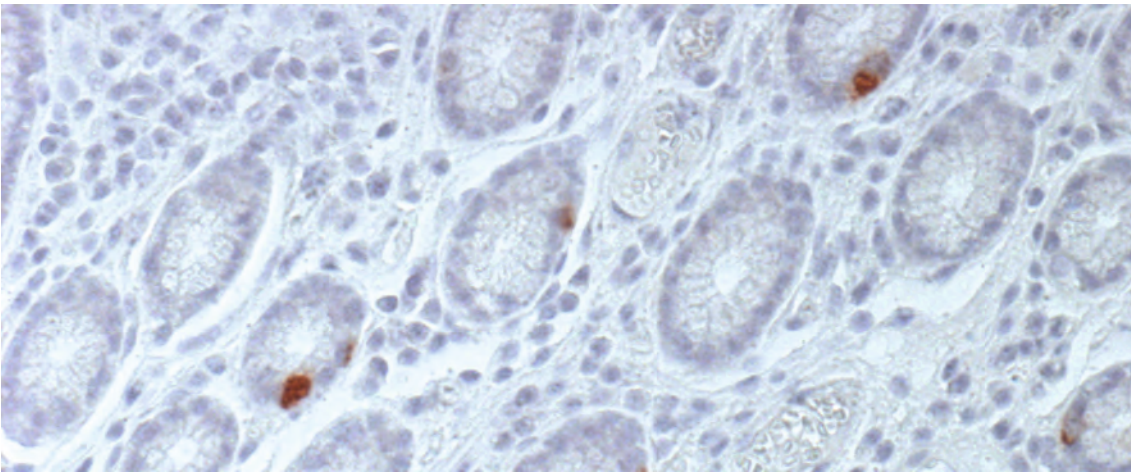
Immunohistochemical detection of gastrin and motilin peptides, their receptors, VIP receptors and caspase activity from the abomasal wall of cattle

AYCAN ÖZCAN



INAUGURAL-DISSERTATION zur Erlangung des Grades eines **Dr. med. vet.**
beim Fachbereich Veterinärmedizin der Justus-Liebig-Universität Gießen

édition scientifique
VVB LAUFERSWEILER VERLAG

1. Auflage 2012

1st Edition 2012

Printed in Germany

édition scientifique
VVB LAUFERSWEILER VERLAG

STAUFENBERGRING 15, D-35396 GIESSEN
Tel: 0641-5599888 Fax: 0641-5599890
email: redaktion@doktorverlag.de

www.doktorverlag.de

Aus dem Klinikum Veterinärmedizin,
Klinik für Wiederkäuer und Schweine
(Innere Medizin und Chirurgie)
und dem
Institut für Veterinär-Anatomie, -Histologie und -Embryologie
der Justus-Liebig-Universität Gießen

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Prof. Dr. Dr. S. Arnhold

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INAUGURAL–DISSERTATION
zur Erlangung des Grades eines
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eingereicht von

Aycan Özcan
Tierarzt aus Meric (Türkei)

Giessen 2012

Mit Genehmigung des Fachbereichs Veterinärmedizin
der Justus-Liebig-Universität Gießen

Dekan: Prof. Dr. Dr. h. c. M. Kramer

Gutachter: Prof. Dr. Dr. habil. K. Doll
Prof. Dr. Dr. S. Arnhold

Tag der Disputation: 25. Juni 2012

Dedicated to my family

Anne ve Babama

Parts of this study have been presented in the following conferences:

A. Özcan, M. Sickinger, S. Arnhold, K. Doll (2011): Zur ätiopathogenetischen Bedeutung von Neurotransmittern bei der Labmagenverlagerung der Milchkuh. Oral presentation, 8th buiatrics conference, 1-3.4.2011, Oberschleißheim

M. Sickinger, A. Özcan, R. Leiser, S. Arnhold, K. Doll (2011): Neuropeptidgehalte und Innervationsdichte in der Labmagenwand von Holstein-Kühen. Ätiopathogenetische Bedeutung hinsichlich Labmagenverlagerung? Proceedings, 3rd annual conference of German Buiatrics Association DVG, 10-13.11.2011, Berlin, 90-92

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Table of abbreviations

ABC	Avidin-Biotin-Complex
ACh	Acetylcholin
AD	Abomasal displacement
AEC	3-Amino-9-ethylcarbazol
AIF	Apoptosis inducing factor
Apaf 1	Apoptosis protease activating factor 1
Blc-2	B-cell lymphoma 2
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
Caspase	Cysteine aspartate proteinase
CCK	Cholecystokinin
CCK-AR	Cholecystokinin A receptor
CCK-BR	Cholecystokinin B receptor
CD-95	Cluster of differentiation 95
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CO	Carbon monoxide
DA	Displaced abomasum
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid
ECL	Enterochromaffine-like cell
EM-A	Erythromycin
FasL	Fas ligand
GI	Gastrointestinal
GFV	German Fleckvieh
GH	German Holstein
GHR	Growth hormone secretagogue receptor
GPCR	G protein coupled receptor
GPR38	G protein coupled receptor 38
GRP	Gastrin releasing peptide
G-17	Gastrin 17
G-34	Gastrin 34

HO-2	Heme oxygenase 2
ICE	Interleukin 1β converting enzyme
IR	Immunoreactive
IL	Interleukin
IL-1β	Interleukin 1β
IP3	Inositol-1,4,5-triphosphate
min	Minute
ml	Millilitre
µl	Microlitre
mg	Milligram
n	Number
MLC	Myosin light chain
µm²	Micrometer square
MMC	Migrating motor complex
mRNA	Messenger ribonucleic acid
N.	Nervus
NANC	Non-adrenergic non-cholinergic
NO	Nitric oxide
OP	Operation
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAP	Peroxidase-antiperoxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHI	peptide histidine isoleucine
PKC	Proteine kinase C
PLC	Phospholipase C
RT	Room temperature
sec	Second
SP	Substance P
VIP	Vasoactive intestinal polypeptide
VPAC1-VIPR1	Vasoactive intestinal polypeptide receptor 1
VPAC2-VIPR2	Vasoactive intestinal polypeptide receptor 2
W	Watt

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1 Introduction and aims of the study

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Abomasal displacement (AD) of cattle is a very serious disorder which causes economical harm including reduced milk production, the cost of treatment or culling (Doll et al. 2009). According to Dirksen (1961) the AD of cattle was observed already in 19th century, but it was not until the 1950s that the first cases were published (Begg 1950, Moore et al. 1954, Müller 1953). Between the years 1948 and 1954 Moore et al. (1954) found 33 cows with AD following exploratory laparatomy of 580 cows. Since this initial publication the reporting of AD has increased. Coppock (1974) supposed that the increase in reporting of AD was the result of new diagnostic techniques and their application, as well as an increase in the occurrence of this disorder.

In the meantime, Doll (2007) reported an average lactation incidence of 2.3% in 12 German Holstein herds from Hessen, Germany. Other reports from Northern Germany found a lactation incidence of 1.6% (Wolf et al. 2001a, b), whereas Poike and Fürll (2000) found a lactation incidence of 1.75% with a large variation at herd level of up to 7.5% in some individual herds. In North America, a lactation incidence of 3% is considered to be “normal” (Whitmore 2004). In some extreme cases it can go up to 20% (Dawson et al. 1992, Pehrson and Stengärde 1998). According to Dirksen (2002), changes in cattle breeding and feeding in the last decades such as the selection of high milk yield, deep bodied cows and high food intake ability are the causes of this enormous increase in the incidence of AD.

The dairy breeds such as German Holstein and Holstein Friesian (Geishauser et al. 1996a, Martin et al. 1978, Wolf et al. 2001a, b), Brown Swiss, Ayrshires, Guernseys (Constable et al. 1992) and Jerseys (Jubb et al. 1991) are affected by AD. Other breeds such as German Fleckvieh (Doll 2007), Brown Swiss (Eicher et al. 1999) and Swedish Red Cattle (Stengärde and Pearson 2002) also suffer from AD, but the incidence in these breeds is very low compared to the classical dairy breeds named above.

There is a variety of risk factors that can lead to AD, but the primary cause of this disease remains unknown. It is supposed that hypomotility or atony of the abomasum is a prerequisite for the occurrence of AD (Dirksen 1961). This impairment of motility results in the accumulation of methane and CO_2, which pass into the abomasum from the rumen or are produced in the abomasum. The constant accumulation of gases in the abomasum distend it like a balloon and cause the abomasum to dislocate either to the left side, where it slides

between the abdominal wall and rumen, or caudo-dorsally to the right side. Motility impairment of the abomasum is accepted as a prerequisite of AD. Some factors held to be responsible for causing this impairment are: metabolic disorders (Holtenius et al. 2000, Pravettoni et al. 2004), high-concentrate low-fiber diets (Shaver 1997, Van Winden 2002), high insulin concentrations as a result of insulin resistance (Pravettoni et al. 2004) and genetic factors (Constable et al.1992, Geishauser et al. 1996a, Wolf 2001).

Geishauser et al. (1998) found an increased activity of neuronal nitric oxide synthase and a decreased sensitivity to acetylcholine in the tissue samples from the abomasal wall of German Holstein cows with AD compared to healthy cows of this breed (Geishauser et al. 1998). They suggested that a dysfunction of enteric neurons may be present in cows with AD. Recent studies have focused on possible disorders of the enteric nervous system. However, there are very few comparison studies between healthy cows and cows with AD regarding neuronal disorders. Furthermore, the immunohistochemical studies on the gastrointestinal tract of ruminants are limited (Balemba et al. 1999, Baltazar et al. 1998, Calingasan et al. 1984, Guilleteau et al. 1997, Kitamura et al. 1985, Pfannkuche et al. 2002a, b, 2003, Sickinger 2007, Soehartono et al. 2002).

Ontsouka et al. (2007, 2010) found with PCR tests that motilin receptor, muscarinic M2, M3 and α-2A adrenergic receptors were significantly lower in different parts of gastrointestinal tract of cows with AD than in healthy cows. Sickinger (2007) found with immunohistochemical ABC method that both substance P and vasoactive intestinal polypeptide concentrations were lower in cows with AD than in healthy cows.

It is unclear why German Holstein cows have an increased incident of AD when comparing them to German Fleckvieh cows. A recent study found significant differences between German Holstein and German Fleckvieh cows regarding the content of substance P (SP) and total innervation density in the abomasum (Sickinger 2007). The concentration of SP was significantly lower in German Holsteins and was accompanied by markedly higher levels of the neurotransmitter vasoactive intestinal polypeptide. These differences could explain why German Holstein cows are more frequently affected by AD than German Fleckvieh cows.

The primary aim of this study is to identify possible factors that may play a role in the occurrence of AD concerning the comparatively high incidence of AD in German Holsteins. Furthermore, the inter-individual differences between healthy German Holstein individuals and those affected by AD are discussed.

The effects of motilin and gastrin on the gastrointestinal motor functions are well known (Dockray 1994, Walsch 1994). These substances are hormones within the gastrointestinal tract which interact with their receptors to exert their effects. Vasoactive intestinal polypeptide (VIP) is a neuropeptide in the gastrointestinal tract which also plays a role in abomasal motility. The immunohistochemical expression of receptors for these peptides has not been studied in cow abomasum until now. An understanding of physiological functions of these gastrointestinal peptides requires precise localization of the peptides and their receptors of cattle with AD and healthy cows. Through the comparison of caspase-3 activity and the concentrations of motilin-, gastrin- and VIP-receptors and peptides, it was examined if the impairment of the abomasal wall has an effect on the abundance of these receptors and peptides. For the demonstration of these peptides and their receptors as well as the caspase-3 activity in the abomasal wall of cattle the immunohistochemical ABC method was used.

In summary, this study aimed to investigate the following questions:

- Are there differences between the German Holstein and the German Fleckvieh breed regarding the abundance of gastrin and motilin peptides, their receptors and also vasoactive intestinal polypeptide receptors in the abomasal wall?
- Are there differences between healthy German Holstein cows and German Holstein cows with AD regarding the substances that have been mentioned above?
- Is there an impairment of the abomasal wall in healthy German Holstein cows and those with AD?
- If such differences exist, are these a cause or a result of AD?

2 Literature review

2.1 Innervation of the gastrointestinal tract

The autonomic nervous system (also called visceral nervous system), hormones and other chemicals control motility and secretion of the digestive system. This system is composed of extrinsic (sympathetic and parasympathetic) and intrinsic (enteric) nervous systems (Dockray 1994).

Normally, the sympathetic and parasympathetic nervous systems act as antagonists (Schemann 2010). The sympathetic system activates and prepares the body for muscular activity, stress and emergencies while the parasympathetic system lowers the activity and is active in the normal situations. Sympathetic and parasympathetic neurons are composed of two neuron chains. Central parts of the autonomic nervous system are located in the spinal cord and brainstem, whereas the peripheral neurons are located in the ganglia outside of the central nervous system. The neurons which are located at the spinal cord and brainstem are called as preganglionic neurons, whereas the neurons in the ganglia are called as postganglionic neurons. The preganglionic neurons of sympathetic nervous system are located in the lateral horn of spinal cord and the preganglionic neurons of parasympathetic nervous system are located in brainstem and sacral spinal cord (Diener 2010). The neurons of both sympathetic and parasympathetic nervous systems use acetylcholine (ACh) as transmitter between preganglionic and postganglionic neurons. ACh binds to nicotinergic receptors in the postganglionic neurons. The transmission between the postganglionic neurons and the target organ occurs differently between both systems. Here, ACh acts as a transmitter in parasympathetic system, whereas noradrenaline is the transmitter in sympathetic system. Binding of noradrenaline to α-2 and β-2 receptors leads to the inhibition of motility while the binding of ACh to muscarinic M-3 receptors leads to motility stimulation (Diener 2010). In cows, M-2 and M-3 muscarinic receptors were found to be the most abundant of five muscarinic receptor subtypes in the gastrointestinal tract (Ontsouka et al. 2007). However, the use of muscarinic antagonists suggests that M-3 receptors play a more important role in smooth muscle contraction in cows (Niederberger et al. 2010). There are also co-transmitters such as nitric oxide (NO) and neuropeptides, which are released from the neurons of autonomic nervous system in addition to classical neurotransmitters ACh and noradrenalin (Diener 2010).

2.1.1 Extrinsic nervous system

2.1.1.1 Parasympathetic nerves

In ruminants, the motility of reticulum, rumen and omasum is highly dependent on parasympathetic activation. There are few thousand efferent fibers of the vagus nerve in the gastrointestinal tract. Therefore, N. vagus uses the enteric nerves as an intermediate amplifier (Schemann 2010). The motility of the gastrointestinal tract is controlled reflexive through the stimulation of digesta and afferent fibers of the vagus nerve in the medulla oblongata (Hofmann and Schnorr 1982). The activation of N. vagus leads to an increased secretion in the gastrointestinal tract and both to stimulation and inhibition of muscle activity. The activation of muscle cells occurs via muscarinic receptors, whereas the inhibition occurs with the release of nitric oxide (NO) and vasoactive intestinal polypeptide (VIP) from the enteric nerves (Schemann 2010).

2.1.1.2 Sympathetic nerves

The sympathetic nerves do not exert their effects on gastrointestinal muscle and mucosal activity directly but they use the enteric nerves. They exert their effect directly only on the blood vessels. The release of noradrenaline from the sympathetic nerves such as N. sympathicus causes an inhibition both on the motility, and the secretion of gastrointestinal tract (Schemann 2010). The visceroafferent nerves of the sympathetic nervous system are known as “pain nerves”. Certain spinal cord segments are responsible for the innervation of certain areas in the body. Therefore, pain from the inner organs is transferred to the certain skin areas which are also called as “head zone” (Diener 2010).

2.1.2 Enteric nervous system

The enteric nervous system (ENS) is responsible for the autonomic regulation of primary gastrointestinal tract functions. It is composed of nerve nets which are located in the organ walls and it has numerous small ganglia distributed from oesophagus to anus in the gastrointestinal tract (Schemann 2010). The main components of enteric nervous system are myenteric and submucosal plexuses which are embedded in the gastrointestinal wall. Myenteric plexuses are located between circular and longitudinal layers of muscle in the gastrointestinal wall and they play a role in the control of gastrointestinal motility. Submucosal plexuses are located in the submucosa and they regulate different mucosa functions such as secretion and resorption (Diener 2010, Phillips and Wingate 2002).

The ENS can activate the muscle and mucosa discretely and regulate motility, secretion, microcirculation, and immune processes in the gastrointestinal tract. In the ENS, there are three types of functionally different neurons for the regulation of complex processes which are: sensory neurons, motor neurons, and interneurons. The sensory neurons are the primary afferent neurons of ENS which respond to mechanical, thermal, osmotic, and chemical stimuli. Interneurons are responsible for the communication between enteric neurons, whereas the motor neurons control gastrointestinal motility and secretion (Schemann 2010). The sympathetic and parasympathetic nerves are connected with the ganglia of ENS and their effects on the innervation of muscle and mucosal activity occur via the ENS (Philips and Wingate 2002). Central nervous system (CNS) is also involved in digesting processes. The interaction between the brain and ENS occurs via visceral afferent neurons which conduct sensory impulses to the CNS, the sympathetic and parasympathetic nerves are the efferent side, which transfers the signals from CNS to gastrointestinal tract (Schemann 2010).

The activities of both excitatory and inhibitory neurons that innervate the muscle are controlled by the neurotransmitters in the ENS. In addition to the classical neurotransmitter ACh and noradrenaline, the release of neuropeptides such as substance P stimulates the motility of gastrointestinal tract, whereas vasoactive intestinal polypeptide (VIP) inhibits the GI motility. These effects of the neuropeptides occur without participation of ACh and noradrenaline, therefore these neuropeptides are called as non-adrenergic non-cholinergic (NANC) transmitters (Schemann 2010).

2.2 Neuropeptides and gastrointestinal hormones

Neuropeptides ranging in size from 3 to 40 amino acid residues constitute a major group of intercellular agents for cell to cell communication, either as messenger hormones or as neurotransmitters and neuromodulators. They are expressed and released by neurons and mediate neuronal communication by acting on cell surface receptors. As they form an extremely large class of communicating molecules, it has been shown that the same neuropeptide may act at one site as a neurotransmitter, at another site as a neuromodulator, and at a third site as a neurohormone (Strand 1999). Some of these peptides can have a function both as hormone and transmitter (Dockray 1994). The classical neurotransmitters are synthesized in the nerve terminals, with the necessary enzymes being transported from the cell body to the terminals. It has been shown that two or more potential transmitters can co-

localize in the same neuron (Pfannkuche et al. 2002a, Vittoria et al. 2000). They are released by nerve stimulation, with the release being dependent on calcium influx. In the gastrointestinal tract, neurotransmitters are involved in controlling the motility, secretion and absorption (Strand 1999).

The effect mechanism of neuropeptides and gastrointestinal hormones occurs through binding of these peptides with their cell surface receptors. Gastrointestinal hormones are molecules which transmit information from one cell to another. They are secreted from endocrine cells upon stimulation into the bloodstream and are distributed to their targets through the general circulation where they elicit an acute response such as secretion or muscle contraction (Dockray 1994, Rehfeld 1998). Furthermore, gastrointestinal hormones may act as neurotransmitters, local growth factors, and fertility factors (Rehfeld 1998).

2.3 Gastrointestinal peptides

2.3.1 Gastrin

Gastrin was first discovered in antral mucosal extracts of the stomach by Edkins in 1905 as a substance, which stimulates the gastric acid secretion (Edkins 1905). 60 years later, Gregory and Tracy showed the precise identity of gastrin, they first chemically described the gastrins as 17 and 34 amino acid residues, C-terminally amidated peptides (G17, G34) (Dockray 2004, Gregory and Tracy 1964). These both G17 and G34 peptides are called as “classical gastrins” because they possess the defining biological property of the hormone: the stimulation of gastric acid secretion (Dockray et al. 2005, Gregory and Tracy 1964).

Gastrin and cholecystokinin (CCK) share a common C-terminal tetrapeptide sequence, which also constitutes the minimal structure essential for biological activity (Johnsen 1998). They together constitute the CCK/gastrin family which are the only members of this family in mammals (Dockray 2004, Johnsen 1998). Gastrin and CCK are thought to have evolved from a common ancestral gene because of their structural and functional similarities (Schulkes and Baldwin 1997). Gastrin and CCK can both bind to gastrin/CCK-B receptors, whereas the CCK-A receptors bind only sulphated CCK peptides (Rehfeld et al. 2007).

The antral G cells are the main site of gastrin synthesis. The distribution of these cells differs among species. In humans and dogs, they are localized between the neck and base of the antral glands, whereas in rats they are localized on the base of the glands (Walsh 1994). Early immunohistochemical studies on ruminant abomasal tissues have also shown a restricted localization of gastrin to the antral mucosa of the abomasum and to some extent in duodenum but not within the corpus (Baltazar et al. 1998, Calingasan et al. 1984, Kitamura et al. 1985). There are different factors which cause gastrin release such as gastric luminal amino acids, neuronal stimulation, circulating catecholamines, and gastrin releasing peptide (GRP) and inhibited by low gastric luminal pH, by several peptides such as somatostatin, VIP, and by certain prostaglandins (Walsh 1994). VIP does not have a direct effect on G cells to stimulate gastrin secretion but it stimulates somatostatin release from D cells, which inhibits gastrin secretion (Schubert and Makhlouf 1992). When acid secretion is reduced or absent, for example as a result of a pernicious anaemia, gastrin release will increase (Jensen 2002).

There are also other cell types than G cells which also release gastrin peptides. But these cells contribute only little to circulating gastrin because the secretion seems to serve local purposes (Rehfeld et al. 2007). These cells, which express progastrin, could be detected in ileum and colon (Friis-Hansen and Rehfeld 1994, Lüttichau et al. 1993), in cerebellar and vagal neurons (Rehfeld 1991, Uvnäs-Wallensten et al. 1977) and in the pancreas (Bardram et al. 1990).

The main circulating forms of gastrin in the plasma of most mammals are G34 and G17 (Walsh 1994). In cats, only gastrin-17 can be detected in the peripheral blood (Rehfeld et al. 2007). The concentration of gastrin in plasma varies between 10-20 pmol/l in basal state and increases to 50 pmol/l after stimulation with a protein rich meal in 15-20 minutes (Rehfeld et al. 2007). However, the concentration of CCK in human plasma is 10 to 20 fold below those of gastrin (Rehfeld 1998). Receptor selection is influenced from this difference because the 10-20 folds lower CCK concentrations in plasma can not compete with gastrin, therefore, the gastrin/CCK-B receptors in periphery are the receptors only for gastrin (Rehfeld et al. 2007).

The gastrin receptor

Peptides of gastrin/CCK family bind at the well characterized CCK1 (also known as CCK-A) and CCK2 (also known as CCK-B) receptors, which belong to the G protein coupled receptor family. These receptors have seven transmembrane segments and in humans 50% of the

amino acids are identical (Shulkes and Baldwin 1997). The purification and cloning of these receptors made it evident that, these two peptides exert their effects on their target cells by binding to their receptors (Kopin et al. 1992, Wank et al. 1992).

Gastrin has a high affinity for CCK2 receptor but not CCK1 receptor, whereas CCK has a high affinity for both CCK1 and CCK2 receptors (Noble et al. 1999). CCK1 receptor has been first described by Sankaran et al. (1980) in rat pancreas, whereas the CCK2 receptor was first found in brain (Innis et al. 1980).

Both gastrin and CCK are secreted from peptide producing cells into the interstitial fluid as well as in the blood. From circulating blood, they may specifically act on their target cells (Walsh 1994). Both CCK1 and gastrin/CCK2 receptors use the same signalling mechanisms to control the secretion. When the CCK2 receptor binds to a G protein, the activation of phospholipase C (PLC) occurs as a result, which catalyzes the formation of inositol- 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 stimulates the release of intracellular calcium, and DAG activates protein kinase C (PKC) (Sawada and Dickinson 1997).

