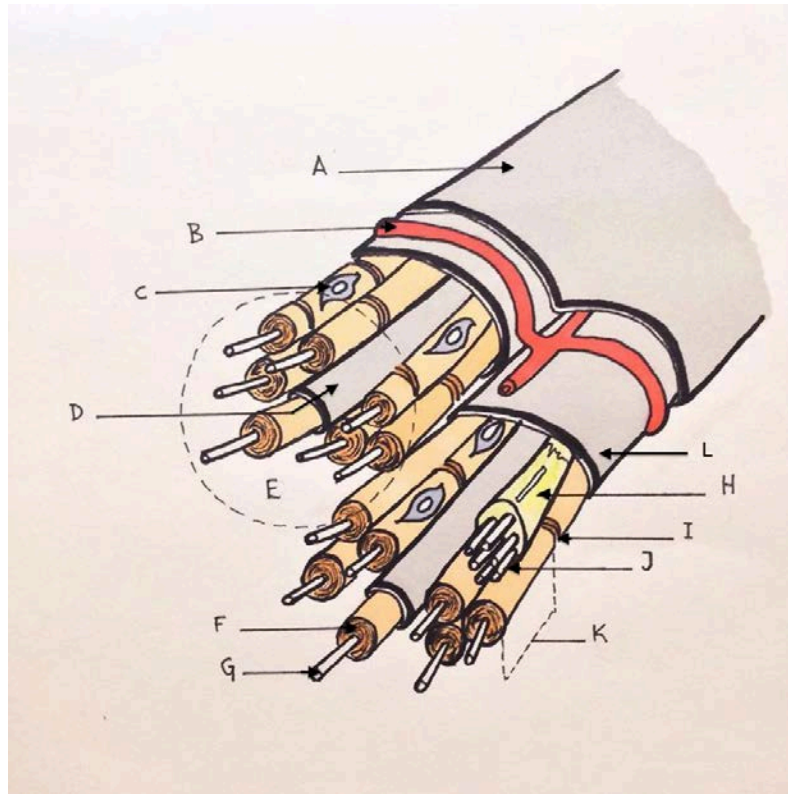


Figure 2. *Histology of a peripheral nerve: A. Epineurium, B. Capillaries, C. Schwann cell, D. Endoneurium, E. Fascicle, F. Myelin, G. Axon, H. Schwann cell, I. Node of Ranvier, J. Unmyelinated axon, K. Internode and L. Perineurium. Illustration created by Johanna Boklund based on Kierszenbaum, 2007.*

### **Autonomic nervous system**

Neurons of the autonomic nervous system are located in ganglia outside the central nervous system, CNS (Kierszenbaum, 2007). Preganglion fibre derives from a neuron in the CNS and postganglion fibres origin from the neuron in the ganglia to innervate the target cell or organ. Nociceptive stimuli from viscera travel with sensory afferent fibres, with neurons in the dorsal root ganglia, to CNS by pathways belonging to either or both of the parasympathetic or the sympathetic nervous system.

### **Pathophysiology of an injured peripheral nerve**

To understand the development of a neuroma we need to understand the pathophysiology of a damaged and severed peripheral nerve. When a peripheral nerve is injured, degenerative processes take place before regeneration can occur (Burnett & Zager, 2004). Hours after injury a degenerative process, Wallerian degeneration (first described by Waller, 1850), starts in the distal segment with axonal fragmentation and myelinolysis. Disintegration of neurofilaments in the axon is mediated by calcium-activated enzymes in the axoplasm (Schlaepfer & Hasler, 1979).

Schwann cells in the distal segment divide and forms dedifferentiated daughter cells. These degrade myelin together with macrophages and remove axonal- and myelin debris by

phagocytosis (Stoll *et al.*, 1989). The macrophages have entered the injury site after passing through capillary walls permeable by mast cells releasing of histamine and serotonin (Burnett & Zager, 2004), as a part of the inflammatory response due to the trauma of the nerve. The Schwann cells, macrophages, mast cells and neutrophils, also entering the injury site, secrete inflammatory mediators, for example; cytokines, prostaglandins and bradykinin (Stoll *et al.*, 2002; Vora *et al.*, 2007). In the distal segment the daughter cells of Schwann cells are also responsible of forming longitudinal “bands of Büngner” (Büngner, 1891: quoted by Ramon y Cajal, 1928), which guide regenerating nerve fibres and assist axonal re-growth and neuronal survival by producing a range of trophic factors, such as nerve growth factor (NGF), together with laminin and fibronectin found in the basal lamina (Lundborg, 2000).

The endoneurial tubes in the distal segment swell initially after the nerve have been injured, but later the diameter becomes smaller (Burnett & Zager, 2004). If the injury have severed nerve fibres these retracts in the fascicle, which impairs axonal regeneration leaving the endoneurial tubes denervated, which cause shrinking of the tubes and if sustained; fibrosis.

In the proximal segment close to the injury site Schwann cells degrade and myelin and axonal diameter is reduced (Burnett & Zager, 2004), which can remain especially if the function of the nerve is not restored. The extent of the degradation depends on the severity of the injury. In severe trauma, degradation of Schwann cells can extend all the way to the nerve cell body. After severe axonal damage the nerve cell body in the dorsal root ganglion reacts with chromatolysis and the cell prepares for regeneration (Lundborg, 2000). Occasionally this instead leads to degeneration and apoptosis. Wallerian degeneration and phagocytosis of the entire proximal segment of the nerve can then take place (Lundborg, 2000; Burnett & Zager, 2004).