CCK1 and CCK2 receptor expression was found on multiple tissues but the tissue distribution shows a variation between species. In human, gastrin/CCK2 expression was found in the parietal and ECL cells of stomach (Kulaksiz et al. 2000, Schmitz et al. 2001, Waldum et al. 2002), pancreas (Morisset et al. 2003, Reubi et al. 2003), smooth muscle of stomach (Reubi et al. 1997) and in brain (Monstein et al. 1996). It was also found in canine stomach mucosa, circular muscle and myenteric neurons (Mantyh et al. 1994), ECL and D cells, submucosal smooth muscle cells (Helander et al. 1997), guinea pig gastric smooth muscle cells (Bishop et al. 1995, Boyle et al. 1993, de Weerth et al. 1997) and parietal cells on the gastric mucosa (Tarasova et al. 1994, 1996). Other tissues that the expression of gastrin/CCK2 receptor was found are rat vagal afferent neurons (Moriarty et al. 1997) and pancreas (Wank et al. 1992).

Studies dealing with ruminants concerning the localization of CCK/gastrin family receptors are very limited. Onaga et al. (2008) found in sheep omasal muscle layer a high expression of CCK2 and CCK1 receptors. It is shown that calf pancreas express both CCK1 and CCK2 receptors (Le Drean et al. 1999, Morriset et al. 2003). Guilloteau et al. (1997) found by mRNA analysis that both CCK1 and CCK2 receptors are expressed in the central nervous system of the calf. An immunocytochemistry study in calf intestinal mucosa showed a co-

localization of CCK1 and CCK2 receptors with neural fibres (Zabielski et al. 2002). These receptors were also examined by mRNA expression on the ventral sac of the rumen and fundus of the abomasum from new born calves, 13 weeks old calves and adult cows (1.5-2 years old). CCK1 receptor expression in the rumen could be detected in 3 week old animals but not in 13 week old and adult animals. CCK2 receptor expression in abomasal wall decreased gradually from 3 week old to adult animals and in adult animals the expression was very weak (Yonekura et al. 2002).

Effects of gastrin

Gastric acid secretion was the first effect of gastrin to be found (Edkins 1905). Stimulation of gastric acid secretion is regulated by gastrin directly by stimulating parietal cells and indirectly by stimulating the release of histamine from enterochromaffin-like (ECL) cells, which then binds to H2 receptors on parietal cells and stimulate the gastric acid secretion (Schubert 2007). The release of somatostatin from gastric D cells is also stimulated by gastrin, which is a potent inhibitor of gastric acid secretion (Walsh 1994). The exocrine effect of gastrin on pancreas tissues of calves (Le Drean et al. 1999) and human (Valenzuela et al. 1976) has also been shown.

In humans, it has been shown that the duodenal acidification inhibits gastric motility and may induce phase III-like complexes in duodenum (Woodtli and Owyang 1995). In another study, patients with gastric acid hypersecretion showed a suppression of interdigestive gastrointestinal motility (Bortolotti and Barbara 1988). Verkijk et al. (1998) found that infusion of gastrin to postprandial plasma levels prolongs the reoccurrence of phase III motor activity in both antrum and duodenum and this effect is a result of increased intraluminal acidity rather than gastrin itself. A study in canine also showed that intraluminal acidification can interrupt gastric emptying (Lin et al. 1990). However, Gielkens et al. (1998) showed that acute inhibition of gastric acid secretion in healthy human patients does not affect interdigestive antroduodenal motility.

It has already been shown that the cows with left displaced abomasum have a smaller pH value in their abomasum compared to the healthy cows (Geishauser et al. 1996, Krey 2005, Meermann and Aksoy 1983).

There are also reports that gastrin is a trophic/mitogenic peptide for normal and neoplastic gastrointestinal cells (Majumdar and Johnson 1982, Watson et al. 1989, Yassin 1999). Higher levels of gastrin in the circulation in human and rodents caused hypertrophy of gastric mucosa, hyperplasia of parietal and ECL cells, whereas its absence cause reduced numbers of parietal and ECL cells (Bordi et al. 1995, Hakanson et al. 1986, Wang and Dockray 1999).

Todisco et al. (2001) have proposed a cellular apoptosis inhibiting function for gastrin which induced by serum withdrawal through the activation of gastrin/CCK2 receptor. Another study has also shown CCK2 receptors in the regenerative gastric mucosa in the repair zone adjacent to the ulcer crater of rats (Schmassmann and Reubi 2000). This result suggests a role for gastrin and CCK2 receptor in the wound healing. There is also another report which suggests an antiapoptotic role for gastrin in human oesophagus tissues (Harris et al. 2004).

The effect of gastrin on smooth muscle has also been studied on different species. In dogs, increased gastrin secretion elevates caudal oesophageal sphincter pressure, delays gastric emptying, and promotes pyloric muscular hypertrophy (Hall et al. 1989). El-Sharkawy and Szurszewski studied also canine stomach and found an enhanced mechanical response to pentagastrin and they stated that this effect is a direct action on smooth muscle cells (El-Sharkawy and Szurszewki 1978).

In humans, pentagastrin has been found to stimulate circular muscle cells of corpus region of human stomach (Hara 1980) and slower gastric emptying (Jin-Zhang et al. 1996). Another study in humans reported that gastrin by itself does not seem to have a major role in human antroduodenal motility regulation (Dooley et al. 1984). In rats, gastrin induces also a contraction on gastric smooth muscle cells (Wang and Zhou 2000). A study reported in rats without CCK2 receptor an enhanced gastric emptying of a non-nutrient liquid load (Miyasaka et al. 2004).

McLeay and Wong (1989) showed on the sheep omasum and abomasum that pentagastrin stimulates both muscle layers of body and antral regions of the abomasum. In contrast, pentagastrin showed inhibitory effects on reticuloruminal motility. They concluded that this stimulating action of pentagastrin on the abomasum conducted by direct effect on the muscle and by cholinergic neurons.

Although Vlaminck et al. (1986) found no significant differences between serum gastrin levels of healthy cows and cows with abomasal displacement, Sen et al. (2002) found that the mean plasma gastrin concentration was significantly higher in cows with abomasal displacement than in healthy cows.

2.3.2 Motilin

Motilin was first isolated by Brown et al. in 1971 from hog duodenal mucosa and named as motilin because of its biological capacity to stimulate motility of digestive organs (Brown 1971). Two years later, the complete 22 amino acid sequence of motilin was announced which was corrected again a year later by Schubert and Brown (Brown 1973, Schubert and Brown 1974). The structure of motilin was also identified in other species like the cat (Depoortere et al. 1993), man (Dea et al. 1989), sheep (De Clercq et al. 1997), rabbit (Banfield et al. 1992), cow (Huang et al. 1999), horse (Huang et al. 1999), canine (Poitras et al. 1983), and differences in amino acid sequences have been found. The N-terminal amino acid sequence 1-9 of motilin was found to be responsible for the contractile activity of motilin (Huang et al. 1999, Poitras et al. 1992).

Motilin was the only representative in its class until the discovery of ghrelin in 1999 (Kojima et al. 1999). The identification and characterization of ghrelin and motilin precursors showed almost 50% similarity in their amino acid sequences. As a result of this similitude, ghrelin has been identified as “motilin related peptide” (Tomasetto et al. 2000). Now, motilin and ghrelin together constitute the newly discovered motilin-ghrelin family (Poitras and Peeters 2008, Tomasetto et al. 2000). A recent study of upper human intestine showed that ghrelin and motilin are co-secreted from the same endocrine cells (Wierup et al. 2007).

Motilin is produced by duodenojejunal endocrine cells (Walsh 1994). After the discovery of motilin, the immunolocalization of motilin secreting cells has been demonstrated in the upper small intestine of dog (Smith et al. 1981, Yanaihara et al. 1978), cat (Depoortere et al. 1993), rabbit (Satoh et al. 1995), man (Heitz et al. 1978, Helmstädter et al. 1979, Smith et al. 1981), monkey (Helmstädter et al. 1979), pig (Agungpriyono et al. 2000), rat (Sakai et al. 1994), and in ruminants such as cow (Kitamura et al. 1985), sheep (Calingasan et al. 1984), and Philippine Carabao (Baltazar et al. 1998). Some authors could also locate motilin cells in the antrum of rabbit (Satoh et al. 1994) and man (Bryant et al. 1983). Recently Xu et al. (2005)

could show motilin in the myenteric plexuses of guinea pig where it is co-localized with nitric oxide synthase. Other than gastrointestinal tract, motilin immunoreactivity was also detected in brain of man and rabbit (Depoortere et al. 1997). Nilaver et al. (1982) detected motilin immunoreactivity also in cerebellum purkinje cells of rat, mouse and human. Feng et al. (2007) could also locate motilin and motilin receptor in the rat amygdala. In contrast, Bond et al. (1988) could not detect any motilin mRNA in the brain. However, recent studies found motilin and motilin receptor in the rat and guinea pig brain (Feng et al. 2007, Liu et al. 2005, Xu et al. 2001).

Motilin plays an important role in the initiation of phase III of the migrating motor complex (MMC) in man (Peeters et al. 1980) and dog (Itoh et al. 1978, Poitras 1984). It is released at 100 min. intervals during the interdigestive state when no nutrient is present, at least in the duodenum and the upper jejunum, and moreover the release of motilin is inhibited by feeding in dog (Itoh et al. 1978) and in man (Rees et al. 1982, Boivin et al. 1990). In ruminants, there is a continuous inflow of digesta from the forestomachs into the abomasum, therefore abomasum is never empty. Because of this condition, it does not exist any interdigestive period in the abomasum. Therefore, the emptying of the abomasum and cleaning of intestines does not occur simultaneously in ruminants (Ehrlein 2010). MMC of sheep is not abolished by feeding like in dogs and man (Ruckebusch 1977). Ruckebusch (1977) showed that MMC is always present in ruminants and can only be seen during the fasting state in dogs and rats. The irregular spike activity of MMC begins in the sheep at the pylorus and there is an antral inhibition during the periods of duodenal bulb activity. A study in man showed that motilin can induce phase III contractions only in antrum (Luiking et al. 2003). In dog, the plasma motilin concentrations coincide with the end of phase III contractions in the stomach (Itoh et al. 1978) and the presence of nutrient in duodenum strongly suppresses the endogenous release of motilin (Mori et al. 1981). Motilin induced contractions can be obtained through stimulation of motilin receptors located directly on smooth muscle cells (Miller et al. 2000).

Tanaka et al. (2001) found that the action of motilin in dogs is not mediated via the vagal afferent nerve since exogenous motilin still induced a premature gastric MMC in the interdigestive state after vagotomy. In humans, the premature phase III contractions induced by exogeneous motilin could be blocked by atropine in the antrum but not in duodenum, indicating the involvement of muscarinic mediation in human antrum and a non-cholinergic pathway in duodenum (Boivin et al. 1997).

Motilin receptor

Feighner et al. (1999) identified the orphan G protein-coupled receptor GPR38, originally isolated from thyroid gland, as the human motilin receptor. Recently, the motilin receptor has also been cloned from other species like rabbit (Dass et al. 2003) and dog (Ohshiro et al. 2008).

Motilin receptors are mainly found in the gastrointestinal tract, but their exact localization is species dependent. Recent studies located motilin receptor mRNA and immunoreactivity in human stomach and upper intestine (Takeshita et al. 2006). In the same study, motilin receptor mRNA was found on the mucosal and muscle layers of stomach and upper intestine, whereas the immunoreactivity of this receptor was found only in the muscle layer and myenteric neurons. Another study found motilin receptor immunohistochemical staining being strongest in the antrum but they could also show it in colon and ileum muscle layers (ter Beek et al. 2008). Miller et al. (2000) has also found that the motilin receptors are in maximum concentration in human antrum and they are located in muscle and nerves in the antral wall. In rabbits, the highest concentrations of motilin receptor were found in the antrum and colon (Miller et al. 2000b, Van Assche et al. 1998). A functional motilin receptor does not exist in the gastrointestinal tract of rodents (Aerssens et al. 2004). Outside of the gastrointestinal tract, they could also be shown in the central nervous system (Feng et al. 2007, Liu et al. 2005) and in thyroid (Feighner et al. 1999). Localization studies of motilin receptor in ruminants are very scarce. Ontsouka et al. (2007b) found motilin receptor mRNA in abomasal wall and upper intestines of cattle.

The amino acid sequence of human motilin receptor was found to be 52 % identical to the human receptor for growth hormone secretagogues (Feighner et al. 1999). GPR38/motilin receptor was identified as an orphan receptor belonging to growth hormone secretagogue receptor (GHR-S) family (Holst et al. 2004). Motilin receptor is a G protein coupled receptor (GPCR) and it has an amino terminus located at the extracellular side and a carboxyl terminus located at the intracellular side (Feighner et al. 2009). Motilin shows a high affinity to receptors on the smooth muscle cells (Huang et al. 2004). There are two signalling pathways that mediate Ca^{2+}-dependent and independent contraction by motilin receptors on smooth muscle cells. Motilin initiates $G\alpha_q$ – mediated cascade, Ca^{2+} /calmodulin activation of myosin light-chain (MLC) kinase and transient MLC20 phosphorylation, and contraction as well as a

sustained Gαq- and Gα13-mediated Rho-A dependent cascade leading to inhibition of MLC phosphatase, sustained MLC20 phosphorylation, and contraction (Huang et al. 2004).

GPCRs stimulated by their agonist are susceptible to tachyphylaxis (De Smet et al. 2009). After the finding of erythromycin A (EM-A) as a motilin receptor agonist (Peeters et al. 1989), it became also clinically relevant when it is discovered that EM-A accelerated delayed gastric emptying in patients with diabetic gastroparesis (Janssens et al. 1990). It has also been shown in cows that preoperative administration of a single dose erythromycin increases abomasal emptying rate (Wittek et al. 2008). However, recently the motilin receptor agonist (ABT-229) has failed to improve the gastric emptying and caused a rapid desensitization of motilin receptor (Thielemans et al. 2005). It was shown that the stronger desensitization properties of ABT-229 are due to a higher degree of internalization and a delayed recycling of internalized receptors to plasma membrane (De Smet et al. 2009). Tack and Peeters (2001) discussed that motilides with a short half-life such as EM-A may be less likely to induce tachyphylaxis. There are also reports that chronic use of erythromycin causes a down regulation of motilin receptors (Bologna et al. 1993, Depoortere et al. 1991).

Effects of motilin

After the discovery of motilin, it was shown that the exogenous administration of motilin initiates phase III contractions in the stomach which are similar to spontaneously occurring phase III contractions in dogs (Itoh et al. 1976) and man (Tomita 2009, Vantrappen et al. 1979). Itoh et al. (1978) showed also that the phase III contractility is associated with the plasma motilin peaks in dogs, and Lee et al. (1983) reported that the immunoneutralization of circulating motilin suppresses phase III contractions. It was also shown that motilin and its agonist erythromycin-A accelerates gastric emptying in man (Janssens et al. 1990, Urbain et al. 1990) and cow (Wittek et al. 2008). Coulie et al. (1998) suggested that the antral motor effects of erythromycin-A (EM-A) in humans were mediated via different pathways. A low dose of EM-A induced a premature antral activity which was mediated through activation of an intrinsic cholinergic pathway, whereas a higher dose produces prolonged antral contractile activity which did not require activation of a cholinergic pathway (Coulie et al. 1998). Bouvin et al. (1997) showed also the effect of motilin on the antrum and duodenum is exerted through different stimulatory pathways in humans. The finding that the effect of motilin on phase III activity in dogs was blocked by a 5-hydroxytryptamine-3 (5-HT3) antagonist suggests that the

effect of motilin may be mediated via motilin receptors located on 5-HT neurons in the medulla (Itoh et al. 1991).

In vitro studies have shown that motilin induces a contractile activity of smooth muscle of man (Cuomo et al. 2006, Lüdtke et al. 1989), guinea pig (Harada et al. 1992), dog (Mizumoto et al. 1993) and rabbit (Adachi et al. 1981, Moummi et al. 1989). The contractile effects of motilin were not antagonized by muscarinic blockade or by administration of tetrodotoxin to inhibit Na^+-channels which suggest a direct action of motilin on smooth muscle cells. Both motilin and EM-A has also a direct excitatory action on circular smooth muscle of left and right human colon in vitro (Van Assche et al. 2001). Tanaka et al. (2001) showed that after vagotomy in dogs, the premature gastric MMC was still present.

Motilin was also shown to have effect on gallbladder emptying. Intravenous administration of EM-A induced a plasma motilin peak followed gallbladder emptying (Fiorucci et al. 1992). This effect is blocked by atropine and ondansetron indicating that cholinergic and serotonergic mechanisms are involved (Fiorucci et al. 1992, Fiorucci et al. 1993). However, Luiking et al. (2002) could not confirm motilin induced gallbladder emptying in healthy volunteers. It has also been shown that the intravenous administration of motilin agonists increases the motility of lower esophagal sphincter (Chaussade et al. 1994, Korimilli and Parkman 2010).

2.3.3 Vasoactive intestinal polypeptide

Vasoactive intestinal polypeptide (VIP) was discovered by Drs. Victor Mutt and Sami Said in 1970 from porcine intestinal extracts which displays a 28 amino acid sequence (Said and Mutt 1970). After the isolation and characterization of this peptide in different species, it has been shown that VIP is highly conserved between vertebrate species and belongs to the secretin/glucagon/pituitary adenlylate cyclase activating peptide (PACAP) family (Carlquist et al. 1979, Dimaline et al. 1984, Dockray 1994). This family has structurally related peptides which also include several other peptides like growth hormone releasing hormone and peptide histidine isoleucin (PHI). PHI shares structural and functional properties with VIP and it was shown that it binds preferentially to VIP receptors (Huang and Rorstad 1990, Tatemoto and Mutt 1981). Among these members, VIP and PACAP are structurally and functionally the most closely related peptides in this family (Lelievre et al. 2007).

The largest amounts of neurons containing VIP are found in the gastrointestinal tract of mammals (Dockray 1994). VIP is present in the intrinsic neurons of the stomach which mainly innervate the smooth muscle layer (Fahrenkrug 1993). Other than digestive tract, it could also be found in the brain (Benagiano et al. 2009, Fahrenkrug 1993), central and peripheral nervous system (Said and Rosenberg 1976), spinal cord, urinary system (Dockray 1994), and in the smooth muscles of respiratory system (Lundberg et al. 1984). Studies in ruminants located VIP containing neurons in the myenteric neurons in the abomasal wall and pancreas of sheep (Arciszewski and Zacharco-Siembida 2007, Arciszewski et al. 2009) but not in the endocrine glands of gastroenteropancreatic cells (Calingasan et al. 1984). Recent studies could also locate VIP in the nerves in smooth muscle of the abomasal wall of cows (Sickinger 2007, Vittoria et al. 2000) and in the smooth muscle of rumen and reticulum of lambs (Pfannkuche et al. 2004). Some authors showed that nitric oxide (NO) and VIP are colocalized in the myenteric neurons of bovine gut like several other mammalian species (Pfannkuche et al. 2003, Vittoria et al. 2000).

VIP in gastric motility is a putative non-adrenergic non-cholinergic (NANC) transmitter of the enteric nervous system. Feeding does not affect the systemic plasma concentrations of VIP and normally, the systemic plasma concentrations are low (Dockray 1994). Electrical stimulation of vagal efferent fibres in the presence of atropin and adrenergic blockers or gastric distention induces VIP mediated gastric relaxation (Chayvialle et al. 1980, D'amato et al. 1992, Gaginella et al. 1981, Ohta et al. 1990). It was also shown that the release of VIP is calcium dependent (Angel et al. 1983). Stimulation of Nervus splanchnicus was shown to inhibit vagally provoked release of VIP in the cat via α-adrenergic receptors (Fahrenkrug et al. 1978). Edwards et al. (1978) found that stimulation of Nervus vagus in the calf without splanchnic nerves causes a rise both in arterial plasma VIP and in the VIP concentration of intestinal lymph. Also in dog, it was shown that vagal stimulation causes an increase in VIP concentrations of portal and gastrin venous blood and gastric relaxation (Ito et al. 1988, Ohta et al. 1990).

VIP receptors

VIP and PACAP share two common G protein-coupled receptors VPAC1 and VPAC2 while PACAP has an additional specific receptor PAC1 (Harmar et al. 1998, Laburthe and Couvineau 2002). The PAC1 receptor shows lower affinity for VIP, whereas the VPAC1 and

VPAC2 receptors exhibit similar affinities for VIP and PACAP (Laburthe and Couvineau 2002, Schulz et al. 2004). However, VIP binds to VPAC1 and VPAC2 in peripheral organs including gastrointestinal tract of human (Schulz et al. 2004). The relaxation of smooth muscle occurs when VIP interacts with its receptors coupled to cAMP- or cGMP- signalling pathways. It was also shown that human fundic smooth muscle is affected by the activation of cGMP-pathway, whereas the smooth muscle of antrum is affected by the activation of cAMP-pathway (Severi et al. 2006).

VPAC2 receptor was mainly found in the smooth muscle of gastrointestinal tract of human (Schulz et al. 2004), mouse (Harmar et al. 2004), and rabbit (Murthy et al. 1997), whereas the VPAC1 receptor was found in mucosa and myenteric neurons throughout the gastrointestinal tract of human (Schulz et al. 2004) and mouse (Harmar et al. 2004). Binding of VIP and PACAP released from mucosal nerve fibers and neuroendocrine cells to VPAC1 possibly regulates the secretion, whereas binding of VIP released from myenteric nerve fibres to the VPAC2 receptor in the smooth muscle of gastrointestinal wall probably regulates the motor activity (Schulz et al. 2004). Outside of the gastrointestinal tract, VPAC1 and VPAC2 were found in urogenital system, lympathic system of mouse, however, VPAC2 receptors were not detected in heart and skeletal muscle. The human lung expresses predominantly VPAC1 receptors, whereas mouse lung express VPAC2 receptors (Busto et al. 2000, Harmar et al. 2004, Reubi et al. 2000). VPAC2 receptor distribution shows a difference between mouse and man (Harmar et al. 2004). The VPAC1 receptor was also found in smooth muscle cells in cerebral arteries and arterioles of rat (Fahrenkrug et al. 2000). In ruminants, a study indicates the presence of VPAC1 receptor in calf pancreas membranes (Guilloteau et al. 1997, Le Meuth et al. 1991).

VPAC1 receptor was expressed predominantly in most frequently occurring human tumors (Reubi et al. 2000). Breast cancer tissues in rats also express VPAC1 receptors predominantly (Dagar et al. 2001). VPAC2 receptors were only found in the benign leiomyoma (Reubi et al. 2000).

Effects of VIP

VIP is an important inhibitory non-adrenergic non-cholinergic (NANC) neurotransmitter of peripheral neurons. It has a relaxant effect on both vascular and non-vascular smooth muscle

and stimulates the secretion from pancreas and intestines (Dockray 1994). An interplay between neuronal NO synthase (nNOS) and the peptide neurotransmitter VIP causes the relaxation of smooth muscle in gastrointestinal tract. Stimulation of smooth muscle eNOS by VIP causes the production of NO (Murthy 2006). A study suggested also that VIP induce its relaxation effect indirectly by stimulating heme oxygenase 2 (HO-2) to form CO, which in turn mediates the relaxation (Watkins et al. 2004). In vitro studies have also shown a relaxing effect of VIP on smooth muscle preparations of gastrointestinal tract from different species including guinea pig, cat, pig, and man (Bitar and Makhlouf 1982, Grider et al. 1985, Lundberg et al. 1984) and a vasodilatation effect (Bardrum et al. 1986). However, it was also shown that VIP induces an excitatory effect by causing release of ACh in the ileum of guinea pigs (Gordon et al. 1990). Exogeneous infusion of VIP was shown to cause a cessation of the electromyographic activity of the abomasum in lamb (Reid et al. 1988).