Regeneration leads to an increase of axonal diameter and starts when chromatolysis is reversed (Lundborg, 2000; Burnett & Zager, 2004). Functional recovery can be restored in milder injuries and the regeneration process starts almost immediately. In severe injuries this process does not start until Wallerian degeneration is complete and can be initiated up to several months after the injury (Burnett & Zager, 2004). Multiple sprouting fibres (fine processes) grow from the proximal segment and can connect with their original endoneurial tube in the distal segment (Wall & Melzack, 2013). Regenerating axons in severe injuries can fail to innervate the end organ if they meander into other endoneurial tubes or the surrounding tissue. This phase of reparation and regeneration can persist for months (Burnett & Zager, 2004).

### ***Neuroma development***

When the sprouting axons attempt of regeneration fails to reach the distal end of the nerve, if the gap between the proximal and distal end is too long or if the distal end is missing such as after amputation (and consequently after castration), these fibres can form tangled bundles in connective (scar) tissue. These irregular fibres capsulated in connective tissue are a neuroma. This type of neuroma is referred to as traumatic-, nerve-end- or amputation neuroma.

When the cut ends are not separated a “neuroma in continuity” can be formed from fibres failing to regenerate and individual sprouting fibres can form micro-neuromas when they try to reach the distal segment but does not succeed (Devor, 2013).

All mechanisms for neuroma development are not known. One hypothesis is that the proliferating nerve fibres are entrapped in scar tissue during wound healing and especially during healing by second intention, as during infection and/or inflammation (Foltán *et al.*, 2008). Where the endoneurial- and perineurial tissue proliferating around axons is a reaction to the contraction of myofibroblast in scar- and wound tissue, to protect nerve fibres from being injured by the pressure.

A neuroma typically consists of irregularly distributed nerve fibres, Schwann cells and fibroblasts. Often surrounded by fibrous connective tissue prolonged from the nerve sheaths perineurium (Foltán *et al.*, 2008). Inflammatory cells such as macrophages and lymphocytes can be seen inside a neuroma (Vora *et al.* 2007).

### **Peripheral neuropathic pain and hyperalgesia**

Evoked and spontaneous neuropathic pain is due to electrical hyperexcitability of injured afferent neurons. Hyperalgesia can occur after injury or inflammation in both somatic and visceral tissue, including testicle tissue (Devor, 2013).

### ***Neuroma discharge patterns and pain***

The full explanation of why a neuroma becomes painful is not yet fully understood. In traumatic neuromas electrically excitable afferent nerve fibres with a low conduction velocity and spontaneous electrical hyperexcitability have been found (Wall & Gutnick, 1974; Blumberg & Jänig, 1984). Changes in features or dispersion of potassium and sodium ion-channels can lead to ectopic activity of the axons, causing abnormal discharge patterns and thereby pain or paraesthesia (Devor, 1983; England *et al.*, 1996). Mechanical stimulation of neuromas activates both response, spontaneous and after-discharge activity of the afferent axons leading to hyperalgesia (Blumberg & Jänig, 1984; Dorsi *et al.*, 2008). Wound contraction compressing axons in the neuroma have been conjectured to be a trigger of pain or abnormal sensation (Foltán *et al.*, 2008).

Traumatic neuromas and pain symptoms have been associated with inflammatory cell accumulation (Vora *et al.*, 2007), but no correlation with symptoms could be seen in the mentioned study due to pain symptoms from a neuroma existing even without inflammation. Studies have shown a higher expression of NGF in painful neuromas comparing to relocated non-painful neuromas and control nerves (Atherton *et al.*, 2006).

### **Traumatic neuromas in animals**

In addition to the studies of neuroma models on animals, where the purpose is to create a neuroma for research, there are some studies where neuroma formation have been investigated on animals with trauma to peripheral nerves. Some of them also include investigation of neuroma formation in relation to pain. Either as pain symptoms or as discharge patterns of the nerves. A brief review of the research in this area is presented below.

### ***Tail docking - dogs***

Gross and Carr (1990) investigated six dogs with docked tails after severe self-trauma to the amputation site. The distal stump was removed during surgery and examined grossly and histologically. The dogs were one to four years old and the self-trauma had developed several months to a year after docking of the tail. Causes for the self-trauma had been investigated for up to several years and four dogs have had anal sacculotomy. All six dogs had severe pain response on mild pressure of the self-trauma caused lesions on the tail.

Gross examination of the removed distal tail-stump revealed thickened and firm skin and underlying subcutis. Histologically neuromas were found; dense collagenous connective tissue with randomly distributed proliferated nerve bundles containing myelinated axons encircled by perineurium. This appearance was not shown in an additional tail docked dog without pain symptoms but with similar gross appearance, nor in two intact tail docked stumps of dogs collected post mortem, further reinforce the pain originating from the neuromas.

### ***Tail docking - pigs***

There are several studies that have shown neuromas in docked tails of pigs. In the first one by Simonsen *et al.* (1991) tail tips from 10 day-old piglets, undocked tails from 10 fattening pigs and tail docked tips from 20 fattening pigs were examined. All docked tails were amputated with an emasculator, a pair of forceps used to crush the tissue to create haemostasis with or without a cutting blade. In tail docked pigs they found traumatic neuromas. In how many cases is however not specified. The neuromas contained constricted and eliminated myelin sheaths and axons by the proliferation of fibroblasts and Schwann cells in a twirling appearance. Generally the docked tails contained an uneven and irregular distribution of peripheral nerves, showing regressive changes, uneven myelin distribution, thick perineural sheaths and a proliferation of fibroblasts and Schwann cells. These findings did not occur in the undocked tails.