VIP also acts as presecretory neurotransmitter in the gastrointestinal system. Intravenous administration of VIP stimulates intestinal secretion (Schwartz et al. 1974). It has also been shown that VIP producing tumors may cause increased fluid secretion and diarrhea which is particularly evident in the watery diarrhea syndrome (Bloom et al. 1973). The regulation of blood flow in intestines and salivary gland may also be mediated by VIP (Dockray 1994). VIP may also stimulate somatostatin release from D cells, which in turn inhibits gastric secretion (Sawada and Dickinson 1997).

The frequent overexpression of VIP receptors, especially VPAC1 in human tumors suggest that VIP may have a growth stimulating effect (Schulz et al. 2004). VIP receptor antagonists have been shown to inhibit this growing effect in various tumor models (Maruno et al. 1998, Moody et al. 2001).

There are also reports about the antiapoptotic effect of VIP (Gonzales-Rey et al. 2005). VIP and PACAP have been shown to inhibit the production of TNF-α, IL-1β, IL-6, NO and several pro-inflammatory chemokines (Delgado et al. 2002, Kim et al. 2000). They also stimulate the expression of anti-inflammatory factors IL-10 and IL-1Ra and this action is mediated by VPAC1 receptor in mouse (Delgado et al. 2003). Vaudry et al. (2000, 2002) showed in rat cerebellar granule cells that PACAP exerts a neuroprotective effect and protect dopaminergic neurons from oxidative stress-induced apoptosis death by inhibiting caspase-3 activation. Said and Dickman (2000) also revealed that the reduction in lung injury by VIP

was associated with the inhibition of caspase activation which suppress cell death and promote cell survival and with the upregulation of bcl-2 anti-apoptotic protein.

2.4 Caspases

Apoptosis is the major form of cell death in the organism (Krammer 2000). It is a complex biological process which enables an organism to kill and remove unwanted, old, and injured cells during animal development, normal homeostasis, and disease (Jacobson et al. 1997, Porter and Jänicke 1999, Thompson 1995).

Early studies on apoptosis have been carried out in nematodes. Mutation analysis identified in the nematode “Caenorhabditis elegans” that the genes ced-3 and ced-4 are essential for the apoptosis, whereas ced-9 antagonizes their function and prevents cell death (Ellis and Horvitz 1986). During the development of this nematode 131 of 1090 somatic cells die because of apoptosis. These deaths are precisely controlled because in every animal the same cells die and each at its own characteristic time (Ellis et al. 1991). Since the recognition that ced-3 has sequence identity with the mammalian cysteine protease interleukin 1-β converting enzyme (ICE or Caspase-1), a family of 14 related cysteine proteases has been identified (Fan et al. 2005).

Cysteine-aspartic proteases (Caspase) are a family of cysteine proteases that play essential roles in apoptosis and inflammation (Alnemri et al. 1996, Thornberry and Lazebnik 1998). Fourteen caspases have been identified so far (caspase 1-14) and they share similarities in amino acid sequence, structure, and substrate specificity (Nicholson and Thornberry 1997). Caspases are highly conserved through evolution and can be found from humans all the way down to insects, nematodes, and hydra (Budihardjo et al. 1999, Cikala et al. 1999, Earnshaw et al. 1999). Caspases are synthesized as inactive proenzymes (procaspases) which contain an N-terminal peptide together with one large and one small subunit (Cohen 1997). When cells undergo apoptosis, these caspases become activated through one or two sequential proteolytic events that cleave the single peptide precursor into the large and small fragments that constitute the active enzyme (Budihardjo et al. 1999, Thornberry and Lazebnik 1998). Caspases can be classified into two main groups according to the length of their N-terminal prodomain. Procaspases such as procaspase-2, -8, -9, and -10 are characterized by their long prodomain. These enzymes are responsible for initiating caspase activation cascades.

Procaspases with a short prodomain such as procaspase-3, -6, and -7 are called as effector caspases. These caspases are the main effectors of apoptotic cell death by cleaving cellular substrates, in other words, they are thought to be responsible for the actual demolition of the cell during apoptosis (Launay et al. 2005). The apoptosis is irreversible when the effector caspases (caspase 3, 6, 7) exert their proteolytic activity (Thornberry and Lazebnik 1998). Caspase-3 is one of the most important members of the family due to its important position in the apoptotic processes (Porter and Jänicke 1999).

The initiation of apoptosis may be divided into two phases, an initiation and an execution phase. In the initiation phase, caspases become initially active, whereas in the execution phase these enzymes act to cause cell death. In mammals, there are two main pathways for caspase activation which are called intrinsic (mitochondrion-mediated) and extrinsic (death receptor-mediated) pathways. Both pathways converge to activate caspases (Logue and Martin 2008).

The extrinsic pathway to caspase activation involves the interaction of death receptor ligands such as FasL and tumor necrosis factor-α (TNF-α) with the death receptors. The best characterized death receptors are Fas (also called CD95) and tumor necrosis factor 1 (TNF1). Additional receptors such as death receptor 3 (DR3), DR4, and DR5 have also been identified (Ashkenazi and Dixit 1998). Signalling by CD95 plays an important role in peripheral deletion of activated mature T-cells at the end of immune response and killing virus-infected or cancer cells by cytotoxic T-cells and by natural killer cells (Nagata 1997). Briefly, after the binding of FasL with Fas, the activation of initiator enzyme caspase-8 occurs, which in turn triggers a cascade of caspase activation by cleaving and activating other procaspases and the active enzymes (caspase-3) mediate the execution phase of apoptosis (Kumar et al. 2005).

The intrinsic pathway of apoptosis is the result of increased mitochondrial permeability and release of proapoptotic molecules into the cytoplasm after a wide variety of extracellular and intracellular stimuli including hypoxia (Malhotra et al. 2001, Nishimura et al. 2008), ischemia and reperfusion injury (Iwata et al. 2002, Rowe et al. 2003, Shah et al. 1997), viral infection (Lin et al. 2009), trauma (Newcomb et al. 1999), bacterial toxins (Cherla et al. 2003, Menzies and Kourteva 2000), DNA damage (Kiang et al. 2010), and oxidants (Maenpaa et al. 2007). In vitro studies have also shown that mechanical stretch may induce apoptosis in myoblasts and vascular smooth muscle cells of rat and swine, furthermore, the magnitude and duration of the mechanical stretch was important for the rate of apoptosis (Edwards et al. 1999, Liu et al.

2010, Su et al. 2006). Effects of mechanical stretch have also been studied on the rat lung membrane surface cells and alveolar type II cells in vitro. These studies have shown that induction of a lung inflation (30 % increase in the membrane surface area) leads to a significant increase in the percentage of apoptotic and necrotic cells (Hammerschmidt et al. 2004, Raaz et al. 2010). When the permeability of mitochondrial membrane increases, several proteins can leak out and activate the caspase cascade, especially cytochrome c. Cytochrome c binds to the apoptosis protease activating factor-1 (Apaf-1, ced-4 homologue) in the cytoplasm and forms a complex called apoptosome which includes cytochrom c, apaf-1, and procaspase-9. This complex is responsible for the activation of caspase-9 (Li et al. 1997). Other mitochondrial proteins, such as apoptosis inducing factor (AIF) can also leak out to the cytoplasm where they bind to and neutralize various inhibitors of apoptosis, whose normal function is to block caspase activation (Kumar et al. 2005). The activation of caspase-9 cause further activation of executioner caspases-3 and -7 and results in apoptosis (Li et al. 1997).

B cell lymphoma (Bcl-2) family proteins are also very important in the regulation of apoptosis. This family has both pro- and anti-apoptotic effects. They normally reside in the mitochondrial membranes and the cytoplasm. Bcl-2 is also homologous to the Caenorhabditis elegans protein, ced-9. There are more than 20 proteins in this family. The two main anti-apoptotic members of this family are bcl-2 and bcl-x (Fan et al. 2005). Overexpression of bcl-2 or bcl-x blocks cytochrome c release in response to a variety of apoptotic stimuli (Yang et al. 1997). However, the proapoptotic members of the family such as Bax (Jürgensmeier et al. 1998) and Bid (Luo et al. 1998) have been shown to induce cytochrome c release from the mitochondria.

3 Materials and methods

3.1 Tissue material

As mentioned above, the main purposes of this study are to compare the occurence of gastrin and motilin peptides, their receptors as well as VIP receptors in the abomasal wall of German Holstein and German Fleckvieh cows regarding the breed and inter-individual disposition.

In order to examine the question why German Holstein cows have a higher disposition to abomasal displacement than German Fleckvieh cows, paraffin embedded tissue samples were used from corpus abomasi and antrum pylori regions of the abomasum from 20 clinically healthy German Holstein and German Fleckvieh cows.

Another purpose of this study was to compare the occurence of these peptides and their receptors between the individuals of German Holstein cows in order to examine the inter-individual disposition in this breed and to clarify if these peptides and receptors play a role in the occurrence of abomasal displacement. For this purpose, biopsy samples were examined from the corpus abomasi and antrum pylori of the abomasal wall of 20 German Holstein cows with left-sided AD.

To clarify if the differences found in the occurence of peptides and/or receptors is a cause or a result of AD, tissue samples from 3 healthy German Holstein cows (4, 4.5, 5 years old) were examined. During a first laparotomy, biopsy samples from the corpus abomasi and antrum pylori of the abomasal wall had been taken. In order to induce an abomasal impaction, a variable gastric band had been placed to the proximal part of the pylorus, around the abomasum after extracting the biopsy samples and left there for three days (Sickinger 2007). After 72 hours, these animals had been operated again and biopsy samples from the corpus and pars pylorica of abomasal wall had been extracted.

Details of sample collection, operation techniques, treatment of animals and animal experiment approvals have been published (Sickinger 2007).

3.2 Fixation of the samples

Fixation and further treatment of tissue samples have been published before (Sickinger 2007).

As positive control tissues for antibodies, calf pancreas and cow duodenum biopsy samples were extracted and processed to immunohistochemical staining using the same method as Sickinger (2007).

The paraffin embedded biopsy samples were cut 6 µm thick using a microtome for the immunohistochemical experiments. After cutting, the tissues were fixed on a microscope slide and immunohistochemically stained. For counterstaining, haemotoxylin was used. Finally, the tissue samples were covered with glycerinegelatine, a protective substance, in order to protect the immunohistochemical reaction of the samples from being weakened due to environmental effects. Three samples were taken from both antrum and corpus abomasi of every subject and the best maintained sample was used in the immunohistochemical analysis and examination.

Preparation and analysis of samples, as well as capturing digital images of these samples were carried out at the Institute for Anatomy, Histology and Embryology of Justus-Liebig-University, Giessen. The analysis of sample images was carried out using a half automatic analysis software program "AnalySIS®, Olympus" in Clinic for Ruminants and Swine (Internal Medicine and Surgery) of Justus-Liebig-University, Giessen.

3.3 Immunohistochemistry

Immunohistochemistry is based on the binding of antibodies to a specific antigen in tissue sections. There are two types of detection systems which are called direct and indirect methods. The direct method is a one-step process in which the primary antibody is conjugated with a label. It is an easy and quick method but lacks sufficient sensitivity for the detection of antigens (Ramos-Vara 2005). Therefore, Coons et al. (1955) developed a two step method called the indirect method. In the indirect method, the primary antibody is not labeled but a secondary antibody which binds to the primary antibody is labeled. In comparison to the direct method, the indirect method is more sensitive and the intensity of the reaction is increased, which are important advantages (Mulisch 2010, Ramos Vara 2005).

There are different indirect methods such as the avidin-biotin methods, peroxidase-antiperoxidase (PAP) method, and tyramine amplification method. In this study a common avidin-biotin method called avidin-biotin-complex (ABC) method was used. Avidin is a large glycoprotein with a high affinity for biotin. In this method the secondary antibody is biotinylated and the third reagent is a complex of avidin mixed with biotin and an appropriate label (Ramos Vara 2005). In this ABC method the primary antibody binds to the epitope and is marked by a biotinylated secondary antibody which is produced against the primary antibody. Following, an avidin-biotin-peroxidase complex binds to the biotinylated secondary antibody. The color reaction will occur after adding a chromogen into this reaction. For this study an AEC-substrate-kit was used to give red color reaction (Fig. 1) (Mulisch 2010).

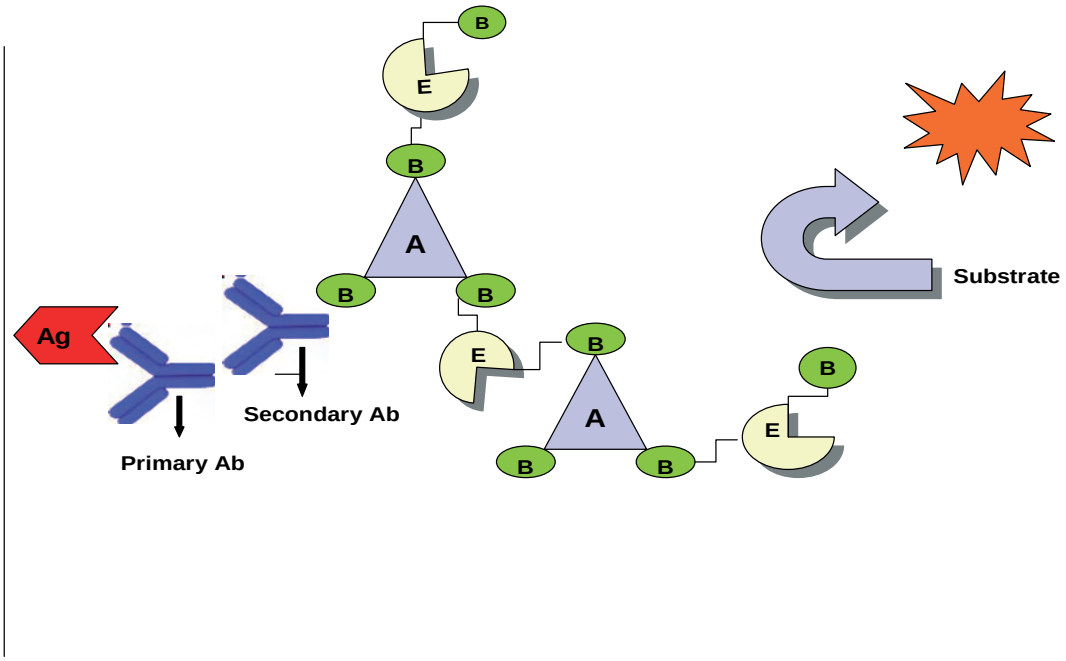

Figure 1: Schematic illustration of the immunohistochemical ABC method. The abbreviations A, Ab, Ag, B and E represent avidin, antibody, antigen, biotin and enzyme respectively.

3.4 Antibodies and controls

The exact definitions of the antibodies used in this study are found in Table 1. The specificity of motilin and caspase-3 antibodies has been proven (Kitamura et al. 1985, Townson et al. 2010). To control the specificity of other antibodies, appropriate blocking peptides for these antibodies were incubated with the original motilin and caspase-3 antibodies in room temperature for an hour in order to block their binding sites.

Both positive and negative control protocols were performed with all antibodies that were used in this study. Positive control tissue is a tissue on which the proven staining of the antibody was shown before [duodenum (motilin receptor) (Fig. 14), human stomach (gastrin and VIP receptors) (Fig 2, 4)]. Abomasal biopsy samples were used as negative control tissues where the primary antibody was replaced by a PBS solution.

A negative control was also carried out in order to ensure that these antibodies do not react with other antigens. For this, each abomasal tissue slice was left without any primary antibody but PBS and received all other components of a regular immunohistochemical ABC method (Fig. 3). The antibodies that could not fulfill all these criteria have not been used in this study.

Table 1: Antibodies used in this study

Primary antibody	**Company**	**Species**	**Dilution**
Anti- Gastrin A 0586 polyclonal	Dakocytomation (Denmark)	Rabbit	1: 500
Anti- Gastrin receptor-B EB06767	Everest Biotech (Great Britain)	Goat	3.5 µg/ml
Anti-Motilin R-1104	Yanaihara Inst. Japan	Rabbit	1:8000
Anti-Motilin receptor LS_A134 polyclonal	MBL Int. Corp. (USA)	Rabbit	1:1000
VIP receptor 1 LS_A1298 polyclonal	MBL Int. Corp. (USA)	Rabbit	1:80
VIP receptor 2 ABN-MAB 1821-C100 monoclonal clone AS 69	Abnova (USA)	Mouse	1:120
Caspase 3 antibody Ab4051 polyclonal	Abcam (England)	Rabbit	1:75
Secondary antibody			
Anti Rabbit	Vector Laboratories (USA)	Goat	1:250
Anti Goat	Dakocytomation (Denmark)	Rabbit	1:400
Anti Mouse	Vector Laboratories (USA)	Horse	1:250

Blocking peptide	Company	Dilution
Blocking peptide for LS_A134 anti motilin receptor (GPR38)	MBL Int. (USA)	10x centrifuge before use
Anti CCKBR blocking peptide	Everest Biotech (England)	10mg/ml
Gastrin peptide	Abbiotec	25x
Blocking peptid for LS-A1298 anti VPAC1	MBL Int. (USA)	20x

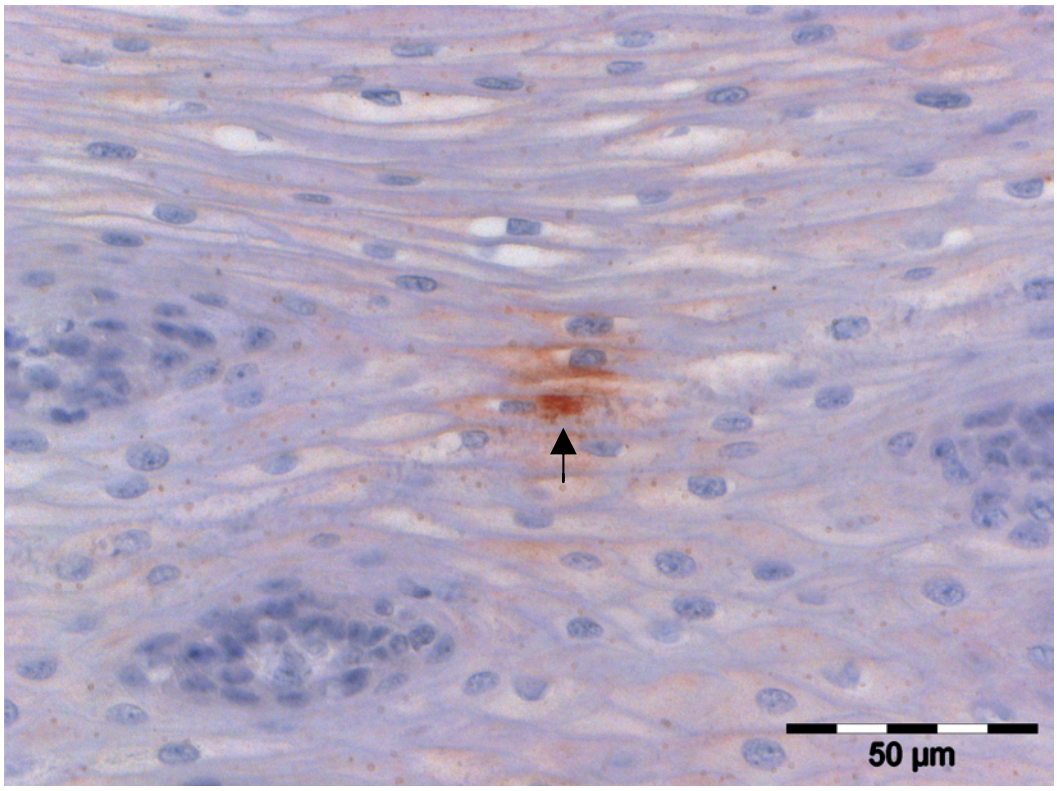

Figure 2: Positive control staining for VPAC2 receptor in smooth muscle of human stomach. Black arrow indicates immunoreactive areas for VPAC2 in the neurons of tunica muscularis of the stomach.

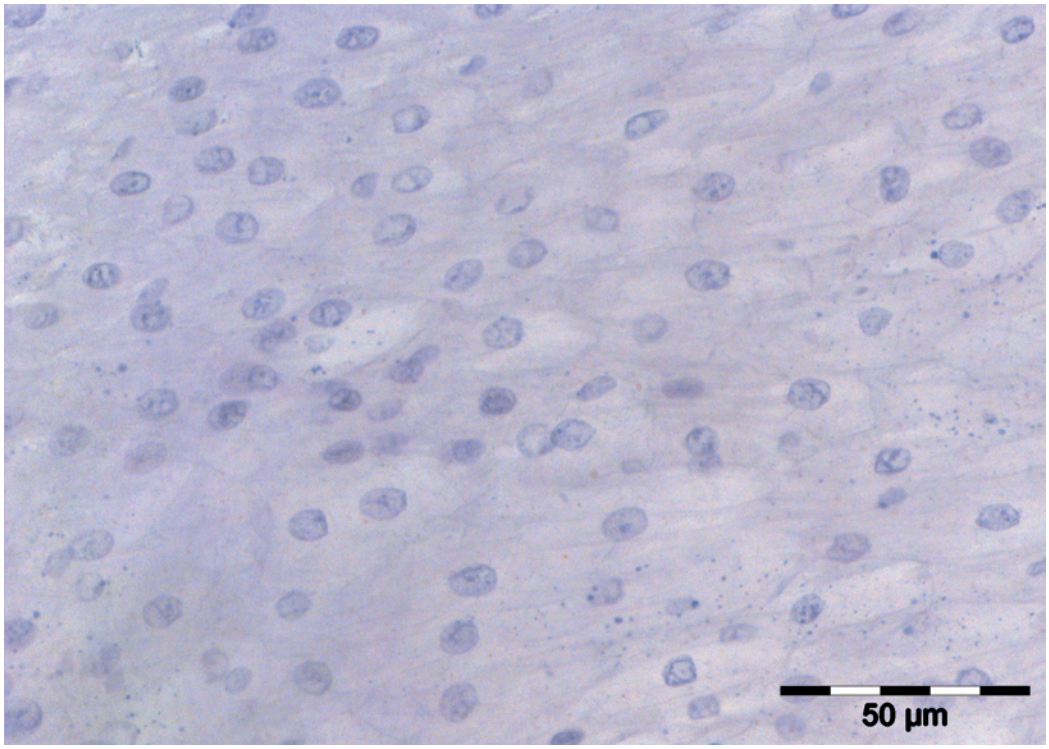

Figure 3: Negative control in the tunica muscularis of human stomach for VPAC2 receptor. For the negative control, the primary antibody is replaced with phosphate buffered saline (PBS) and the rest of the immunohistochemical protocol performed as in the positive control staining.

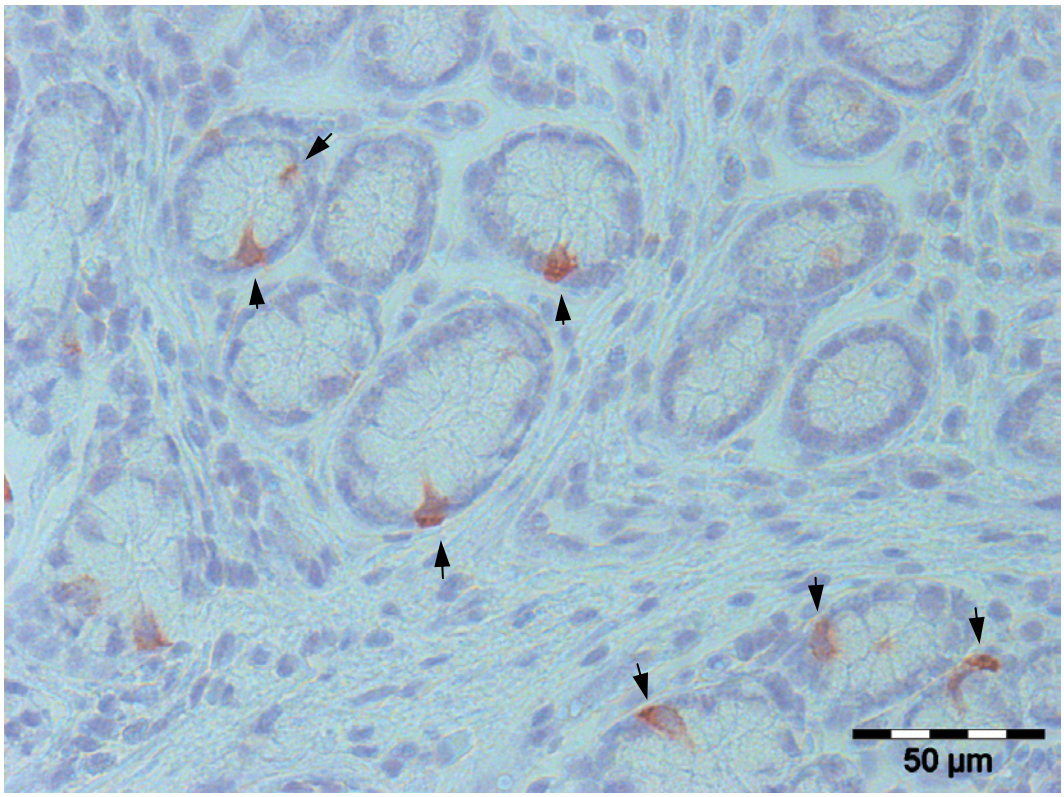

Figure 4: Positive control staining for gastrin peptide in the tunica mucosa of the human stomach. Black arrows show the positive staining of gastrin in the antral glands of tunica mucosa.