Another study on tail docked pigs (Herskin *et al.* 2015) investigated neuroma formation when tails were amputated with hot iron cautery on different docking lengths. The tails were docked at 2-4 day old piglets and divided in groups depending on docking length; leaving 75%, 50% and 25% of the length of the tail. One group were left undocked. The tails were then collected for macroscopically and histologically examination at 22 weeks of age. In all docked groups neuromas were found. No neuromas were found in the undocked group. Histology of the neuromas was only described in the legend of a figure of a cross section from one of the tail docked pigs. The neuroma in the picture was described as “an enormous granuloma – that is, aggregates of hypertrophic axons ensheathed by Schwann cells – is seen to be intermingled and surrounded by multiple layers of fibrous tissue” (Herskin *et al.*, 2015 p. 679). This study confirmed that tail docking with hot-iron cautery led to the formation of neuromas and that the length of tail did not influence the rate of formation.

The latest study on neuroma formation in tail docked pigs is by Sandercock *et al.* (2016). In this study tails were collected from 16 pigs evenly distributed in groups that were euthanized at 1, 4, 8 and 16 weeks after tail docking with hot iron cautery, at 3 days of age. One week after tail docking, no neuromas were found. Four weeks after taildocking two of four tails contained early neuromas consisting of axonal and nerve sheath proliferation forming new nerve endings trying to re-innervate. After two months all four investigated stumps contained neuromas in

granulation tissue. In the last group 16 weeks after tail docking incomplete neuromas, with ongoing re-innervation, of different size and appearance was found in all tails. The neuromas where either expressed as several axons spread into granulation tissue or as limited neuromas.

### ***Tail docking - lambs***

Tail docking in lambs has also been investigated (French & Morgan, 1992) for the presence of neuroma. The study included twelve tail docked lambs and six undocked lambs at 4 to 6 months old. The tails were collected after slaughter so the exact method or age for tail docking was not known. But described was that tail docking generally is performed within 2 days after birth and the methods; hot iron cautery, necrosis induced by rubber rings, or by using a knife or clipper. Gross examination was performed on six docked and two undocked tails. The distal end of the nerves in the docked tails where adherent to scar tissue and three tails had one nerve stump each with a visually obvious swelling. These findings could not be seen in the undocked tails. Six docked tails and four undocked tails was examined histologically. Neuromas where found in two docked tails and described as swellings “contained swirling, tortuous knots of axons surrounded by fibroblasts” (French & Morgan, 1992 p. 390). In connective and scar tissue of two other docked tail stumps many distinct nerve bundles irregularly distributed was found.

### ***Neurectomy - horse***

In a study (Said *et al.*, 1984) two different techniques of neurectomy were investigated on 46 equids in a total of 63 operations. The two techniques comprised the traditional method where a piece of the nerve is removed and the Fackelman and Clodius method (Fackelman & Clodius, 1972; quoted by Said *et al.*, 1984 p 442). In this latter method the proximal and distal stump of the transected nerve is electrocoagulated with an electrocoagulation device, and the nerve endings separated from each other as the proximal stump were bend upwards.

The equids were euthanized 1, 2, 4, 8, 12, 16 or 24 weeks after the neurectomy (Said *et al.*, 1984). There was a distinct difference in frequency of formation of neuromas linked to which method being used. Totally 31 cases of neuroma formation occurred. In the group using the traditional method 24 of 28 neurectomies developed a neuroma, compared to the other group using the Fackelman and Clodius method where only 7 of 35 neurectomies led to neuromas. The study also divided the neuromas into painful and non-painful neuromas. Painful neuromas were defined as neuromas painful to digital pressure. In the group using the traditional technique 7 of 24 neuromas was painful, and in the group using the Fackelman and Clodius method there where noteworthy no painful neuromas.

The histological appearance of the painful and non-painful neuromas differed in appearance. The non-painful consisted of mostly degenerated nerve fibres and a massive proliferation of scar tissue with fibroblasts and collagen fibres. The painful neuromas showed instead a prevalence of nervous tissues with an irregular distribution of nerve fibres and proliferated epi- and perineurium, Axonal degeneration or regeneration in the painful neuromas apparently were depending on how long ago the neurectomy was performed.

### **Shoulder ulceration - pigs**

One study (Dahl-Pedersen *et al.*, 2013) has investigated neuroma formation in shoulder ulcerations in sows. Shoulders from 155 sows 3-4 weeks postpartum were collected for gross- and microscopically evaluation. The material was divided in groups due to visibility and severity of the shoulder sores. Neuromas were found in all groups, also in the group with healed lesions. In total 50 (32%) of the shoulders had traumatic neuromas. The neuromas were described as "...large disorderly bundles of nerve tissue with diameters up to 25 times the size of a normal nerve in the region. Characteristically, the traumatic neuromas were embedded in GAGs and surrounded by concentric layers of connective tissue" (Dahl-Pedersen *et al.*, 2013, p.668). Some of the pigs with healed ulcerations containing neuromas had shown a rubbing behaviour of the shoulder, as a response to palpation. This manner did not exist in the same extent in pigs without neuromas. It was suggested that the behaviour was a response to pain.

## **MATERIAL AND METHODS**

### **Tissue samples and preparation**

Tissue samples were harvested from horses sent for post-mortem examination from the University Animal Hospital in Uppsala, Sweden, after owners consent. Spermatic cords from right and left sides were collected from six geldings and one stallion. Age, breed or circumstance for euthanasia was not relevant for inclusion. The samples were collected between 1-48 hours after euthanasia, for specification see table 1.

Tissue samples were fixed in 10% neutral buffered formalin for at least 24 hours, and then cut out in a proximal-, mid- and distal segment for longitudinally sections (fig 3). Tissue samples from the stallion were cut out in a proximal- and mid segment for cross sections and a mid-segment for longitudinally section. All segments were dehydrated, treated with xylene, embedded in paraffin and cut in 4 µm sections for histologic examination.



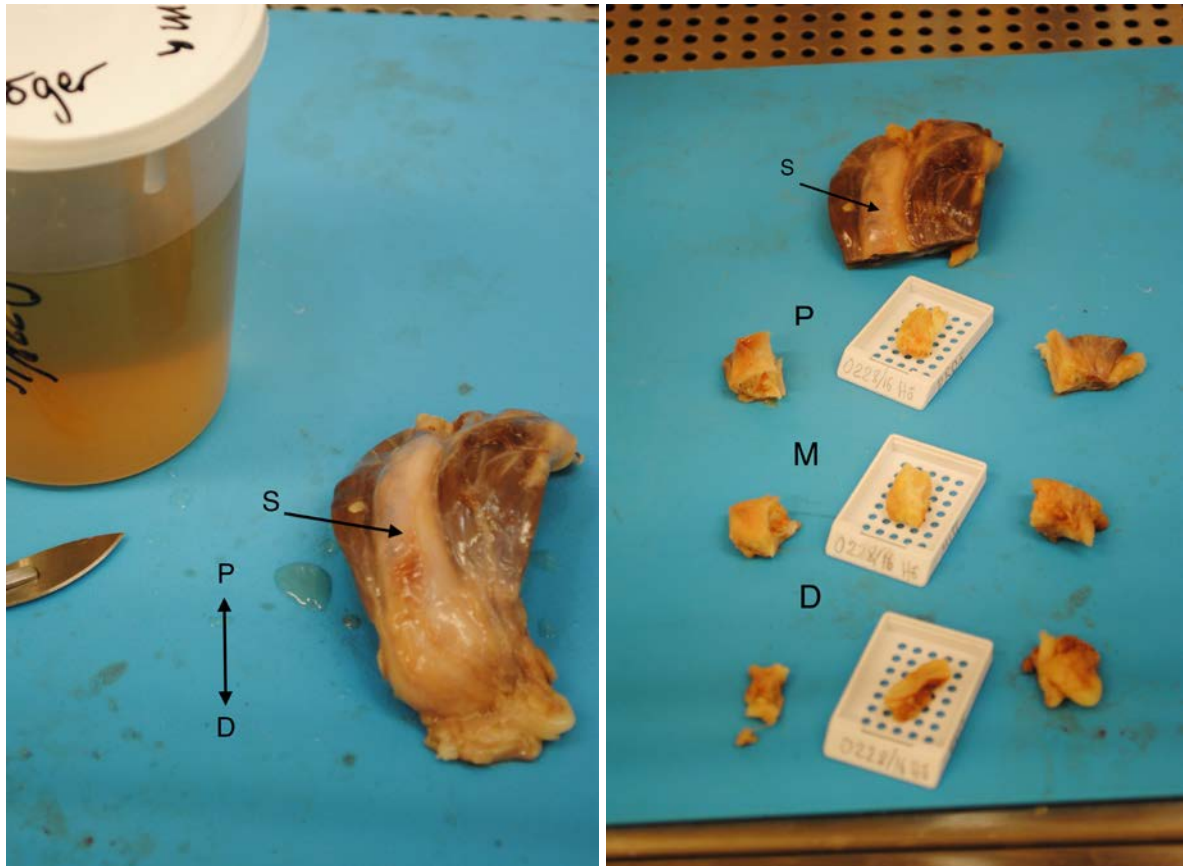


Figure 3: Left – spermatic cord (S) after fixation in formalin. Right – cut out in distal-, mid-, and proximal segment for longitudinally sections. S. Spermatic cord, P. Proximal, M. Mid, D. Distal.

### Histology and immunohistochemistry

Two tissue slides from each cut out segment were stained with haematoxylin and eosin (HE) and Masson’s trichrome (MT) respectively, by laboratory personnel. Slides were then examined in light microscope and slides with presumptive neuromatous tissue/neuroma formation (for definition see “Definition of neuroma and classification” below) were selected for further investigation.

Selected slides were labelled immunohistochemically with antibodies against: a) S-100 protein for visualisation of Schwann cells, b) nerve specific enolase (NSE) for visualisation of neuronal cells, and c) nerve growth factor (NGF) as a potential pain marker. See appendix for immunohistochemical protocols for S-100, NSE and NGF. All slides were then examined in light microscope and evaluated together with the assistant supervisors.

### Definition of neuroma and classification

The definition of neuromas was an abnormal appearance and/or distribution of nervous tissue with an irregular distribution of axons surrounded by, or scattered in, connective tissue. The neuromas were visible in HE and MT and confirmed by the expression of NSE. Graded with + if the slide contained only a focal neuroma or few neuromas, ++ if there was multiple neuromas, or a single neuroma, extended in <50% of the tissue slide and +++ if multiple neuromas, or a single neuroma, extended in >50% of the tissue slide.