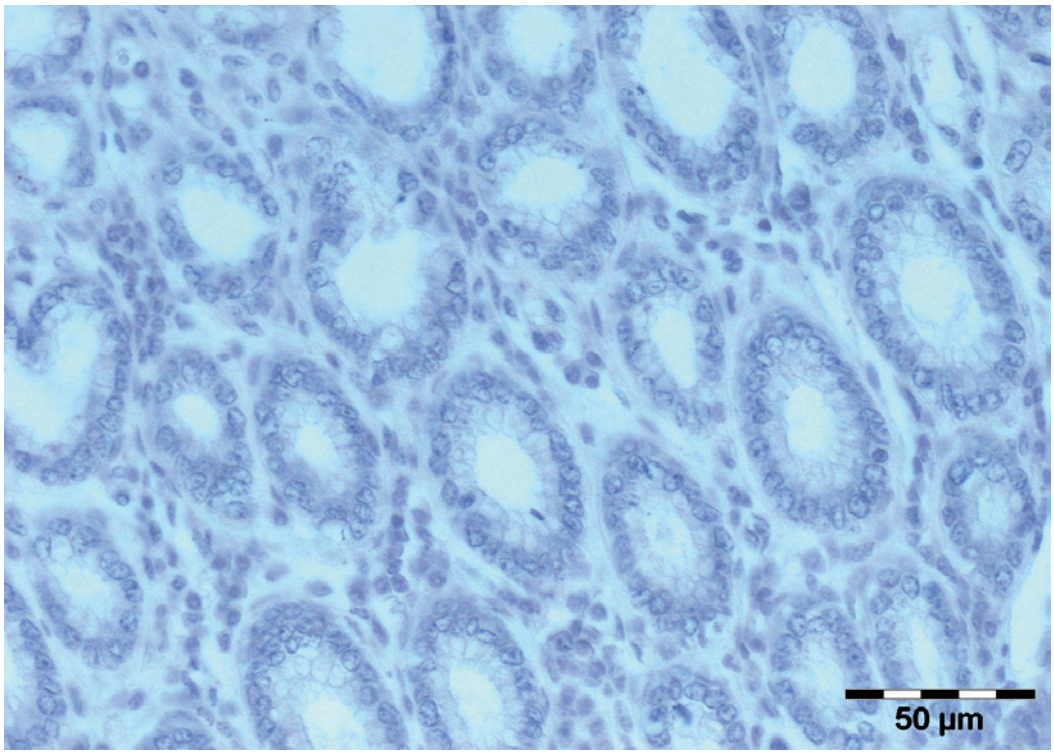

Figure 5: Blocking of the gastrin peptide antibody in the tunica mucosa of the human stomach. To prove the specifity of the gastrin antibody, an incubation of the antibody with a correspondent blocking peptide (gastrin) was performed. Due to the fact that the binding areas of gastrin antibody were occupied by gastrin blocking peptide, no gastrin staining was observed.

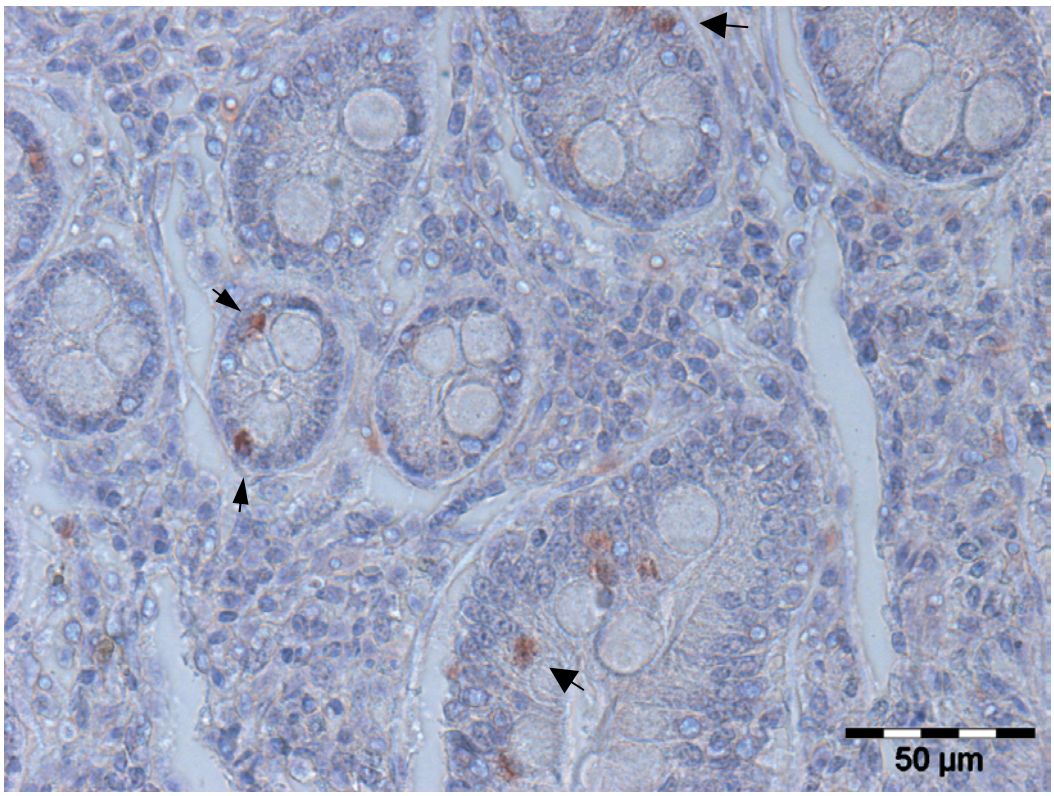

Figure 6: Positive control staining for the motilin receptor antibody (GPR38) in tunica mucosa of human duodenum. Black arrows indicate IR-positive signals.

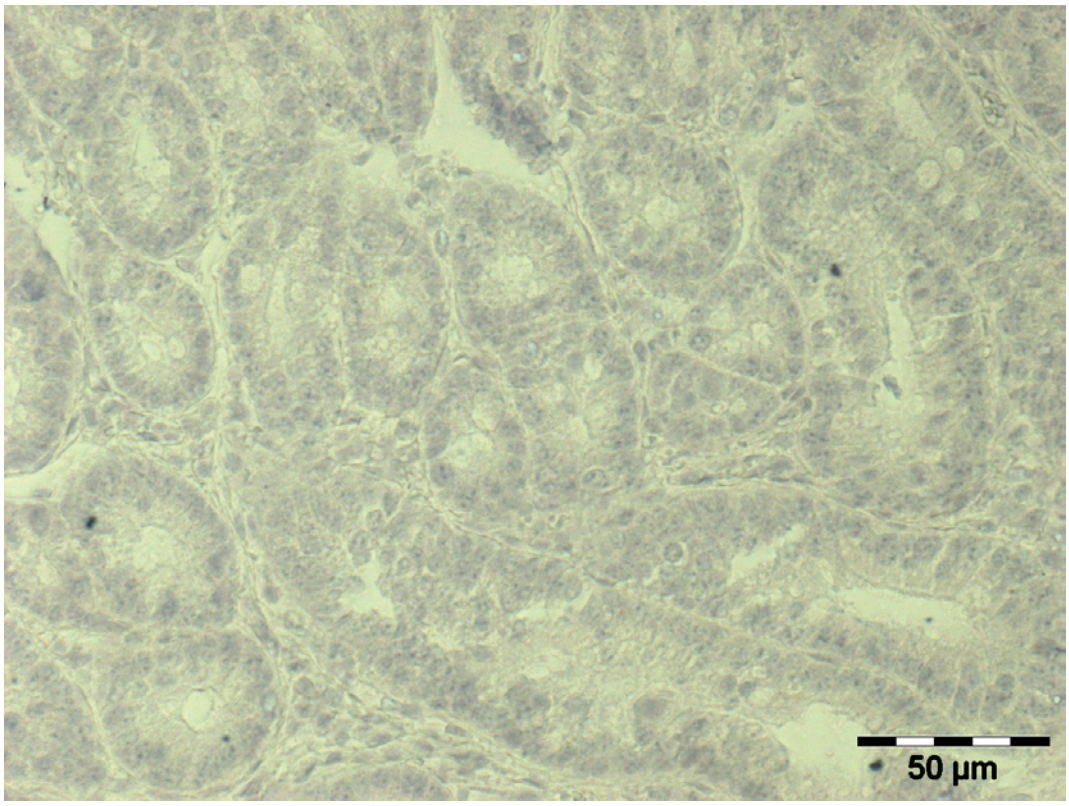

Figure 7: Motilin receptor antibody blocking with a corresponding peptide. No staining was observed after incubating the primary antibody with its corresponding blocking peptide.

3.5 Immunohistochemical staining protocols for the peptides, receptors and caspase activity

The original data specifications for the antibodies that were used in this study can differ in these tables concerning their dilution or incubation time. In pre-tests of these antibodies, optimal staining dilution and time are adjusted to obtain the best results.

Table 2: Immunohistochemical protocol for gastrin

Primary antibody:	Gastrin antibody (A058; rabbit polyclonal; Dakocytomation; Denmark)
Antibody dilution:	1:500
Secondary antibody:	Goat anti-rabbit (BA-1000, Vector)
Secondary antibody dilution:	1:250
Method:	Avidin-Biotin-Complex method
Chromogen:	AEC (3-amino-9-ethylcarbazol)

	Step	Reagent	Time	Approach
1.	Deparaffinization	Xylol	10 min	Rack, RT
		Xylol	10 min	
		Xylol	10 min	
2.	Water removal	100% ethanol	5 min	
		96% ethanol	5 min	
		80% ethanol	5 min	
		70% ethanol	5 min	
		50% ethanol	5 min	
		Distilled water	5 min	
3.	Rinse	PBS	5 min	
4.	Antigen retrieval	Citrat buffer	5 min	Microwave 800W
			10 min	Microvawe 485W
5.	Rinse	PBS	5 min	Shaker
6.	Blocking	0.3% H2O2 in -18 C° methanol	30 min	Cuvette
7.	Rinse	PBS	3x5 min	Shaker
8.	Permeabilization	PBS/BSA/Triton X 100	5 min	Cuvette
9.	Protein blocking	5% goat serum in PBS	30 min	Humid chamber at RT
10.	Incubation	Primary antibody in PBS (1:500)	30 min	Humid chamber at RT
11.	Rinse	PBS	3x5 min	Shaker
12.	Incubation	Secondary antibody in PBS	40 min	Humid chamber at RT
13.	Rinse	PBS	3x5 min	Shaker
14.	Incubation	ABC-Elite-Kit (Vectastatin)	40 min	Humid chamber at RT
15.	Rinse	PBS	3x5 min	Shaker
16.	Detection	AEC (Biologo)	10-12 min	Humid chamber at RT
17.	Rinse	Distilled water	3x5 min	Shaker
18.	Counterstaining	Haemotoxylin	10 sec	
19.	Rinse	Tap water	10 min	Cuvette
20.	Coverslip	Glycerinegelatine(Merck)		

ABC-Kit: 5 ml PBS + 2 drops reagent A + 2 drops reagent B

AEC-Kit: 900 µl substrate buffer + 3 drops AEC-dilution

Table 3: Immunohistochemical protocol for gastrin receptor-B

Primary antibody:	Gastrin receptor-B antibody (EB06767; goat polyclonal; Everest Biotech; England)
Antibody dilution:	3.5 mg/ml
Secondary antibody:	Goat anti-rabbit (Dakocytomation: Denmark)
Secondary antibody dilution:	1:400
Method:	Avidin-Biotin-Complex method
Chromogen:	AEC (3-amino-9-ethylcarbazol)

	Step	Reagent	Time	Approach
1.	Deparaffinization	Xylol	10 min	Rack, RT
		Xylol	10 min	
		Xylol	10 min	
2.	Water removal	100% ethanol	5 min	
		96% ethanol	5 min	
		80% ethanol	5 min	
		70% ethanol	5 min	
		50% ethanol	5 min	
		Distilled water	5 min	
3.	Rinse	PBS	5 min	
4.	Antigen retrieval	Citrat buffer	5 min	Microwave 800W
			10 min	Microvawe 485W
5.	Rinse	PBS	5 min	Shaker
6.	Blocking	0.3% H2O2 in -18 C° methanol	30 min	Cuvette
7.	Rinse	PBS	3x5 min	Shaker
8.	Permeabilization	PBS/BSA/Triton X 100	15 min	Cuvette
9.	Protein blocking	5% rabbit serum in PBS	30 min	Humid chamber at RT
10.	Incubation	Primary antibody in PBS	Overnight	Humid chamber at 4C°
11.	Rinse	PBS	3x5 min	Shaker
12.	Incubation	Secondary antibody in PBS	60 min	Humid chamber at RT
13.	Rinse	PBS	3x5 min	Shaker
14.	Incubation	ABC-Elite-Kit (Vectastatin)	60 min	Humid chamber at RT
15.	Rinse	PBS	3x5 min	Shaker
16.	Detection	AEC (Biologo)	10-15 min	Humid chamber at RT
17.	Rinse	Distilled water	3x5 min	Shaker
18.	Counterstaining	Haemotoxylin	10 sec	
19.	Rinse	Tap water	10 min	Cuvette
20.	Coverslip	Glycerinegelatine(Merck)		

ABC-Kit: 5 ml PBS + 2 drops reagent A + 2 drops reagent B

AEC-Kit: 900 µl Substrate buffer + 3 drops AEC-dilution

Table 4: Immunohistochemical protocol for motilin

Primary antibody:	Motilin antibody (R-1104 rabbit polyclonal; Yanaihara Inst.; Japan)
Antibody dilution:	1:8000
Secondary antibody:	Goat anti-rabbit (BA-1000, Vector)
Secondary antibody dilution:	1:250
Method:	Avidin-Biotin-Complex method
Chromogen:	AEC (3-amino-9-ethylcarbazol)

	Step	Reagent	Time	Approach
1.	Deparaffinization	Xylol	10 min	Rack, RT
		Xylol	10 min	
		Xylol	10 min	
2.	Water removal	100% ethanol	5 min	
		96% ethanol	5 min	
		80% ethanol	5 min	
		70% ethanol	5 min	
		50% ethanol	5 min	
		Distilled water	5 min	
3.	Rinse	PBS	5 min	
4.	Antigen retrieval	Citrat buffer	5 min	Microwave 800W
			10 min	Microvawe 485W
5.	Rinse	PBS	5 min	Shaker
6.	Blocking	0.3% H2O2 in -18 C° methanol	30 min	Cuvette
7.	Rinse	PBS	3x5 min	Shaker
8.	Permeabilization	PBS/BSA/Triton X 100	5 min	Cuvette
9.	Protein Blocking	5% goat serum in PBS	30 min	Humid chamber at RT
10.	Incubation	Primary antibody in PBS (1:8000)	Overnight	Humid chamber at 4C°
11.	Rinse	PBS	3x5 min	Shaker
12.	Incubation	Secondary antibody in PBS	40 min	Humid chamber at RT
13.	Rinse	PBS	3x5 min	Shaker
14.	Incubation	ABC-Elite-Kit (Vectastatin)	40 min	Humid chamber at RT
15.	Rinse	PBS	3x5 min	Shaker
16.	Detection	AEC (Biologo)	10-15 min	Humid chamber at RT
17.	Rinse	Distilled water	3x5 min	Shaker
18.	Counterstaining	Haemotoxylin	10 sec	
19.	Rinse	Tap water	10 min	Cuvette
20.	Coverslip	Glycerinegelatine(Merck)		

ABC-Kit: 5 ml PBS + 2 drops reagent A + 2 drops reagent B

AEC-Kit: 900 µl Substrate buffer + 3 drops AEC-dilution

Table 5: Immunohistochemical protocol for motilin receptor (GPR38)

Primary antibody:	Motilin receptor antibody (LS_A134 rabbit polyclonal; MBL Int; USA)
Antibody dilution:	1:1000
Secondary antibody:	Goat anti-rabbit (BA-1000, Vector)
Secondary antibody dilution:	1:250
Method:	Avidin-Biotin-Complex method
Chromogen:	AEC (3-amino-9-ethylcarbazol)

	Step	Reagent	Time	Approach
1.	Deparaffinization	Xylol	10 min	Rack, RT
		Xylol	10 min	
		Xylol	10 min	
2.	Water removal	100% ethanol	5 min	
		96% ethanol	5 min	
		80% ethanol	5 min	
		70% ethanol	5 min	
		50% ethanol	5 min	
		Distilled water	5 min	
3.	Rinse	PBS	5 min	
4.	Antigen retrieval	Citrat buffer	5 min	Microwave 800W
			10 min	Microvawe 485W
5.	Rinse	PBS	5 min	Shaker
6.	Blocking	0.3% H2O2 in -18 C° methanol	30 min	Cuvette
7.	Rinse	PBS	3x5 min	Shaker
8.	Permeabilization	PBS/BSA/Triton X 100	15 min	Cuvette
9.	Protein blocking	5% goat serum in PBS	30 min	Humid chamber at RT
10.	Incubation	Primary antibody in PBS (1:1000)	Overnight	Humid chamber at 4C°
11.	Rinse	PBS	3x5 min	Shaker
12.	Incubation	Secondary antibody in PBS	50 min	Humid chamber at RT
13.	Rinse	PBS	3x5 min	Shaker
14.	Incubation	ABC-Elite-Kit (Vectastatin)	40 min	Humid chamber at RT
15.	Rinse	PBS	3x5 min	Shaker
16.	Detection	AEC (Biologo)	10 min	Humid chamber at RT
17.	Rinse	Distilled water	3x5 min	Shaker
18.	Counterstaining	Haemotoxylin	10 sec	
19.	Rinse	Tap water	10 min	Cuvette
20.	Coverslip	Glycerinegelatine(Merck)		

ABC-Kit: 5 ml PBS + 2 drops reagent A + 2 drops reagent B

AEC-Kit: 900 µl substrate buffer + 3 drops AEC-dilution

Table 6: Immunohistochemical protocol for VIP-1 receptor

Primary antibody:	VIP receptor-1 antibody (LS_A1298 rabbit polyclonal; MBL Int; USA)
Antibody dilution:	1:80
Secondary antibody:	Goat anti-rabbit (BA-1000, Vector)
Secondary antibody dilution:	1:250
Method:	Avidin-Biotin-Complex method
Chromogen:	AEC (3-amino-9-ethylcarbazol)

	Step	Reagent	Time	Approach
1.	Deparaffinization	Xylol	10 min	Rack, RT
		Xylol	10 min	
		Xylol	10 min	
2.	Water removal	100% ethanol	5 min	
		96% ethanol	5 min	
		80% ethanol	5 min	
		70% ethanol	5 min	
		50% ethanol	5 min	
		Distilled water	5 min	
3.	Rinse	PBS	5 min	
4.	Antigen retrieval	Citrat buffer	5 min	Microwave 800W
			10 min	Microvawe 485W
5.	Rinse	PBS	5 min	Shaker
6.	Blocking	0.3% H2O2 in -18 C° methanol	30 min	Cuvette
7.	Rinse	PBS	3x5 min	Shaker
8.	Permeabilization	PBS/BSA/Triton X 100	5 min	Cuvette
9.	Protein blocking	5% goat serum in PBS	30 min	Humid chamber at RT
10.	Incubation	Primary antibody in PBS (1:80)	Overnight	Humid chamber at 4C°
11.	Rinse	PBS	3x5 min	Shaker
12.	Incubation	Secondary antibody in PBS	40 min	Humid chamber at RT
13.	Rinse	PBS	3x5 min	Shaker
14.	Incubation	ABC-Elite-Kit (Vectastatin)	40 min	Humid chamber at RT
15.	Rinse	PBS	3x5 min	Shaker
16.	Detection	AEC (Biologo)	15 min	Humid chamber at RT
17.	Rinse	Distilled water	3x5 min	Shaker
18.	Counterstaining	Haemotoxylin	10 sec	
19.	Rinse	Tap water	10 min	Cuvette
20.	Coverslip	Glycerinegelatine(Merck)		

ABC-Kit: 5 ml PBS + 2 drops reagent A + 2 drops reagent B

AEC-Kit: 900 µl substrate buffer + 3 drops AEC-dilution

Table 7: Immunohistochemical protocol for VIP-2 receptor

Primary antibody:	VIP receptor-2 antibody (ABN-MAB1821-C100 mouse monoclonal; Abnova;USA)
Antibody dilution:	1:120
Secondary antibody:	Horse anti-mouse (BA-2000, Vector)
Secondary antibody dilution:	1:250
Method:	Avidin-Biotin-Complex method
Chromogen:	AEC (3-amino-9-ethylcarbazol)

	Step	Reagent	Time	Approach
1.	Deparaffinization	Xylol	10 min	Rack, RT
		Xylol	10 min	
		Xylol	10 min	
2.	Water removal	100% ethanol	5 min	
		96% ethanol	5 min	
		80% ethanol	5 min	
		70% ethanol	5 min	
		50% ethanol	5 min	
		Distilled water	5 min	
3.	Rinse	PBS	5 min	
4.	Antigen retrieval	Citrat buffer	5 min	Microwave 800W
			10 min	Microvawe 485W
5.	Rinse	PBS	5 min	Shaker
6.	Blocking	0.3% H2O2 in -18 C° methanol	30 min	Cuvette
7.	Rinse	PBS	3x5 min	Shaker
8.	Permeabilization	PBS/BSA/Triton X 100	10 min	Cuvette
9.	Protein blocking	5% horse serum in PBS	30 min	Humid chamber at RT
10.	Incubation	Primary antibody in PBS (1:120)	Overnight	Humid chamber at 4C°
11.	Rinse	PBS	3x5 min	Shaker
12.	Incubation	Secondary antibody in PBS	40 min	Humid chamber at RT
13.	Rinse	PBS	3x5 min	Shaker
14.	Incubation	ABC-Elite-Kit (Vectastatin)	40 min	Humid chamber at RT
15.	Rinse	PBS	3x5 min	Shaker
16.	Detection	AEC (Biologo)	15 min	Humid chamber at RT
17.	Rinse	Distilled water	3x5 min	Shaker
18.	Counterstaining	Haemotoxylin	10 sec	
19.	Rinse	Tap water	10 min	Cuvette
20.	Coverslip	Glycerinegelatine(Merck)		

ABC-Kit: 5 ml PBS + 2 drops reagent A + 2 drops reagent B

AEC-Kit: 900 µl substrate buffer + 3 drops AEC-dilution

Table 8: Immunohistochemical protocol for caspase activity

Primary antibody:	Caspase-3 antibody (Ab4051 rabbit polyclonal; Abcam, England)
Antibody dilution:	1:75
Secondary antibody:	Goat anti-rabbit (BA-1000, Vector)
Secondary antibody dilution:	1:250
Method:	Avidin-Biotin-Complex method
Chromogen:	AEC (3-amino-9-ethylcarbazol)

	Step	Reagent	Time	Approach
1.	Deparaffinization	Xylol	10 min	Rack, RT
		Xylol	10 min	
		Xylol	10 min	
2.	Water removal	100% ethanol	5 min	
		96% ethanol	5 min	
		80% ethanol	5 min	
		70% ethanol	5 min	
		50% ethanol	5 min	
		Distilled water	5 min	
3.	Rinse	PBS	5 min	
4.	Antigen retrieval	Citrat buffer	5 min	Microwave 800W
			10 min	Microvawe 485W
5.	Rinse	PBS	5 min	Shaker
6.	Blocking	0.3% H2O2 in -18 C° methanol	30 min	Cuvette
7.	Rinse	PBS	3x5 min	Shaker
8.	Permeabilization	PBS/BSA/Triton X 100	5 min	Cuvette
9.	Protein Blocking	5% goat serum in PBS	30 min	Humid chamber at RT
10.	Incubation	Primary antibody in PBS (1:75)	Overnight	Humid chamber at 4C°
11.	Rinse	PBS	3x5 min	Shaker
12.	Incubation	Secondary antibody in PBS	40 min	Humid chamber at RT
13.	Rinse	PBS	3x5 min	Shaker
14.	Incubation	ABC-Elite-Kit (Vectastatin)	40 min	Humid chamber at RT
15.	Rinse	PBS	3x5 min	Shaker
16.	Detection	AEC (Biologo)	10 min	Humid chamber at RT
17.	Rinse	Distilled water	3x5 min	Shaker
18.	Counterstaining	Haemotoxylin	10 sec	
19.	Rinse	Tap water	10 min	Cuvette
20.	Coverslip	Glycerinegelatine(Merck)		

ABC-Kit: 5 ml PBS + 2 drops reagent A + 2 drops reagent B

AEC-Kit: 900 µl substrate buffer + 3 drops AEC-dilution

3.6 Microscopic examination and digital imaging

From every sample and localisation, five visual fields were randomly chosen and images taken. In order to have a standardization of these images, every image was randomly taken using the same scale of enlargement. Each image has an area of 57117.49 µm² and all five images from one animal and one localisation have a total area of 285587.45µm².