The neuromas were classified as delimited or dispersed. Delimited if the nervous tissue was in a coherent area (fig. 5, 6) and dispersed if the nervous tissue was scattered in a large area of the tissue slide (fig. 7). In some of the slides areas with an empty appearance or only thin transparent containment were found, representing necrotic tissue which might be remnants of degenerated nerves. These areas were graded + if the area was focal or the slide only contained few of them, ++ if the areas were multifocal, or extending in <50% of the slide and +++ if multiple areas or a single area were extended in >50% of the slide. The expression of NSE, NGF and S-100 within the neuromas was only graded as + if present and - if not present.

## RESULTS

### Horses and demographic data

The geldings were between 6-25 years old and the stallion was a 3-year-old Icelandic horse euthanized due to acute colitis. For full information about age, breed and circumstance for euthanasia for each gelding, see table 1.

Due to the use of autopsy material the castration age and exact methods for castration were not known. Although in Sweden most castration techniques include a pair of forceps (emasculator), and most horses are castrated before the age of 2 years.

Table 1. Age, breed, circumstance for euthanasia (Eut.) and time between euthanasia and tissue collection – for geldings in the study

Gelding	Age	Breed	Eut.	Time (hours)
1	25 years	Lusitano	Heart failure	1
2	7 years	Quarter horse	Colic	48
3	7 years	Ardennes horse	Chronic lameness	16
4	6 years	Swe. Warmblood	Back problem	1
5	8 years	Standardbred	Chronic illness	16
6	25 years	Welsh. Cob.	Tumor	1

### Gross evaluation

Grossly the spermatic cords differed a lot both in length, thickness and consistency. The length from the external inguinal ring to the distal end of the stump differed from approximately 3 cm up to 15 cm. The thickness and consistency of the cord were in some horses thin, soft and whitish without any visible structures, and in others thick, firm, hypertrophic and with visible vessels and structures. The length and thickness or consistency was not correlated. See Figure 4 for example.

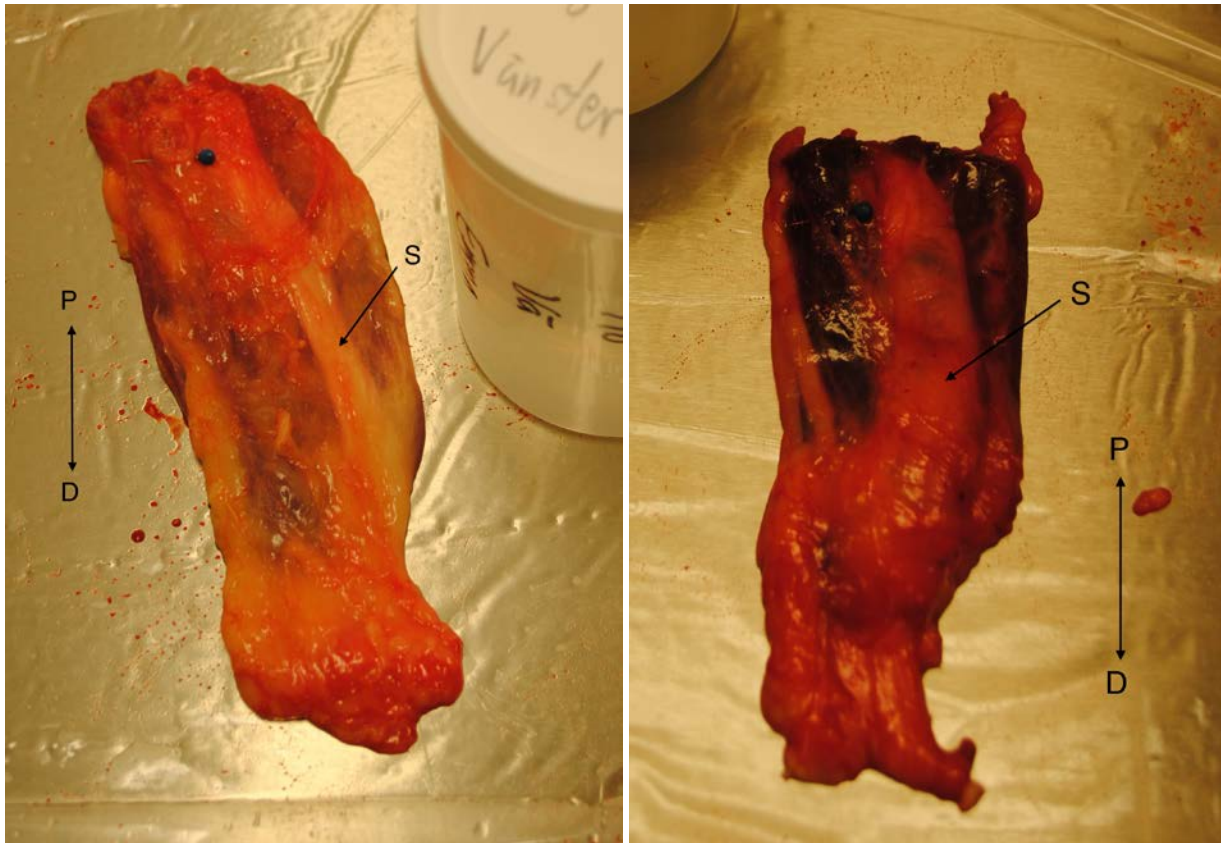


Figure 4. Gross appearance of spermatic cords. S. Spermatic cord, P. Proximal, D. Distal.

### Histology of the spermatic cord from the stallion

The spermatic cord in the stallion contained blood vessels, arteries with thick walls, and a large amount of veins – the plexus pampiniformis. In between the vessels were connective tissue and adipose tissue. The spermatic cord included multiple nerves, individual or in larger bundles, and ductus deferens presented with belonging arteries, veins and nerves. All together surrounded by connective tissue.