The digital capturing of the samples was done in the Institute for Anatomy, Histology and Embryology of Veterinary Medicine, Justus-Liebig-University, Giessen.

3.7 Measurement of the immunoreactive staining

The five images that were taken randomly from each animal and each localization were then analyzed with the help of the program "AnalySIS®", a graphics tablet, and a pen designed for this graphics tablet. The gastrin peptide and motilin receptor immunoreactive areas were encircled with the help of this program and its devices, whereas the VPAC1/VIP-1 receptor was counted regarding its abundance in a total area of 57117.49 µm² from one image and 285587.45 µm² from five images. The cells which show caspase activity were also counted and the number of apoptotic cells was compared to the number of unaffected cells.

All the data gathered from the images taken were recorded in an excel diagram. The results from the areal measurements were acquired as µm², whereas VPAC1 receptor abundance was counted and caspase activity comparison was expressed in percentage.

Immunohistochemical staining protocols for the antibodies and receptors used in this study were performed on samples taken from the same sections of the abomasum from each animal. Therefore, it was possible to compare these peptides and receptors as well as caspase activity with each other.

4 Statistical analysis

The statistical analysis of the data was carried out by the working group Biomathematics and Data processing of the Veterinary Faculty of Justus-Liebig-University Giessen and the statistics program BMDP/Dynamic®, Release 7 was used for the statistical analysis.

The variances of the variables between the groups were compared with each other with Levene- Tests and if no significant difference was found, a mean value comparison with t-Test was performed. If the variances were unsimilar, a t-test with separate variance assessment was used whereas at similar variances, a t- test with pooled variance assessment was performed.

All the data information of German Fleckvieh, German Holstein and German Holstein with abomasal displacement groups was compared at the approximated normal distribution of these data. For gastrin peptide, BMDP7D one way analysis of variance with data screening regarding the localization antrum was used. For the statistical analysis of other substances, BMDP2V program was used for two way analysis of variance with repeated measures regarding the localizations antrum and corpus abomasi.

The statistical analysis of three German Holstein cows with experimentally induced abomasal impaction group was performed using t-test for independent samples for the comparison of this group (n = 3) with the control groups (n = 20). For the comparison between the first and second operation samples, in other words samples which were extracted before and after placing a gastric band, BMDP2V program was performed for two way analysis of variance with repeated measures regarding operation time and localizations.

At the evaluation of the statistical significances, the value $p \leq 0.05$ was interpreted as statistically significant whereas the value $p < 0.001$ was interpreted as statistically high significant.

5 Results

5.1 Results of gastrin immunoreactivity

German Fleckvieh

Gastrin immunoreactivity was detected on the antral mucosa of the abomasal wall. Gastrin immunoreactivity has been shown on antrum mucosa by other authors but not on the corpus mucosa of the stomach. However, the corpus abomasi of four German Fleckvieh and four German Holstein cows have also shown gastrin immunoreactivity in this study. These findings haven't been included in the evaluation of results.

The evaluation of the immunoreactive (IR) area of gastrin peptide on the antrum of the abomasal wall in German Fleckvieh shows a mean value with a standard deviation ($\bar{x} \pm s$) of 1175 ± 655 µm².

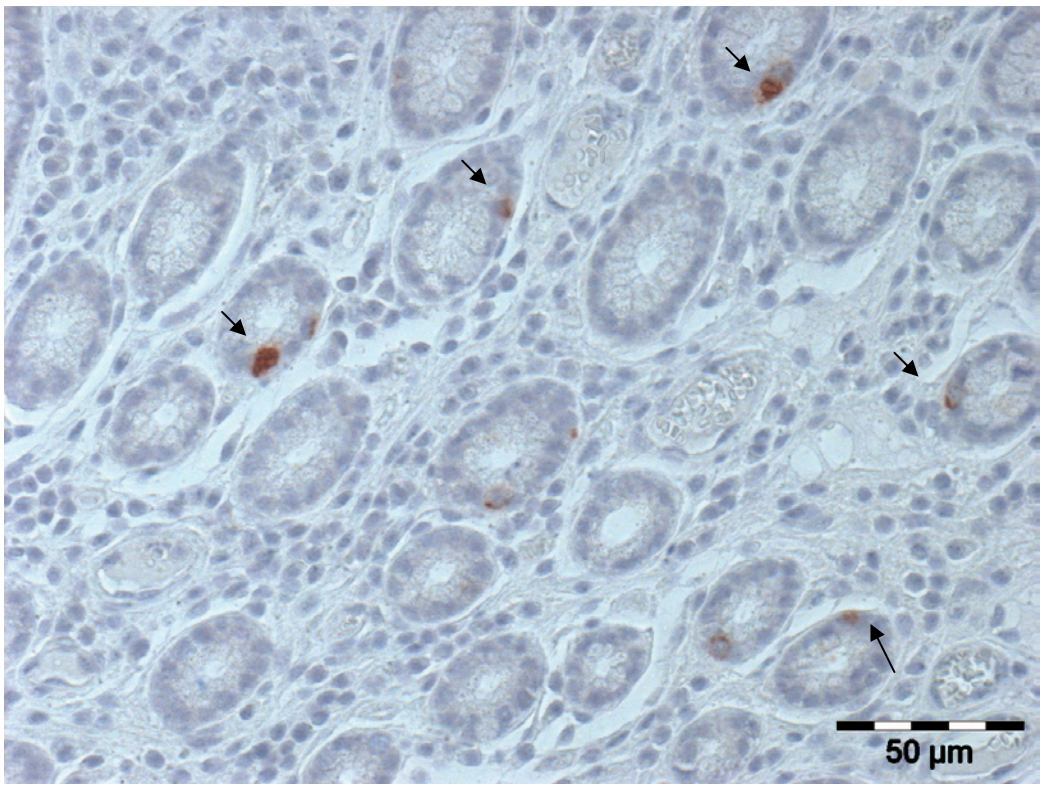

Figure 8: The immunoreactive (IR) staining for gastrin peptide in the antral glands of the abomasum in a German Holstein cow. The gastrin IR positive areas are marked with an arrow.

German Holstein

Samples from the antrum of abomasal wall from German Holstein cows without abomasal displacement show a mean value with standard deviation ($\bar{x} \pm s$) of 1105 ± 930 µm² (Fig. 8).

Comparison of both breeds in respect of the immunoreactive area of gastrin peptide

There is no significant difference between German Holstein and German Fleckvieh cows on the antral mucosa of the abomasal wall regarding IR areas of gastrin peptide (Fig. 9) (p = 0.8).

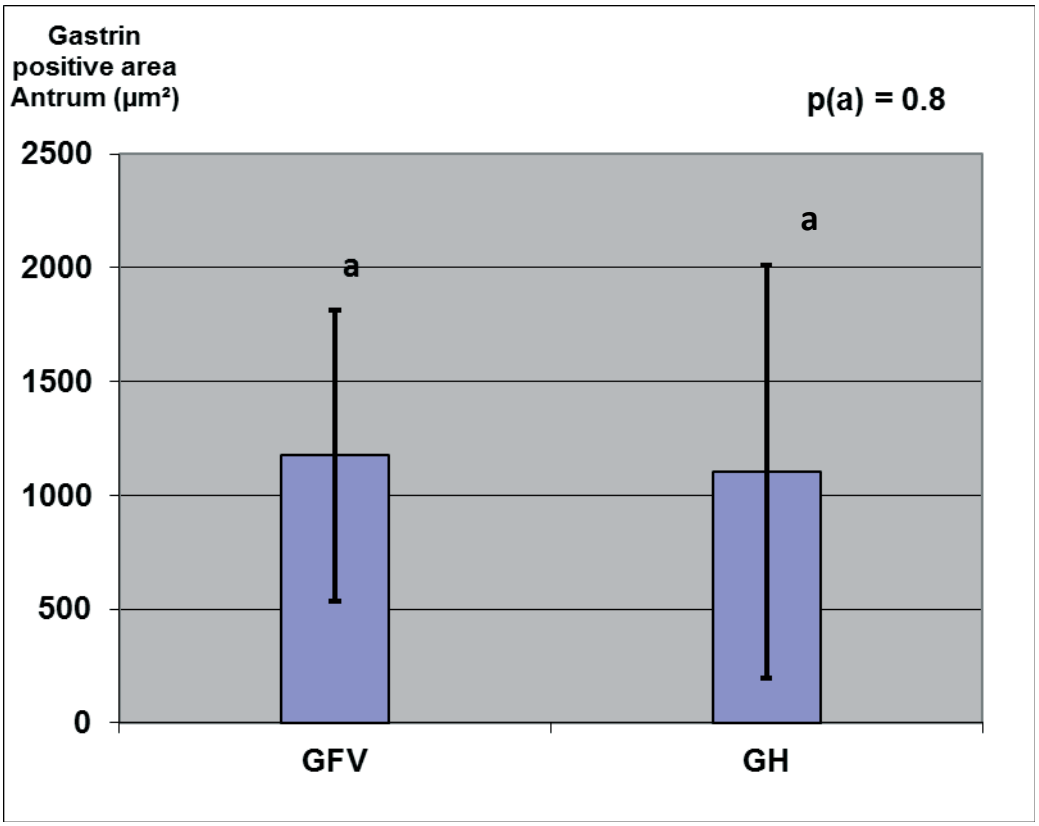

Figure 9: Comparison of the gastrin immunoreactivity in healthy German Fleckvieh (GFV) cows and healthy German Holstein (GH) cows. There is no significant difference between GFV and GH cows concerning their IR-positive area on the tunica mucosa of the antral abomasal wall (p = 0.8). The upper boundries of the boxes represent the mean value ($\bar{x}$) and the horizontal lines represent the standard deviation (s), ($\bar{x} \pm s$).

German Holstein with abomasal displacement

The immunoreactive area of gastrin on the antral mucosa of abomasal wall of German Holstein cows with abomasal displacement shows a slightly higher mean value ($\bar{x} \pm s$) of 1277 ± 863 µm² in comparison to the healthy German Holstein cows (1105 ± 930 µm²). However, this difference is not significant (p = 0.8).

Comparison of immunoreactive area of gastrin peptide in healthy German Holstein cows and German Holstein cows with abomasal displacement

Although the German Holstein cows with abomasal displacement show a slightly higher mean value in comparison to healthy German Holstein cows, this value was found to be not significant (p = 0.8).

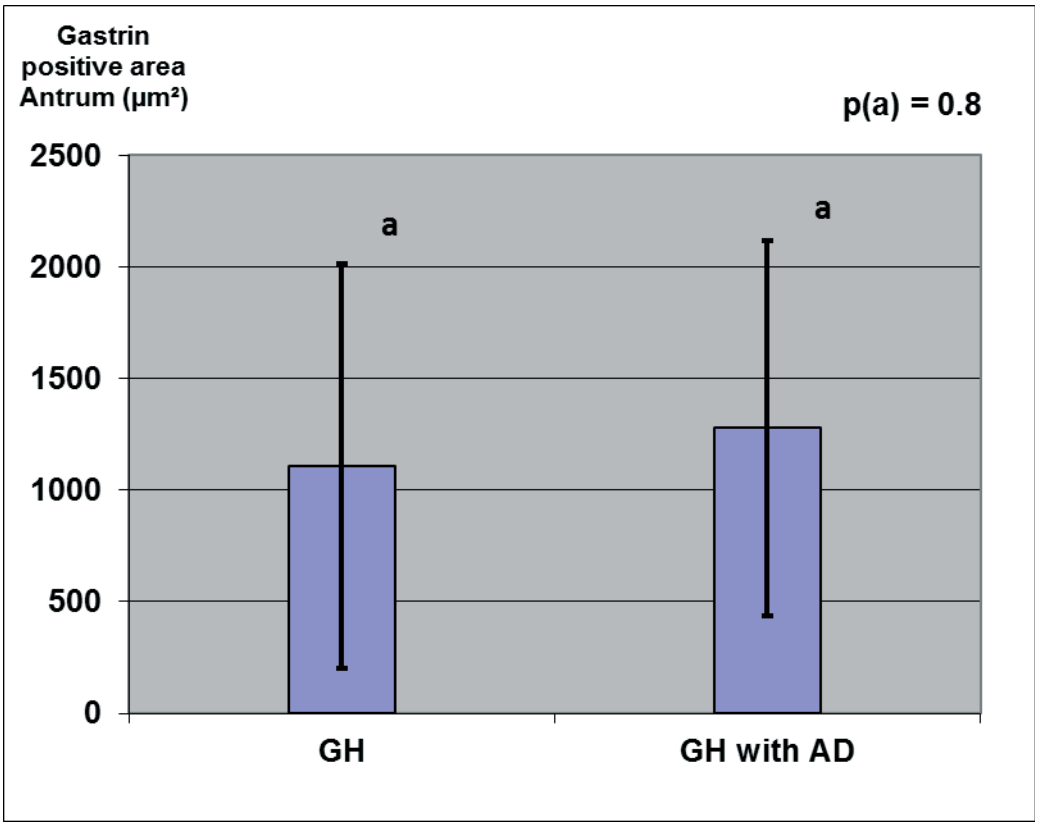

Figure 10: Comparison of the gastrin immunoreactivity in healthy German Holstein (GH) cows and German Holstein cows with abomasal displacement (GH with AD). There is no significant difference between healthy GH cows and GH with AD concerning their IR-positive area for gastrin on the tunica mucosa of the antral abomasal wall (p = 0.8). The upper boundries of the boxes represent the mean value ($\bar{x}$) and the horizontal lines represent the standard deviation (s), ($\bar{x} \pm s$).

5.2 Results of gastrin/CCKB receptor immunoreactivity

Gastrin receptors could not be detected in any layer of neither antrum nor corpus of the abomasal wall with the help of immunohistochemical ABC-AEC method (Fig. 13). Positive control staining of this antibody was performed on calf pancreas and positive IR staining was detected on pancreas acinar cells (Fig. 11). After incubating primary antibody with corresponding CCKB receptor peptide, this immunoreactive reaction disappeared which proves that the antibody was suitable to detect gastrin/CCKB receptor immunoreactivity in bovine tissues (Fig. 12). The biopsy samples of the pancreas were extracted as it is mentioned in material and method chapter.

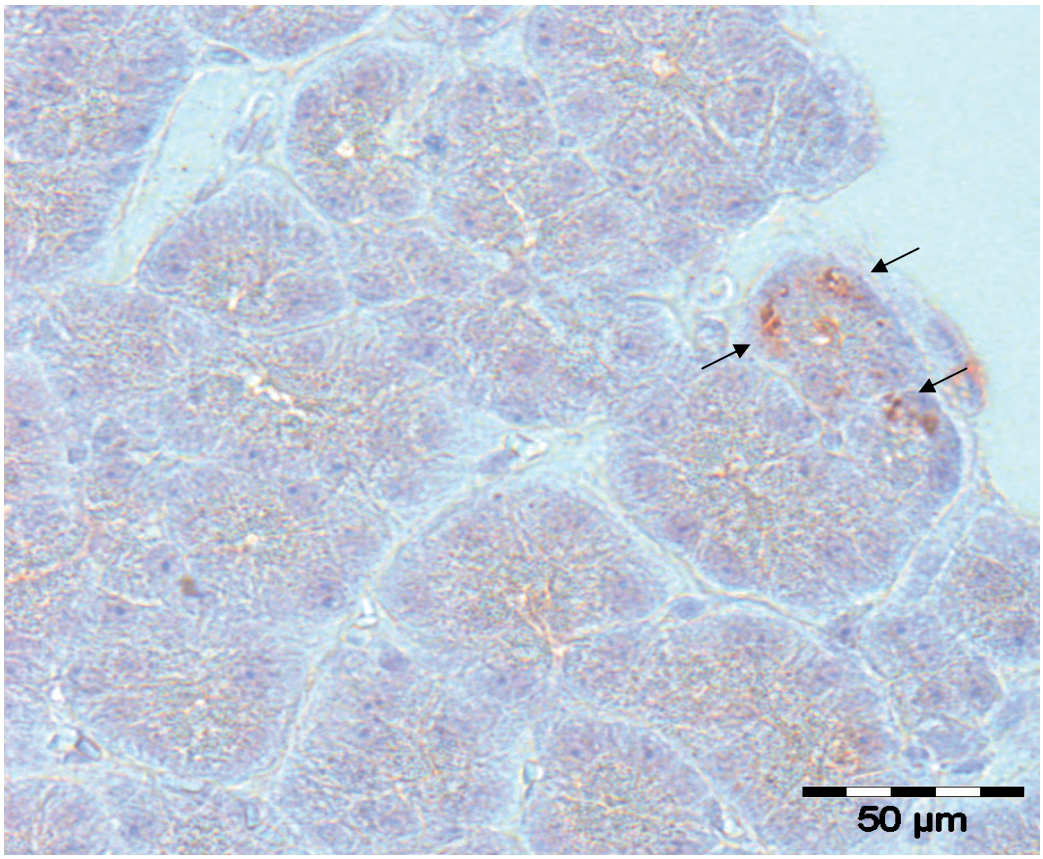

Figure 11: Positive immunoreactive (IR) staining of CCK-B receptor in German Holstein calf pancreas acinar cells. Gastrin/CCKBR IR-positive stained cells are marked with arrows.

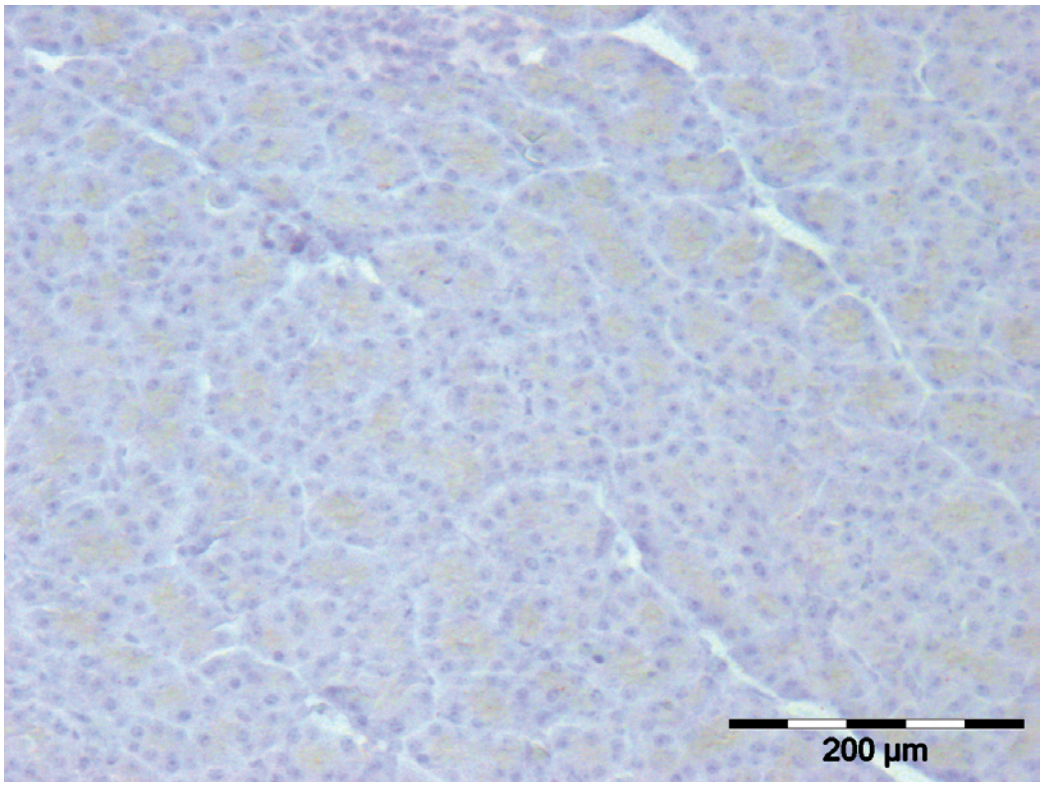

Figure 12: After binding the CCK-B receptor antibody with its blocking peptide, no IR positive staining was detected in pancreatic acinar cells.

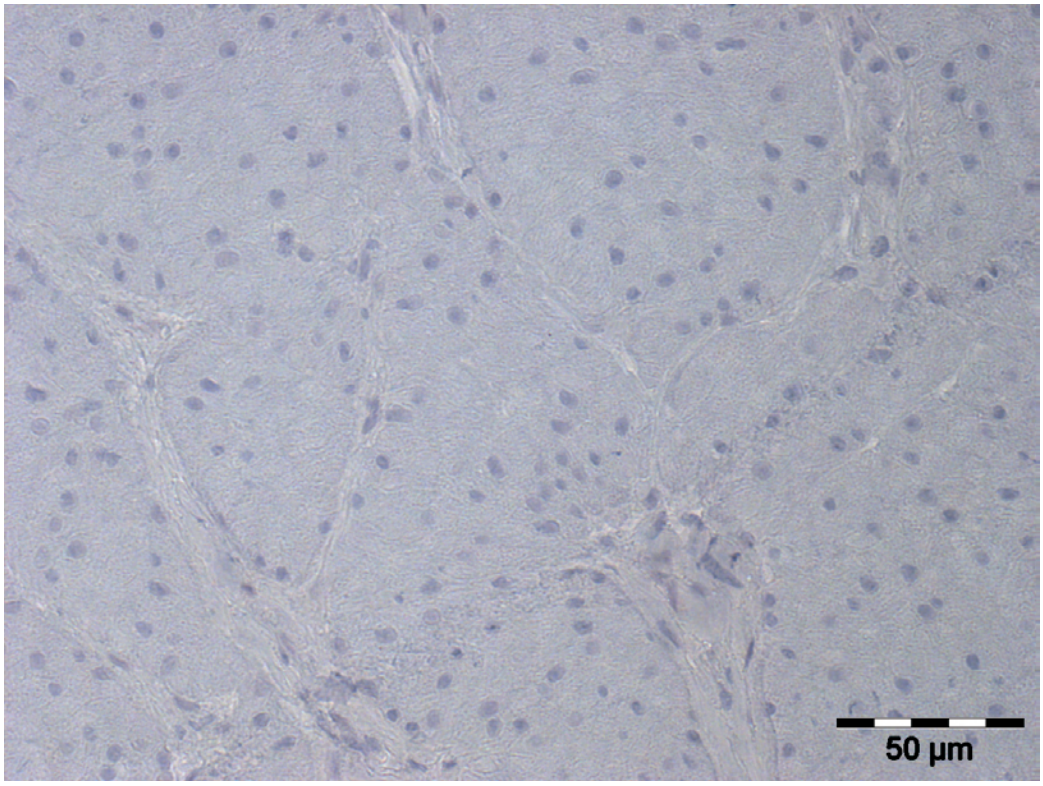

Figure 13: Antral tunica muscularis of a German Holstein cow after CCK-B receptor antibody staining. There is no staining in any part of the tunica muscularis of antrum pylori.