The structures in the spermatic cord were well organised and visible with HE and MT. S-100 protein and NSE were highly expressed in the nerves visualising axons and Schwann cells. Mild immunostaining with NGF was seen in the unharmed adult nerves. The ductus deferens wall had a high expression of NSE, explained by its rich innervation with a nerve plexus in the smooth muscle layer (Kujat *et al.*, 1993).

### Histology of the spermatic cords from the geldings

The spermatic cords of geldings in general contained a lot of connective tissue and adipose tissue. Some of the cords contained a lot of blood vessels, but others very few. Normal nerves were found in some of the cords, but not in all. Neuromas were found in six of twelve spermatic cords belonging to four of six geldings. Both delimited and dispersed neuromas were found although the delimited neuromas were most common (fig. 5-7). Five of twelve spermatic cords, belonging to three of six geldings contained areas of necrosis.

All six neuromas expressed a mild staining with antibodies against NGF. Neuromas in two of the spermatic cords did not express S-100. Histological results for each spermatic cord are presented in Table 2.

Table 2. *Histological results of geldings. Neuroma – presence (+, ++, +++), absence (-). If the neuroma is delimited or dispersed (+ or -). Necrosis – presence (+, ++, +++), absence (-). NSE, NGF and S-100, – if it is expressed in the neuroma (+) or not (-). Not analysed - n.a. Sin – sinister (left), dx – dexter (right)*

Gelding	Neuroma	Delimited	Dispersed	Necrosis	NSE	NGF	S-100
1 sin	++	+	-	+	+	+	+
1 dx	+	+	-	-	+	+	+
2 sin	-	-	-	-	-	-	-
2 dx	-	-	-	-	n.a	n.a	n.a
3 sin	-	-	-	-	n.a	n.a	n.a
3 dx	++	-	+	-	+	+	-
4 sin	+	+	-	++	+	+	-
4 dx	-	-	-	++	-	-	-
5 sin	-	-	-	-	n.a	n.a	n.a
5 dx	-	-	-	-	-	-	-
6 sin	+++	+	-	+	+	+	+
6 dx	+++	+	-	+	+	+	+



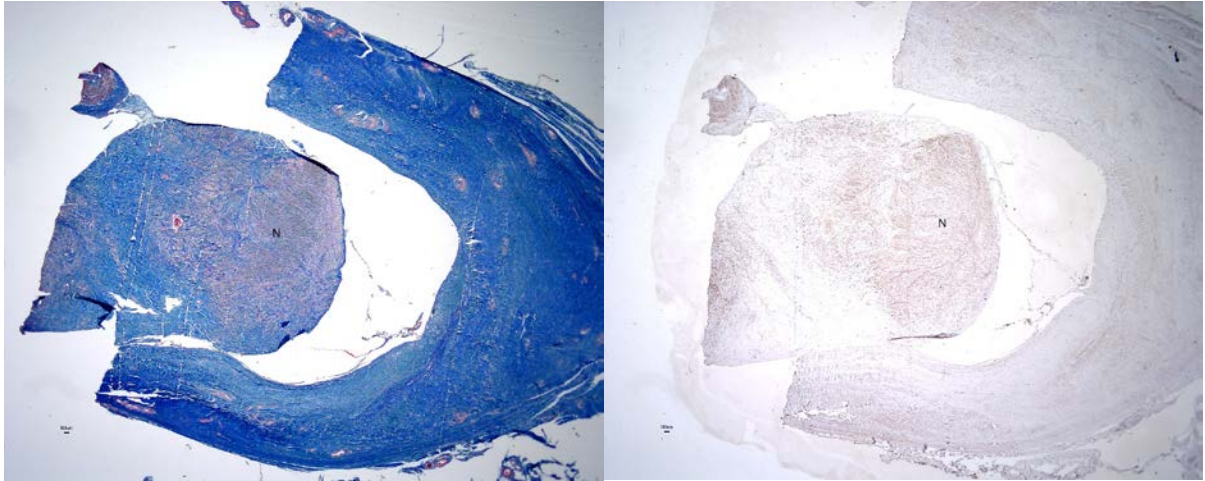


Figure 5. *Delimited neuroma. Left stained with MT and left labeled with NSE. N. Neuroma. Magnification x1.*



Figure 6. *Delimited neuroma, same as above. Labeled with NSE, magnification left x4, right x10. Note the irregularly distributed nerve fibres (brown color).*