5.3 Results of motilin immunoreactivity

Motilin peptide immunoreactivity could not be detected in any layer of the abomasal wall (Fig. 15). It is detected only on the duodenal mucosa of five German Holstein cows (Fig. 14). These duodenum samples were collected at a local slaughterhouse and were taken within 20 minutes after shlaughter. Sample extraction and paraffin embedding was carried out as it is mentioned in material and method chapter.

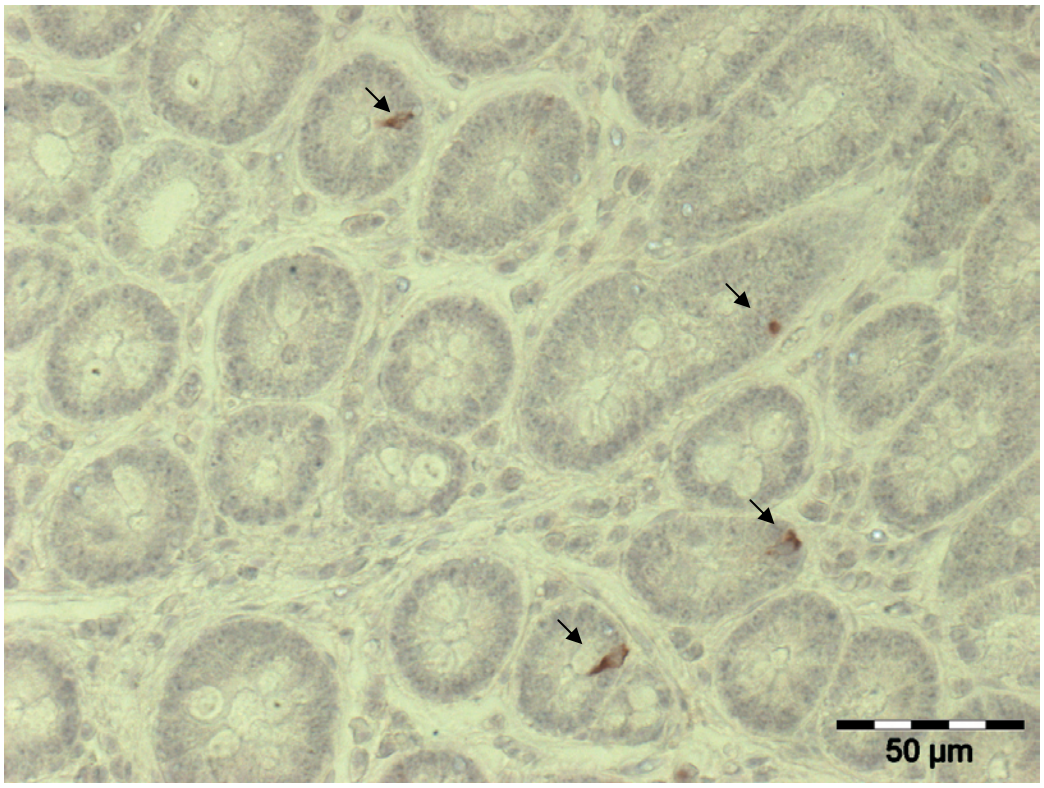

Figure 14: Motilin peptide IR staining in the duodenal glands of a German Holstein cow. IR positive areas were marked with an arrow.

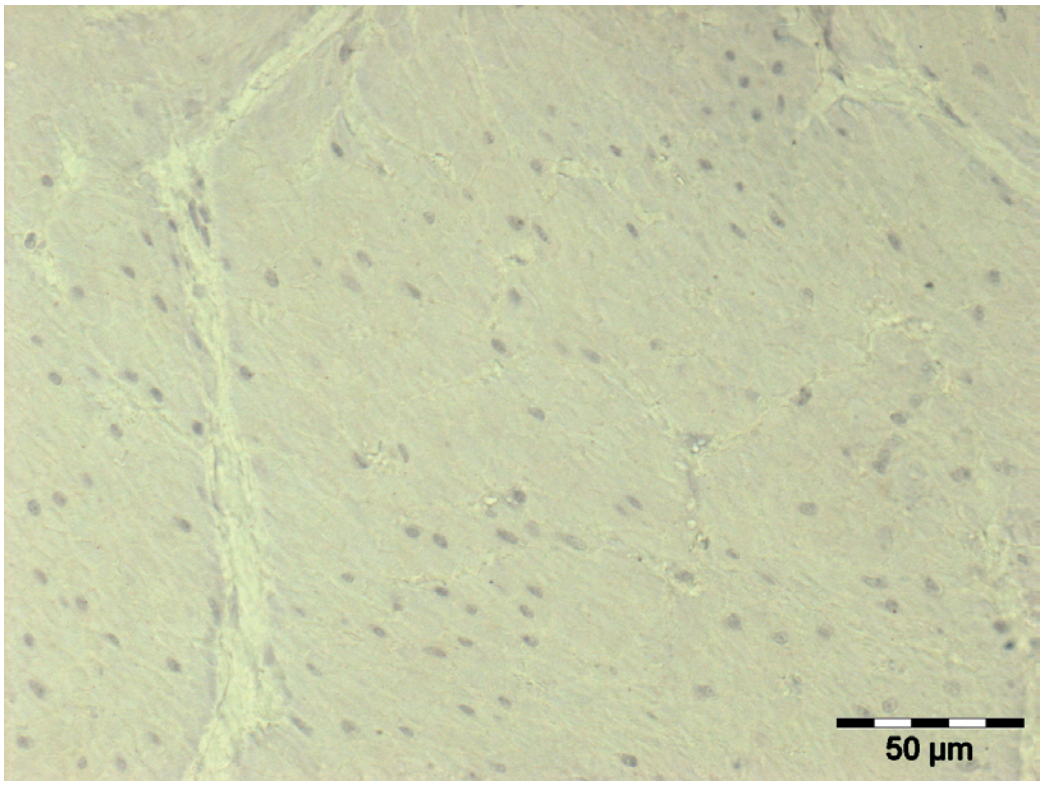

Figure 15: It could not be detected any motilin peptide IR staining in the antral smooth muscle of German Holstein abomasum.

5.4 Results of motilin receptor immunoreactivity

German Fleckvieh

The immunoreactive staining of motilin receptors could be detected on both, antrum and corpus smooth muscle layers of the abomasal wall. In the tunica muscularis of antral part of the abomasal wall, a mean value with a standard deviation ($\bar{x} \pm s$) of 206 ± 57 µm² and in corpus 172 ± 69 µm² has been measured (Fig. 16).

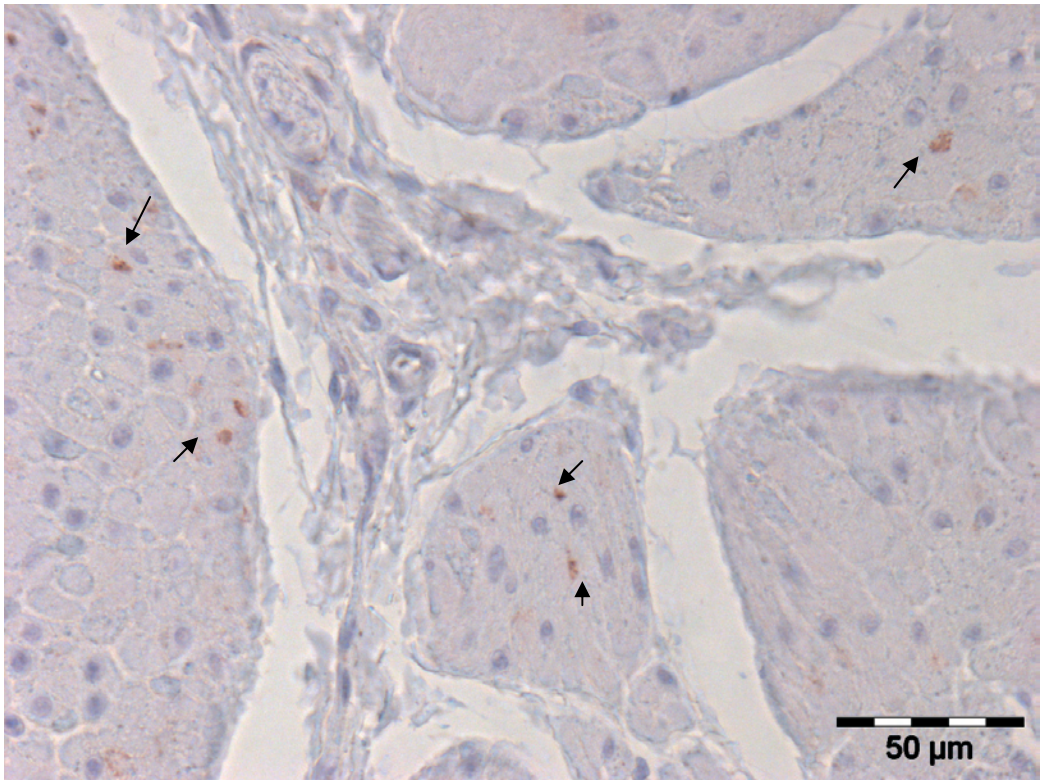

Figure 16: **IR staining of motilin receptor (GPR38) in the antral smooth muscle of abomasal wall. Black arrows show IR-positive stained areas.**

German Holstein

IR area of motilin receptors in the antrum of the abomasal wall represents a mean value with standard deviation ($\bar{x} \pm s$) of 179 ± 53 µm². In the corpus abomasi, the IR area is measured as 152 ± 52 µm².

Comparison of immunoreactive area of motilin receptors in both breeds

In this respect, the German Fleckvieh cows have a slightly larger IR area of motilin receptors in comparison to the German Holstein cows (Fig. 17). Furthermore, this result was not significant (p = 0.11). However, the antral tunica muscularis of the abomasum shows a significant larger IR area for motilin receptors in both breeds compared to the corpus (p = 0.009) (Fig. 18).

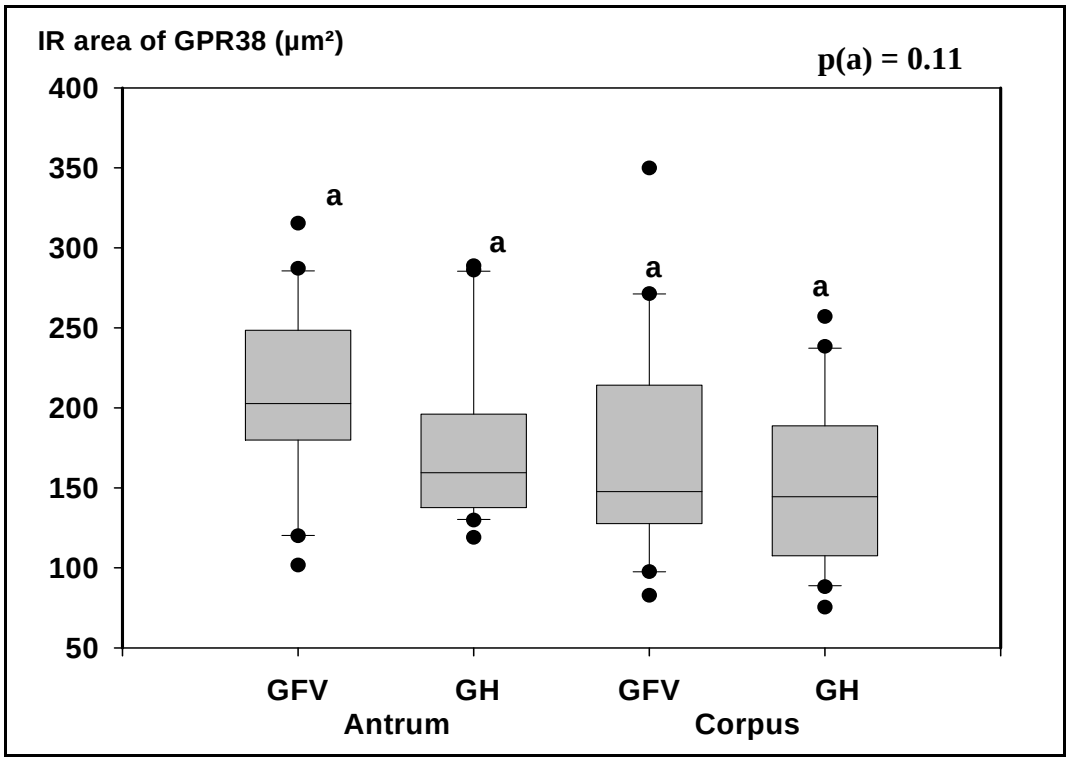

Figure 17: **Comparison of German Holstein (GH) and German Fleckvieh (GFV) cows concerning the motilin receptor (GPR38) immunoreactive area. There is no significant difference between these two breeds but a tendency that German Fleckvieh cows have more motilin receptors than German Holstein cows (p = 0.11). For each box horizontal line represents the median value and the upper and lower boundries represent the 75th and 25th percentiles respectively. Whiskers represent the 10th and 90th percentiles and dots represent outlier values.**

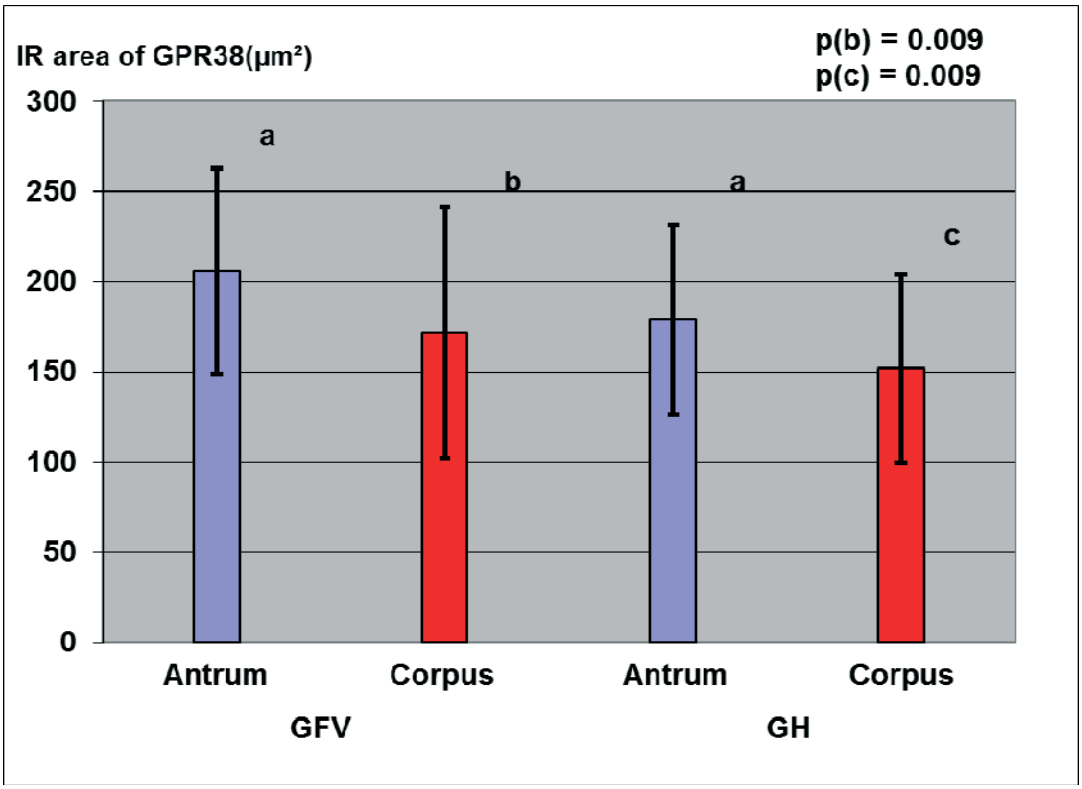

Figure 18: The antrum has a significant larger IR area of motilin receptor (GPR38) than corpus in both breeds (p = 0.009). The different letters show a significant difference between the antrum and the corpus abomasi of both German Fleckvieh and German Holstein cows. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

German Holstein with abomasal displacement

In this group, the antrum has a mean value with a standard deviation ($\bar{x} \pm s$) of 125 ± 55 µm² whereas the corpus abomasi has an area of 124 ± 43 µm².

Comparison of immunoreactive area of motilin receptors in healthy German Holstein cows and German Holstein cows with abomasal displacement

Concerning this criterion, a significant difference is detected between healthy German Holstein cows and German Holstein cows with abomasal displacement (Fig. 19). The German Holstein cows with abomasal displacement have a significant smaller mean IR area of motilin receptors in comparison to the healthy cows of this breed (p = 0.0029).

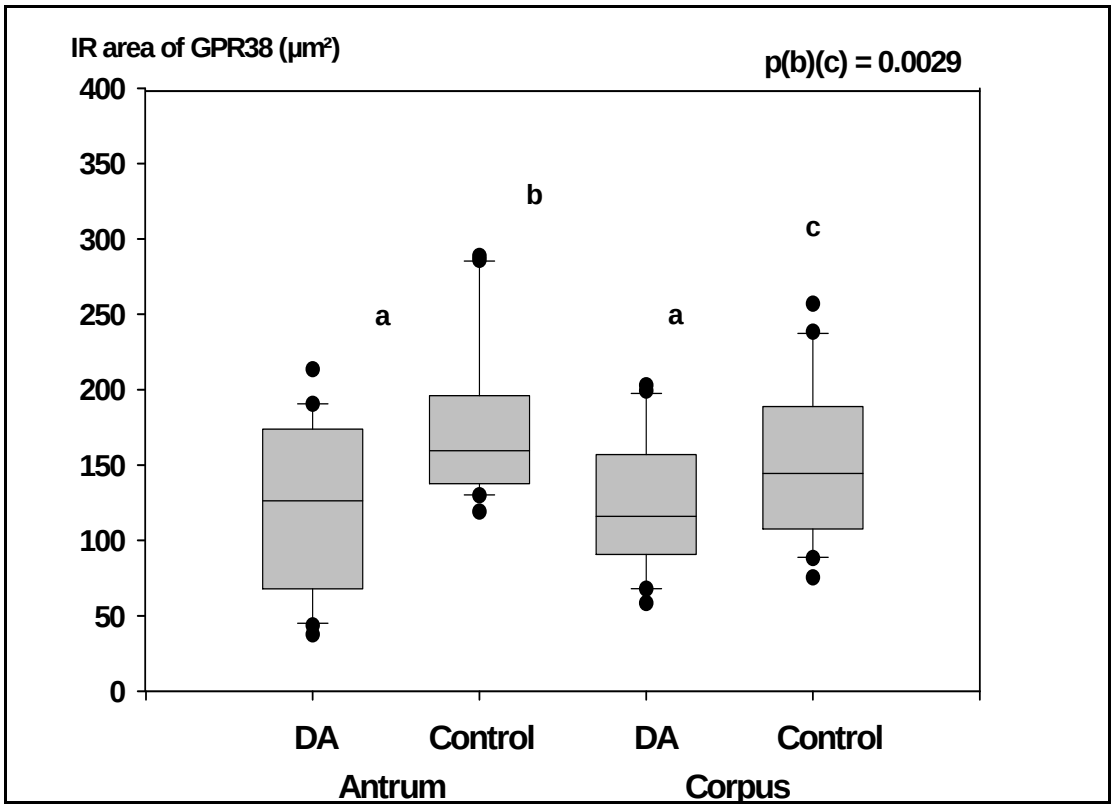

Figure 19: **Comparison of motilin receptor immunoreactive areas in healthy German Holstein cows (Control) and German Holstein cows with displaced abomasum (DA). Different letters show a significant difference between the DA and control groups both in the antrum and the corpus abomasi (p = 0.0029). For each box horizontal line represents the median value and the upper and lower boundries represent the 75th and 25th percentiles respectively. Whiskers represent the 10th and 90th percentiles and dots represent outlier values.**

5.5 Results of VPAC2

VPAC2/VIP-2 receptors could not be detected in any layer of abomasal wall with immunohistochemical ABC-AEC method. VPAC2 positive IR staining is detected in the tunica muscularis of the human stomach which is a positive control tissue for this antibody.

5.6 Results of VPAC1

German Fleckvieh

IR-positive staining for VPAC1/VIP-1 receptor is detected in the tunica muscularis of both antrum pylori and corpus abomasa (Fig. 20). Due to the fact that the IR area of VPAC1 receptors is very small-sized, the number of this receptor is counted within 5 image dimensions of total 285587 µm².

The analysis of vasoactive intestinal polypeptide receptor-1 (VPAC1/VIPR1) revealed in German Fleckvieh cows a mean number with a standard deviation of ($\bar{x}$ ± s) 19 ± 9.8 in antrum and a mean number with a standard deviation of 19.2 ± 10.9 in corpus of the abomasal wall.

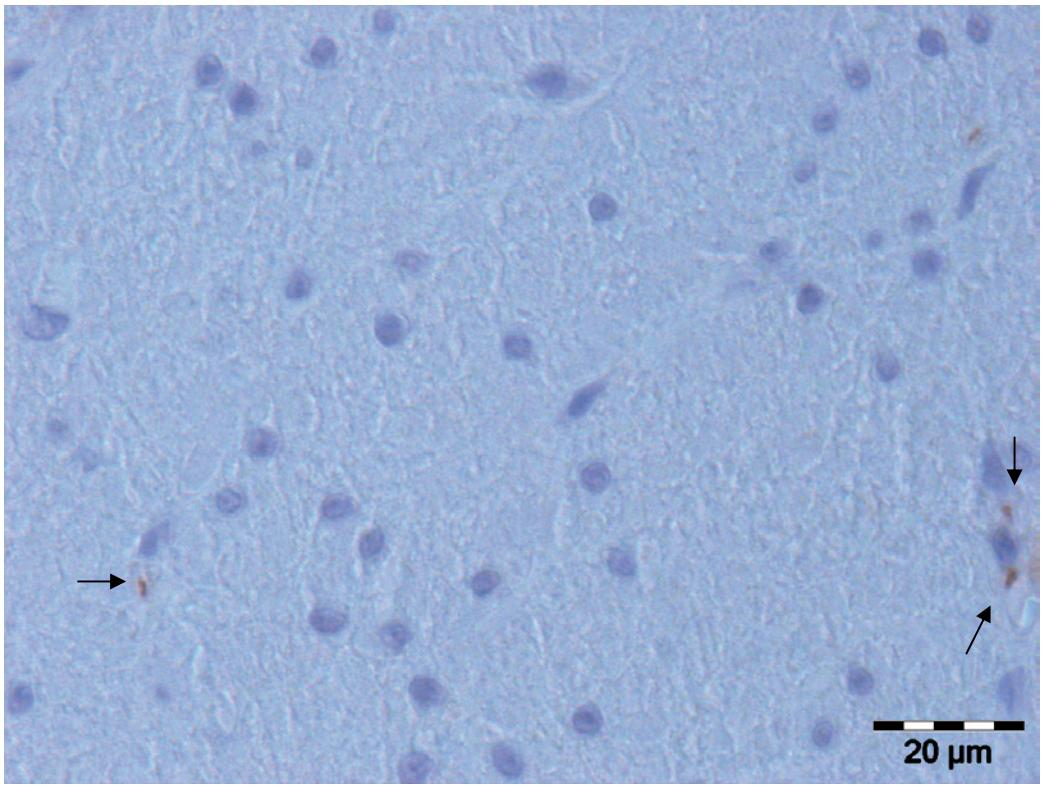

Figure 20: IR-positive staining of VPAC1/VIPR1 receptor on the tunica muscularis of the abomasal wall. The red stained IR positive areas are marked with arrows.

German Holstein

In this group, the antrum pylori represent a mean number with a standard deviation ($\bar{x}$ ± s) of 17.3 ± 6.5 VPAC1/VIP-1 receptors whereas the corpus abomasi exhibits 17.8 ± 8.1.

Comparison of immunoreactive signal number of VPAC1 in both breeds

There are no significant differences between the healthy German Holstein and healthy German Fleckvieh cows regarding the abundance of VPAC1/VIP-1 receptor, neither in antrum nor in corpus of the abomasal wall (Fig. 21).

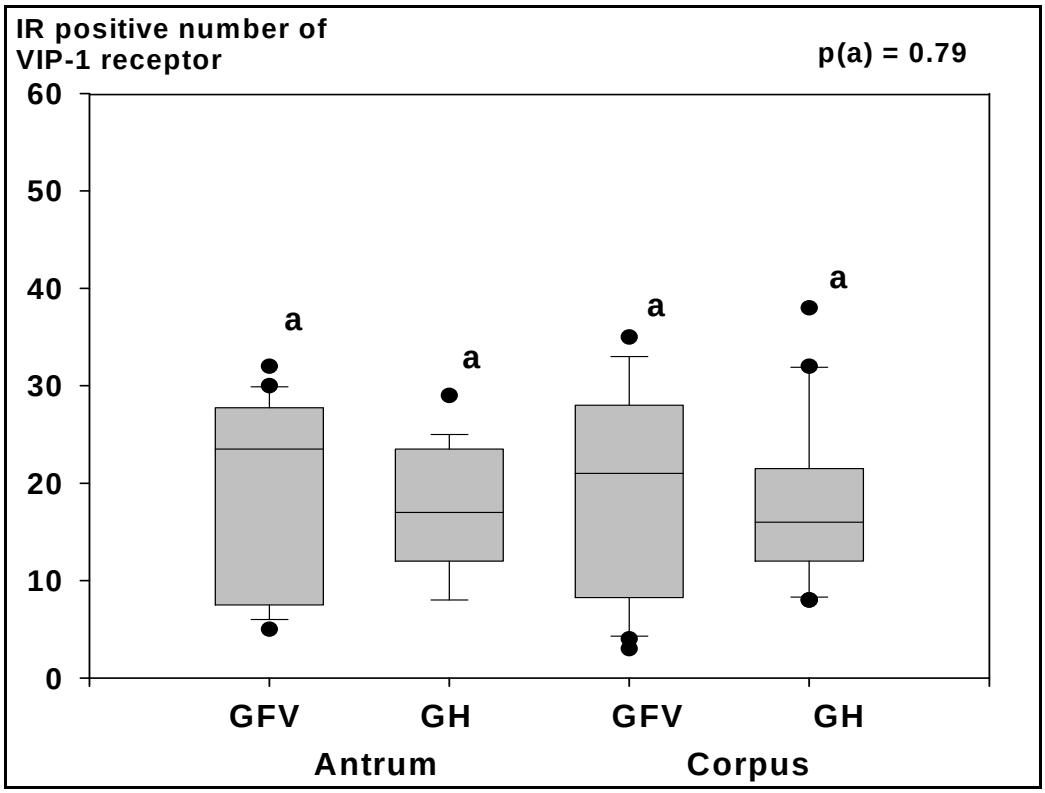

Figure 21: No significant differences are detected between these two German Holstein (GH) and German Fleckvieh (GFV) breeds concerning IR-positive numbers of VPAC1/VIP-1 receptor in the abomasal smooth muscle (p = 0.79). For each box horizontal line represents the median value and the upper and lower boundries represent the 75th and 25th percentiles respectively. Whiskers represent the 10th and 90th percentiles and dots represent outlier values.

German Holstein with abomasal displacement

The German Holstein cows with abomasal displacement present a mean amount with a standard deviation of ($\bar{x}$ ± s) 23.7 ± 11.7 receptors in the antrum and 26.1 ± 11.1 receptors in the corpus of the abomasal wall.

Comparison of immunoreactive signal number of VPAC1 in healthy German Holstein cows and German Holstein cows with abomasal displacement

Regarding the comparison of healthy cows and cows with abomasal displacement, both antrum and corpus of the abomasal wall from German Holstein cows with abomasal displacement show a significant higher number of these receptors compared to the healthy German Holstein cows (Fig. 22) ($p = 0.0053$).

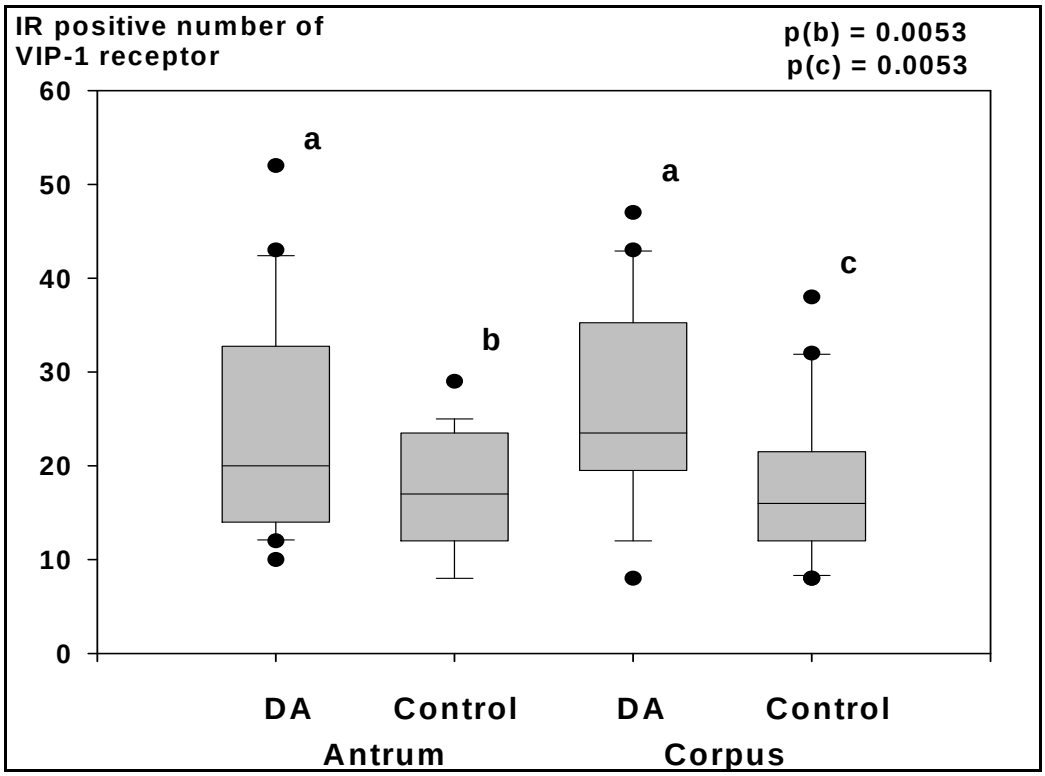

Figure 22: Comparison of VPAC1/VIP-1 receptor in healthy German Holstein cows (Control) and German Holstein cows with displaced abomasum (DA) regarding IR-positive amount of VPAC1/VIP-1 receptor in the abomasal wall. Different letters show a significant difference between the DA and control groups both in the antrum and the corpus abomasi. For each box horizontal line represents the median value and the upper and lower boundries represent the 75th and 25th percentiles respectively. Whiskers represent the 10th and 90th percentiles and dots represent outlier values.

5.7 Results of caspase activity

German Fleckvieh

In the analysis of the caspase activity, the stained cell nuclei were first counted and then proportioned to the entire cell amount of the preparation. Through this process, a percentage of caspase activity showing cells is obtained by comparing the stained cells to the entire cell amount of the preperation. The abomasal antrum of German Fleckvieh cows shows a mean IR ratio with a standard deviation ($\bar{x}$ ± s) of 18.5 ± 8.3 % caspase activity, whereas the corpus shows 21.1 ± 7.9 %.

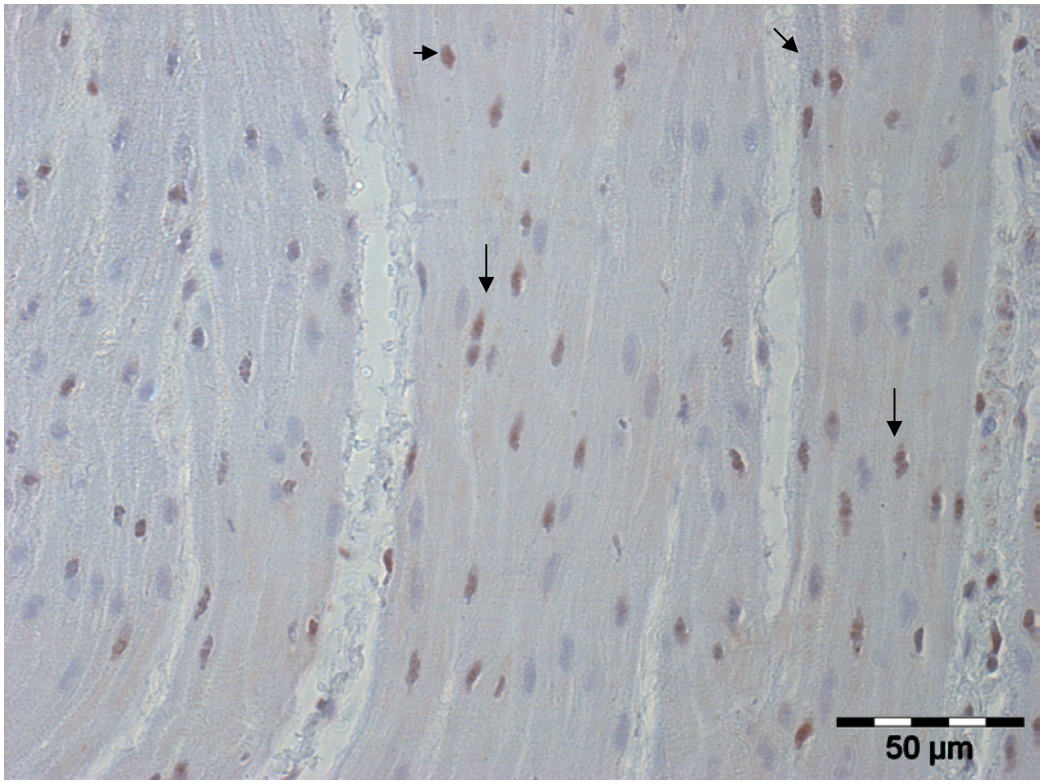

Figure 23: The apoptotic cells are stained with a red color whereas the healthy cells preserve their own blue color. Some of the apoptotic cells are marked with arrows.

German Holstein

In this group, the antrum pylori shows a mean ratio ($\bar{x}$ ± s) of 16.9 ± 5.8 % and the corpus abomasi shows 21.4 ± 6.5 % caspase activity (Fig. 23).

Comparison of immunoreactive ratio of caspase activity in both breeds

A significant difference in the caspase activity between these German Holstein and German Fleckvieh cows could not be demonstrated (Fig. 24). However, in the comparison of antrum and corpus localizations, the corpus abomasi shows a high significant caspase activity than antrum in both breeds ($p < 0.001$) (Fig. 25). Furthermore, the ratio of apoptotic cells between the corpus and the antrum of the abomasal wall of German Holstein cows is significantly higher than German Fleckvieh cows ($p = 0.0013$) (Fig. 25).

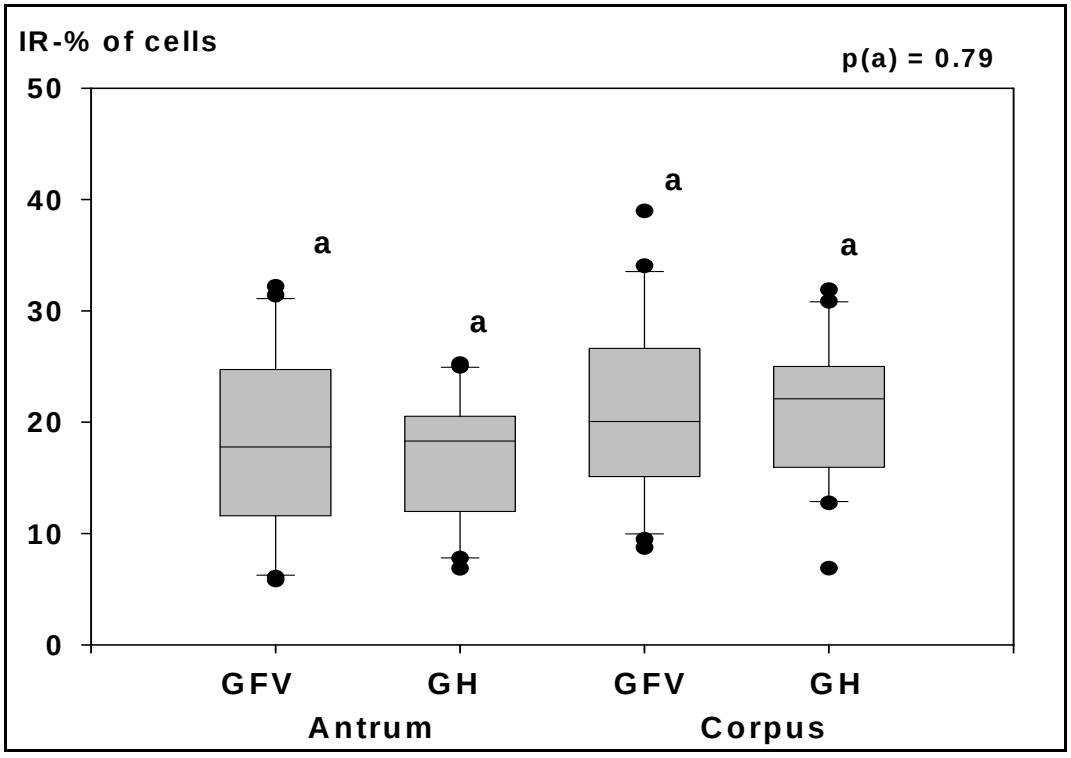

Figure 24: Comparison of two breeds concerning the caspase activity. There is no significant difference between German Holstein (GH) and German Fleckvieh (GFV) cows concerning the IR-% of apoptotic cells (p = 0.79). For each box horizontal line represents the median value and the upper and lower boundries represent the 75th and 25th percentiles respectively. Whiskers represent the 10th and 90th percentiles and dots represent outlier values.

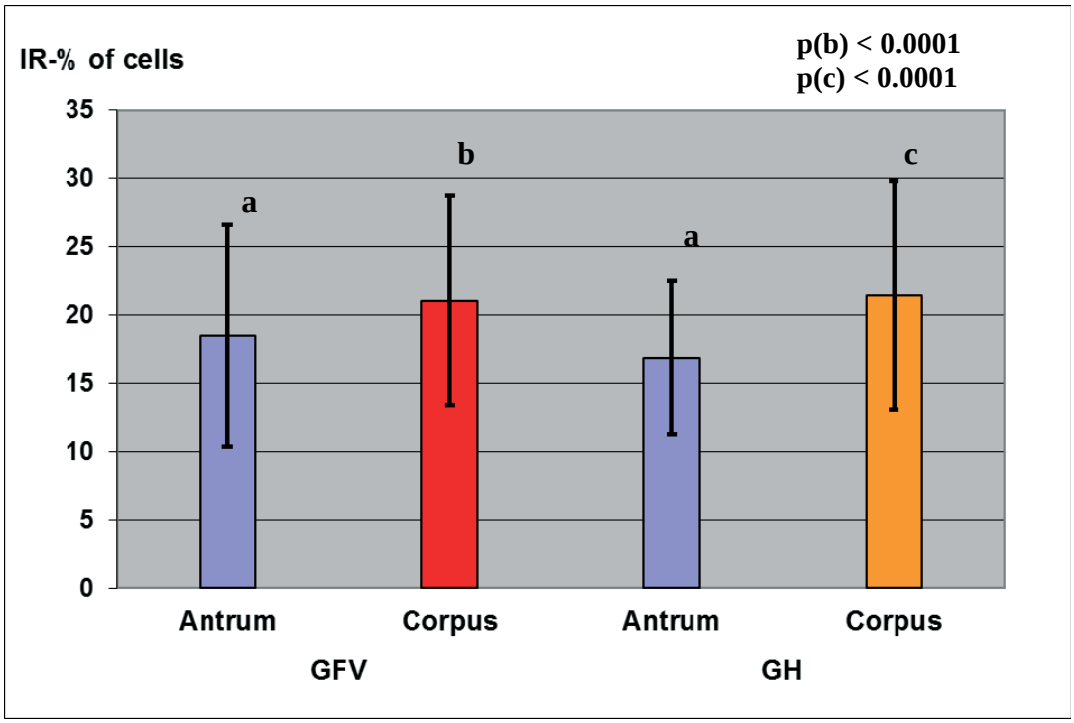

Figure 25: Corpus shows high significant higher % of apoptotic cells than antrum in both breeds ($p < 0.0001$). Furthermore, the ratio of apoptotic cell % between the antrum and corpus of GH cows is significantly higher than the ratio of apoptotic cell % between the antrum and corpus of GFV cows ($p = 0.0013$). Different letters show a significant difference between the antrum and the corpus of both breeds. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

German Holstein with abomasal displacement

German Holstein cows with abomasal displacement show a mean value ($\bar{x} \pm s$) of caspase activity of 26 ± 10.6 % in antrum and 29.4 ± 9.4 % in corpus of the abomasal wall.

Comparison of immunoreactive ratio of caspase activity in healthy German Holstein cows and German Holstein cows with abomasal displacement

The German Holstein cows with abomasal displacement present a significant higher mean ratio of caspase activity compared to the healthy German Holstein cows ($p = 0.002$) (Fig. 26). The comparison between the localizations also shows a high significant higher caspase activity in the corpus than the antrum ($p < 0.0001$) (Fig. 27).

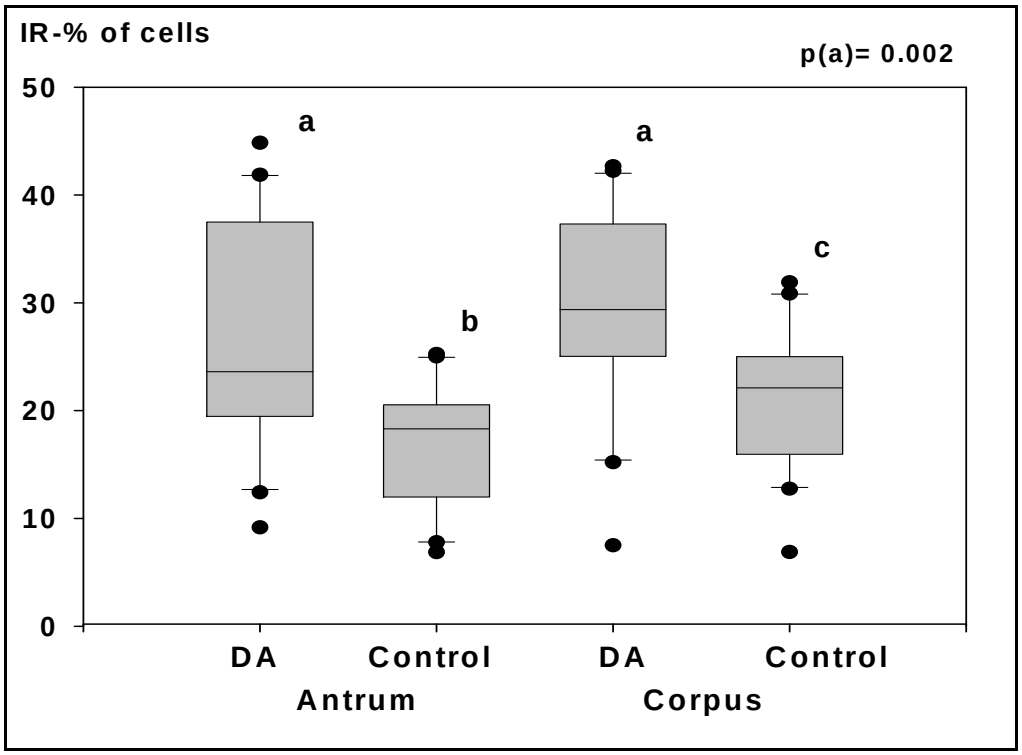

Figure 26: Comparison of healthy GH cows (Control) and GH cows with AD (DA). The percentage of the apoptotic cells is significantly higher in GH cows with AD than healthy GH cows. Different letters show a significant difference between the DA and control group both in the antrum and the corpus concerning the percentage of apoptotic cells. For each box horizontal line represents the median value and the upper and lower boundries represent the 75th and 25th percentiles respectively. Whiskers represent the 10th and 90th percentiles and dots represent outlier values.

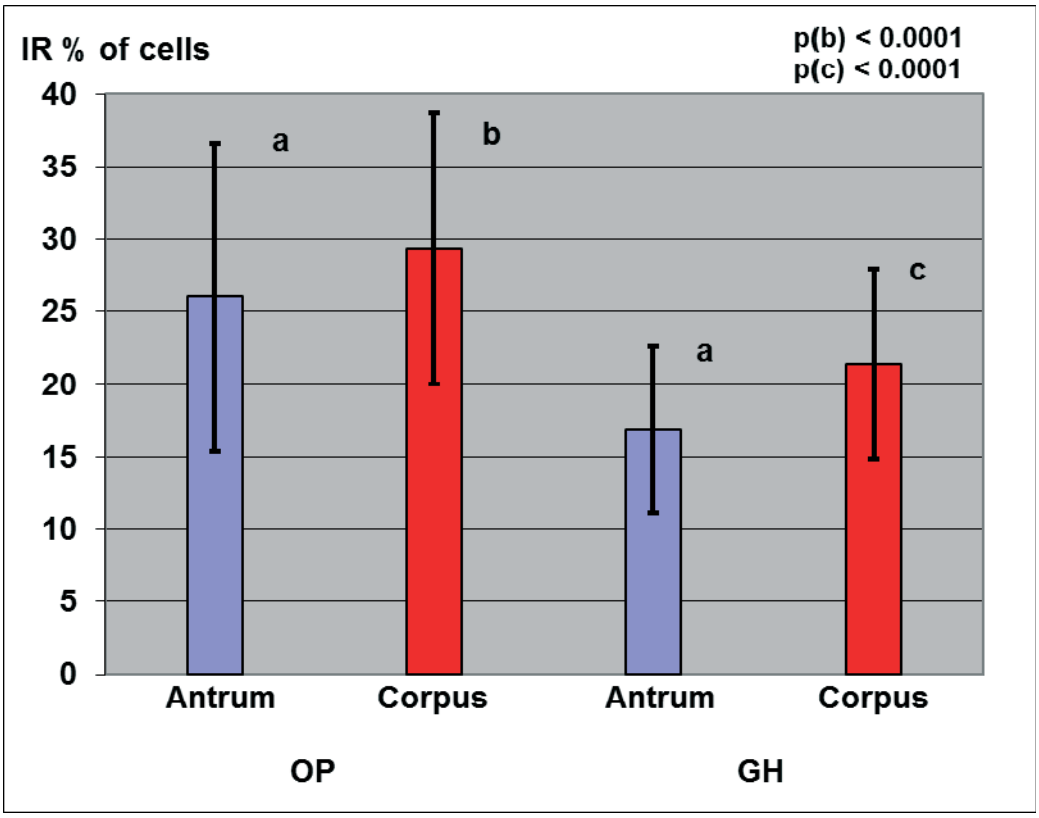

Figure 27: Corpus abomasi of both groups of cows with (OP) and without AD (GH) have a high significantly higher percentage of apoptotic cells compared to the antrum abomasi of these cows. Different letters show a significant difference between the antrum and the corpus abomasi of both OP and GH groups. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x}$ ± s).