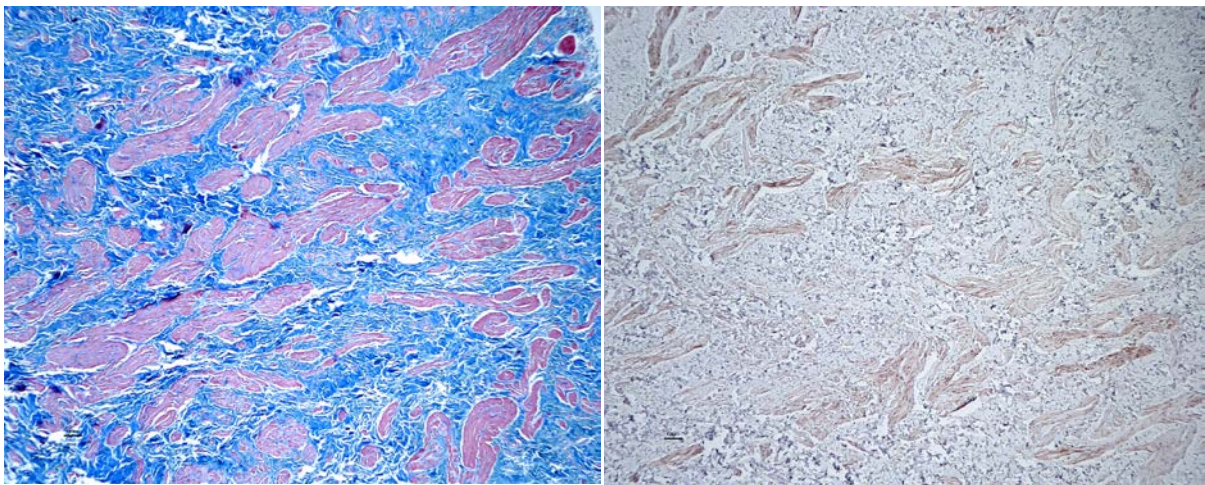


Figure 7. *Dispersed neuroma. Left – stained with MT, red color – nerve tissue, blue color – connective tissue. Right – labeled with NSE, brown color – nerve fibres. Magnification x4 in both pictures. Note the nerve fibres dispersed in connective tissue.*

## **DISCUSSION**

### **Results in the present study**

The results of our study showed that four of six investigated geldings had neuromas in the nerves of one or both spermatic cords. It can however not be excluded that the two horses without neuromas did not have neuromas in their spermatic cords, since the investigated tissue only comprised three areas of the spermatic cords. Serial sections of the tissue were not possible due to time and economical restraints. Only one piece of the spermatic cord in the distal, mid and proximal segments was trimmed and then a maximum of five longitudinal sections per segment were examined. Optimal investigation if time and economy was not a limitation would be to cross section the entire distal part of the spermatic cord (corresponding to the distal, mid and proximal segment), and staining multiple of these sections for examination.

We decided to do longitudinal sections to cover up a bigger area comparing with a cross section, since we looked after multiple nerves and did not know where they ended. Due to this being the first study investigating neuromas in this location we had to try a lot of things out. For example where to cut out the segments of the spermatic cord, decide how to do the sections, which stains we were going to use, test immunohistochemical protocols to see if they worked and how to do the definition and classification of neuromas.

One thing that was surprising and very distinct was the large diversity of gross appearance of the spermatic cords. On forehand we did not established a method on how to investigate the spermatic cords grossly or parameters to include, and this is a recommendation for further studies. Although we had different breeds of all horses included in the study, and some differences in gross appearance of spermatic cords in different breeds is thought to be normal, it would be interesting to study if the gross appearance differ between castration methods and complications during the healing period and how it corresponds to the histological investigation. We had one horse (Gelding 5) with very short stumps, approximately 3 cm. This strongly indicates that this horse have had the procedure done in dorsal recumbence at an equine hospital. It is hard to imagine that it would be possible to crush and sever the spermatic cords so near the external inguinal ring during a castration in the field. This horse did not present any neuromas, so it would have been interesting to know which method had been used.

We also had two geldings with large, thick and firm spermatic cords with visible structures in it, such as vessels. One of those where Gelding 6, whose spermatic cords contained most and largest neuromas of all our horses. Gelding 4, showed the same gross appearance but had a number of areas with necrosis and only one delimited neuroma in one of the cords. Unfortunately we do not know which methods that have been used or if any complications have occurred, so we can only note but not explain the similarities in gross appearance and the differences in histology. The areas of necrosis that we have found could be remnants of degenerated nerves. But due to the necrosis, and thereby the absence of cells, we cannot assure which tissues that underwent necrosis. Another histological notation is that neuromas in two of the spermatic cords did not express S-100 protein. Meaning that they do not contain any

Schwann cells. One explanation of this might be that these neuromas only consist of unmyelinated axons.

The two oldest geldings in the study (25 years) both had neuromas in both spermatic cords. This may indicate that neuromas probably (due to us not knowing the exact castration age) may exist during many years after castration. All of our horses were older than 6 years and if they were castrated as most common before the age of 2 years, all of them have been castrated for more than four years ago. It would be interesting to investigate different time periods after castration to see if there are any histological differences depending on how much time have passed since the procedure was done.

The immunohistochemical protocols used served as desired for NSE and S-100. Using NSE is of great value for identifying nervous tissue. Thereby highly recommended in further studies. S-100 is a protein expressed in more cells than just Schwann cells. If NSE is used S-100 could be used as a complement to investigate if Schwann cells are present or not. Although alone it is not recommended as we have shown not all neuromas contain Schwann cells. We did not get a clear expression of NGF that could be used to draw any conclusions from. However not saying it is not of use. It may be that a change in concentration or staining method gives a clearer result.

### **The nerve injury caused by castration**

When castration is done using forceps to crush the spermatic cord and then severing the cord distal of the crushed area, we end up with both a crushing and a severing injury on the cords and thereby on the testicular nerves. Theoretically we have two different types of injuries on the nerves; one crush injury with a proximal end and a distal end to the injury site, plus a severing injury with a proximal end and a missing distal end. This could theoretically give us both a neuroma in continuity and/or a nerve end neuroma. Which of these types we have found is hard to tell though we are investigating multiple minor nerves and not a single large nerve that we can follow the extent of.

It have been suggested in the literature that neuromas are more likely to form in traumatised and inflamed tissues (Foltán *et al.*, 2008). Most equine traditional castration methods include severe damage of the nerves of the cords and inflammation ought to be common due to the rate of milder complications such as swelling and oedema (Kilcoyne *et al.*, 2013). The castration methods may therefore be questioned, especially if the neuromas are painful. Are forceps necessary to use or are they used by tradition? Is there a more atraumatic way to achieve adequate haemostasis that does not include severe crushing of the spermatic cord? More and more owners choose to castrate their horse in an equine hospital. Even there forceps is used regularly, despite the fact that this environment allows use of different techniques. Would it be possible to only use ligation, ligation devices, diathermy, laser or any other method on the vascular tissues and then conduct the neurectomy in a more atraumatic way? This needs to be investigated. For example diathermy have been shown to reduce the formation of neuromas up to 35% (Tay *et al.*, 2005), and in the study by Said *et al.*, (1984) on neurectomy in horses, using an electrocoagulation device lead to no painful neuromas.

But are neuromas on the testicular nerves then painful? Our study was designed to answer that question. We attempted to use NGF as a pain marker to investigate possible differences between

the neuromas, despite that we did not know the clinical status of the horse. It was not possible to draw any conclusion to the results given. Because no clear expression of NGF was found in the neuromas. This does not exclude the possibility that some of the neuromas might have been painful. A study (van der Avoort *et al.*, 2013) on finger amputations on humans found that 7,3% of the traumatic neuromas were symptomatic (caused pain or altered sensation). In humans neuromas after inguinal hernia repair can cause chronic pain (Bjurstrom *et al.*, 2014). Although it is different nerves, the mechanisms could be similar to a neuroma on the testicular nerves. Even if a corresponding or a lower number is to be found in neuromas on the testicular nerves, more knowledge about this is important. Especially regarding the possible pain, its clinical manifestations and welfare implications.

## **CONCLUSIONS**

This study shows that neuromas can form on the testicular nerves at the castration site in geldings. Despite the small number of horses in this study, four of six horses had neuromas in one or both spermatic cords. The stain for NGF in the neuromas with the methodology used was only mild and any conclusion from it could not be done.

## **Perspectives**

Further studies are needed to investigate the frequency of neuroma formation after castration in horses. When do they occur, are they painful and which methods leads to neuromas? What are the welfare implications for geldings?

This study is the first to document that neuromas can form on the testicular nerves of horses. This should be borne in mind if a gelding is presented with pain behaviour related to the inguinal area, unexplained lameness, lack of hind limb activation, behavioural problems or is unwilling to perform certain movements.



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## **APPENDIX**

### **Protocol for Immunohistochemistry of NSE, S-100 and NGF**

1. Incubate in 60°C for 20 minutes, cool down to approximately 37°C.
2. Deparaffinisation in Histo Lab Clear
  - a. 15 minutes in 37°C
  - b. 15 minutes in room temperature
3. Hydration performed in hood
  - a. Ethanol 99%, 2 x 5 minutes
  - b. Ethanol 95%, 1 x 5 minutes
  - c. Ethanol 70%, 1 x 5 minutes
4. Rinse with de-ionized water 3 x 3 minutes
5. Incubate the slides in Na-citratebuffer (pH6) at 96°C for 20 minutes, cool down in room temperature for 20 minutes.
6. Rinse with de-ionized water 3 x 3 minutes
7. Quench endogen peroxidase with fresh 3% hydrogenperoxide (5 ml 30% H<sub>2</sub>O<sub>2</sub> + 45 ml de-ionized water), 5 minutes in dark.
8. Rinse with PBS (Phosphate Buffered Saline) 3 x 3 minutes, mark with PAP-pen
9. Add primary antibody diluted in PBS. Add negative control diluted in PBS (see dilutions for each label underneath).
10. Incubate for 30 minutes at room temperature in humidity chamber.
11. Rinse with PBS 3 x 3 minutes
12. Add secondary antibody, labelled polymer HRP anti- rabbit/mouse.
13. Incubate for 30 minutes at room temperature in humidity chamber.
14. Rinse with PBS 3 x 3 minutes
15. Incubate with DAB+substrate buffer, check under microscope.
16. Rinse in water for 15 minutes.
17. Stain with Mayers Htx for 1 minute 30 seconds.
18. Rinse in water for 15 minutes.
19. Dehydration performed in hood
  - a. Ethanol 70%, 1 x 1 minute
  - b. Ethanol 95%, 1 x 1 minute
  - c. Ethanol 99 %, 2 x 1 minute
  - d. Ethanol 99 %, 1 x 5 minutes
20. Place in Xylene, 2 x 1 minute, then let it stand in Xylene for 15 minutes.
21. Mount

#### **NSE**

Primary antibody: NSE, mouse monoclonal, Dako M0873, 367 mg/l. Diluted 1:100.  
Negative control: Neg Control Mouse IgG, Dako X0931, 100 mg/l. Diluted 3,5:100

#### **NGF**

Primary antibody: NGF, (H-20), rabbit polyclonal, Santa Cruz sc-548, 200 µg/ml. Diluted 1:100. Negative control: Neg Control Rabbit IgG, Dako X0903, 20 mg/l. Diluted 1:100, then 1:100.

#### **S-100**

Primary antibody: S-100, rabbit polyclonal, Dako Z0311, 4 g/l. Diluted 1:400.  
Negative control: Neg Control Rabbit IgG, Dako X0936, 15 g/l. Diluted 1:100, then 1:15.

Using: Dako EnVision+ system, HRP anti-rabbit/mouse, K5007.

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