5.8 Results of the experimentally induced abomasal dilatation cows

5.8.1 Results from the first operation, before the application of a gastric band

Gastrin

The immunohistochemical staining of these three German Holstein cows before placing a gastric band, in other words cows without an abomasal dilatation or displacement, shows for the IR gastrin peptide staining an average area ($\bar{x}$ ± s) of 211 ± 175 µm² in the antrum of abomasal wall.

Motilin receptor

The antrum of the abomasal wall has an average area ($\bar{x} \pm s$) of 95 ± 46 µm² whereas the corpus has an average area of 101 ± 19 µm², concerning the immunoreactive area of this receptor.

VPAC1 receptor

VPAC1 has a mean number ($\bar{x} \pm s$) of 13.3 ± 5.1 in antrum and 15.7 ± 5.5 in corpus.

Caspase-3

Caspase-3 activity, as an indicator of the damage that occurred by the mechanical stretch or hypoxia, which is caused by the AD, shows a mean apoptotic cell percentage with a standard deviation ($\bar{x} \pm s$) of 19.3 ± 5.9 % in the antrum and 17.9 ± 7.8 % in the corpus of the abomasal wall before placing a gastric band.

5.8.2 Results from the second operation, after the application of a gastric band

Gastrin

The samples which have been extracted after placing the gastric band for 72 hours show a mean immunoreactive area ($\bar{x} \pm s$) of 294 ± 78 µm² for the gastrin peptide. This area is slightly larger than the area before inducing an abomasal impaction (Fig. 28). But this difference is not significant (p = 0.43).

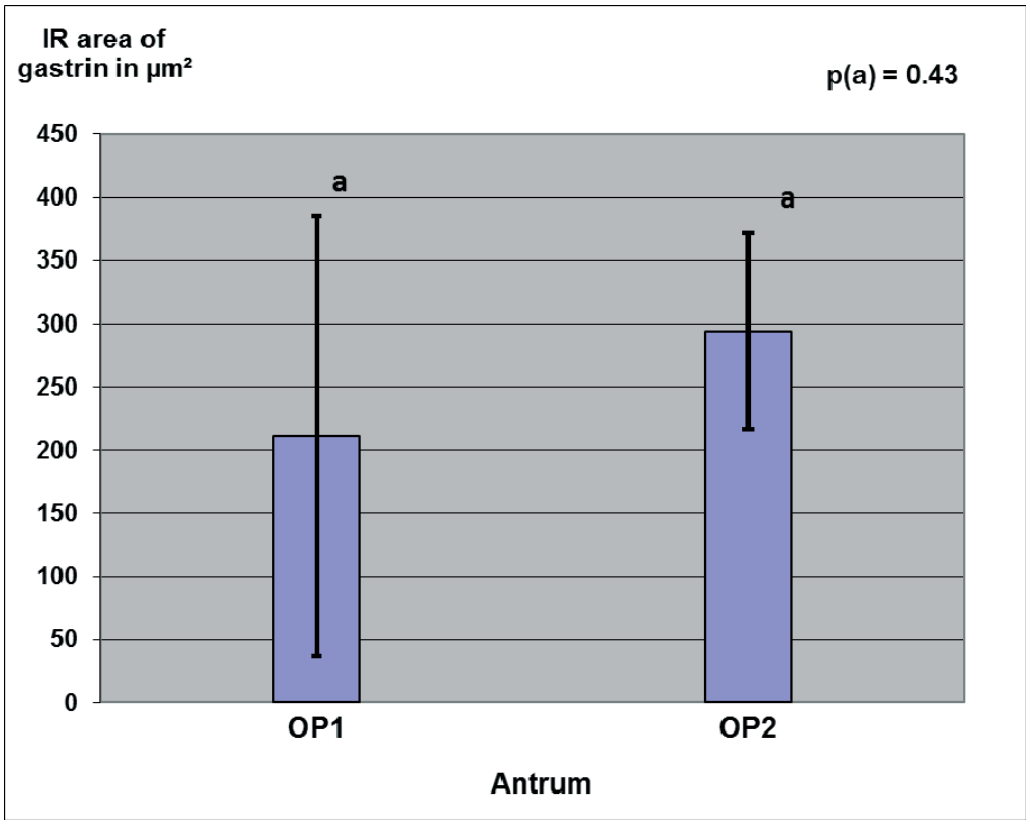

Figure 28: The comparison of the effect of placing a gastric band shows no significant difference before (OP1) and after laying this band (OP2), concerning the IR-area of gastrin peptide in the tunica mucosa of the antrum of German Holstein cows. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

Motilin receptor

The immunoreactive area of the motilin receptor (GPR38) after the experimentally induced abomasal dilatation has an average area ($\bar{x}$) of 47 µm² in the antrum and 80 µm² in the corpus. Such a decrease in the abundance of motilin receptors in abomasal wall was also observed between healthy German Holstein and German Holstein cows with AD (Fig. 19). The same tendency can also be seen between the both groups before and after placing a gastric band (Fig. 29). However, this decrease in both parts of the abomasum is not significant (p = 0.27).

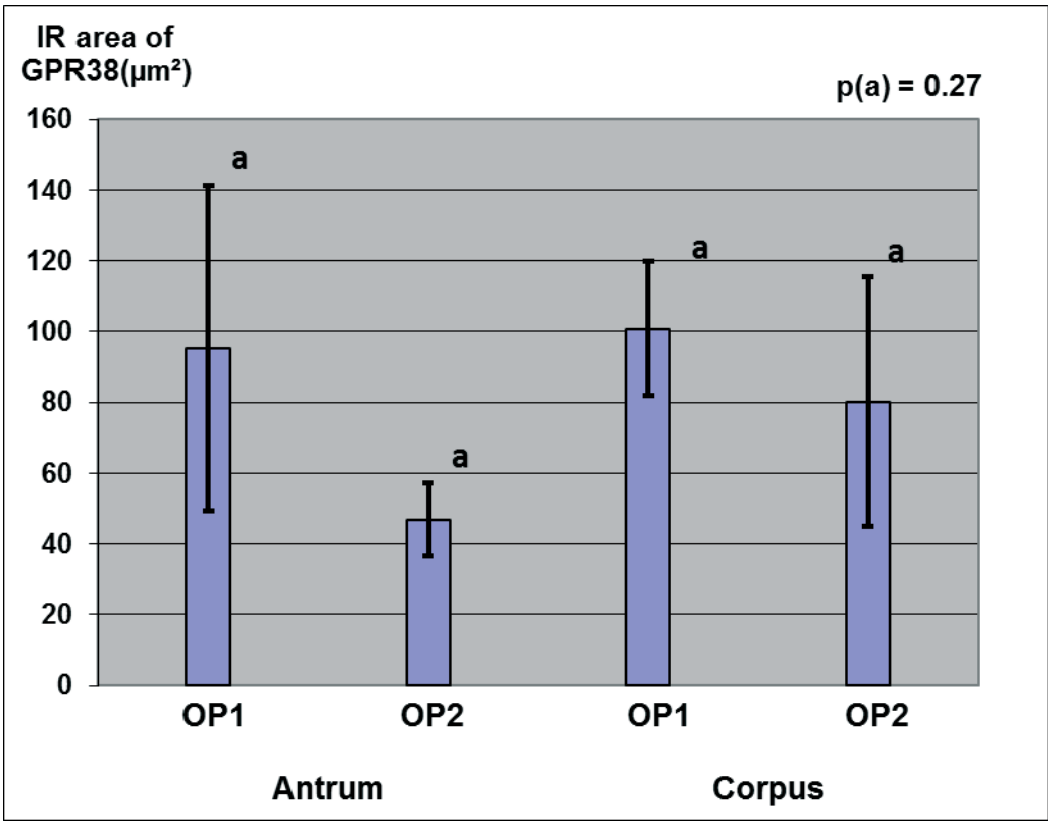

Figure 29: The tendency can be observed that the abomasal impaction (OP2) results in a reduction of the IR-positive area of motilin receptors (GPR38). However, this reduction is not significant between the first (OP1) and second operations (OP2). The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x}$ ± s).

VPAC1 receptor

The number of this receptor changes after placing the gastric band for 72 hours from a mean value ($\bar{x} \pm s$) of 13.3 ± 5.1 to 15.3 ± 3.1 in abomasal antrum and from 15.7 ± 5.5 to 19.7 ± 9.3 in the corpus of abomasal wall. Here, a significant increase after placing the gastric band for 72 hours is not observed (p = 0.6) (Fig. 30).

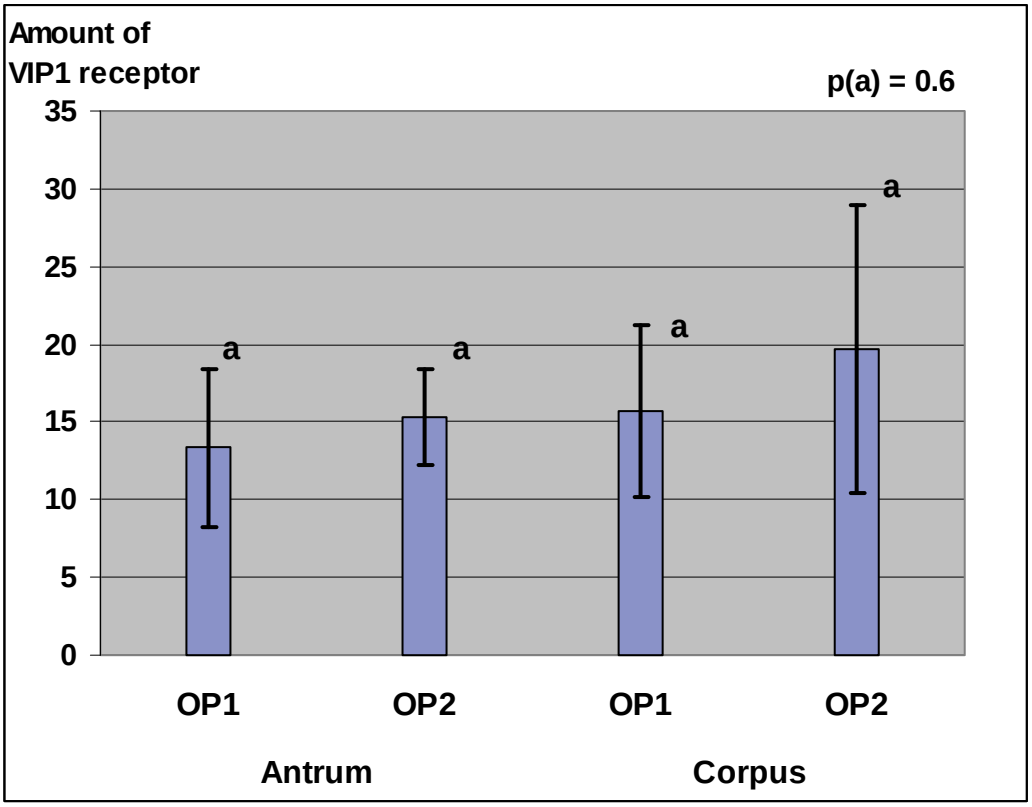

Figure 30: There is no significant difference between the first (OP1) and second operation (OP2) concerning the IR-positive number of VIP-1 receptor. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

Caspase-3

The proportion of the apoptotic cells is increased after the second operation (Fig. 31). In the antrum, the mean ratio of the caspase activity becomes 31.4 ± 8.9 % and in the corpus 32.3 ± 6.4 %. This increase in percentage of the caspase activity is not significant but a tendency can be seen in both localizations of the abomasal wall (p = 0.12).

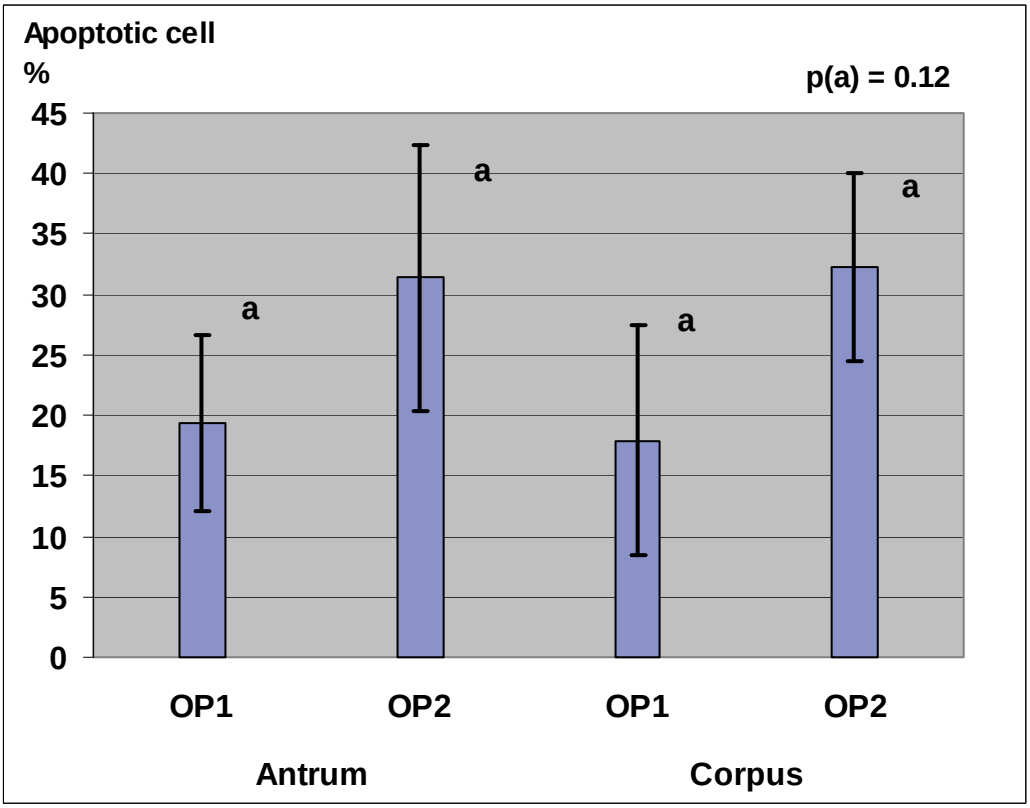

Figure 31: The % of caspase activity is higher in the samples from second operation (OP2) than first operation (OP1) but this increase is not significant. However, a tendency can be observed that the abomasal impaction causes an increase in apoptotic cells in the abomasal wall. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x}$ ± s).

5.8.3 Comparison of the results between the healthy German Holstein cows without an abomasal displacement and the experimentally induced abomasal impaction group before placing a gastric band

Gastrin

There is not any significant difference but a tendency regarding the immunoreactive area between 3 healthy German Holstein cows of the gastric band group, sampled before placing the gastric band and the 20 healthy German Holstein cows (p = 0.22) (Fig. 32).

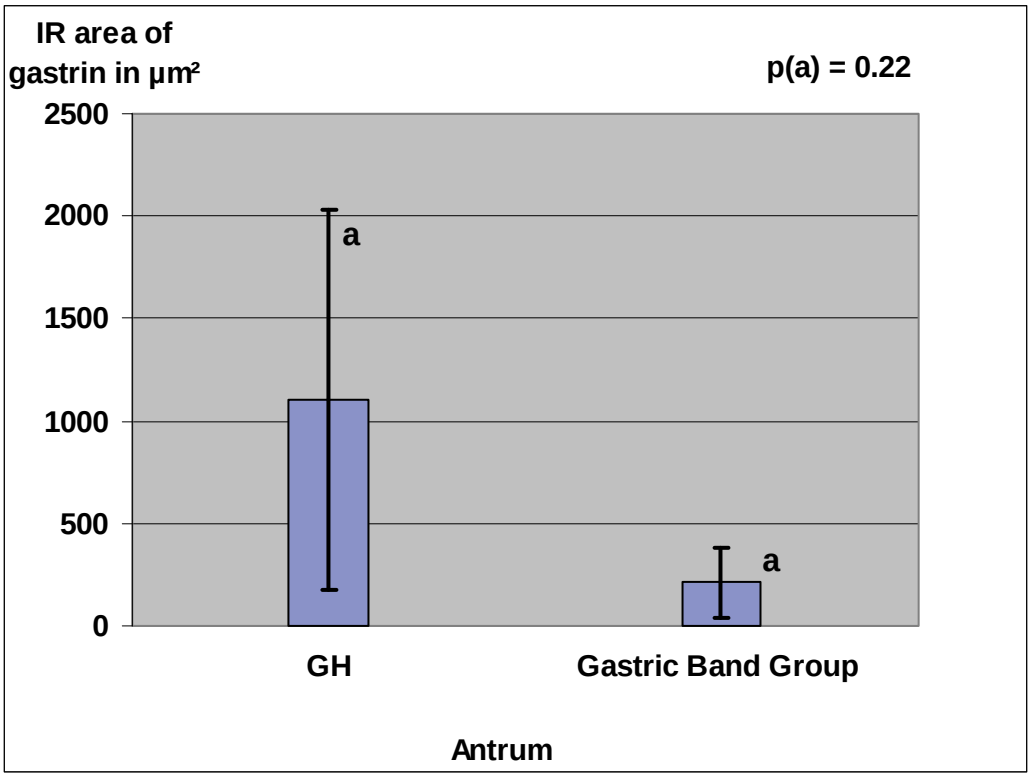

Figure 32: Although the mean IR-positive area of gastrin in the gastric band group is smaller than healthy GH cows, a significant difference could not be detected probably because of high inter- individual variation. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x}$ ± s).

Motilin receptor

In this group, the IR area of motilin receptor in the antrum shows a significant difference between the healthy German Holstein cows and gastric band group (p = 0.0056). The corpus abomasi has also the same tendency but the difference is not significant (p = 0.10) (Fig. 33).

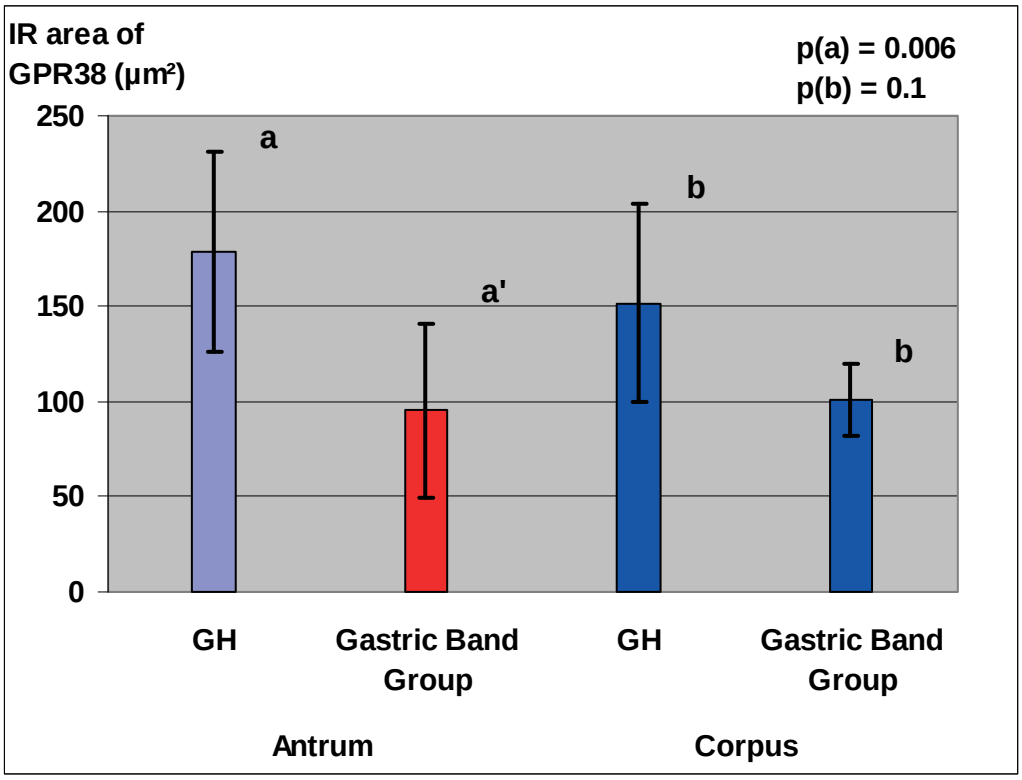

Figure 33: In the antrum, the gastric band group has a significant smaller IR-area of motilin receptor (GPR38) compared to the healthy German Holstein (GH) cows (p = 0.006). The same tendency could also be observed in corpus abomasi but this difference is not significant (p = 0.1). The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

VPAC1 receptor

Neither in antrum nor in corpus of the abomasal wall, there are significant differences between the 20 healthy German Holstein cows and 3 healthy German Holstein cows from the gastric band group regarding the abundance of IR VPAC1/VIP-1 receptor (Antrum p = 0.35, Corpus p = 0.71) (Fig. 34).

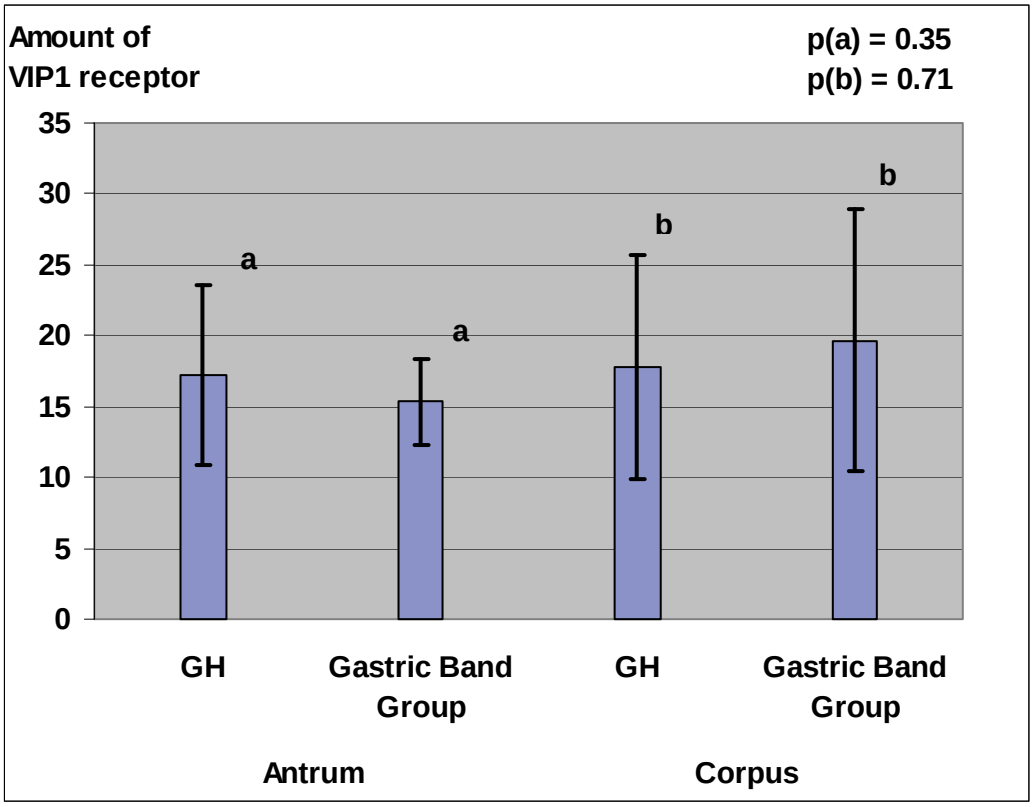

Figure 34: No significant differences are detected between the gastric band group and healthy German Holsteins regarding the IR abundance of VPAC1/VIP-1 receptor. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

Caspase-3

The comparison between healthy German Holstein cows and gastric band group shows no significant difference concerning the caspase activity neither in antrum nor in corpus of the abomasal wall (Antrum p = 0.51, Corpus p = 0.42) (Fig. 35).

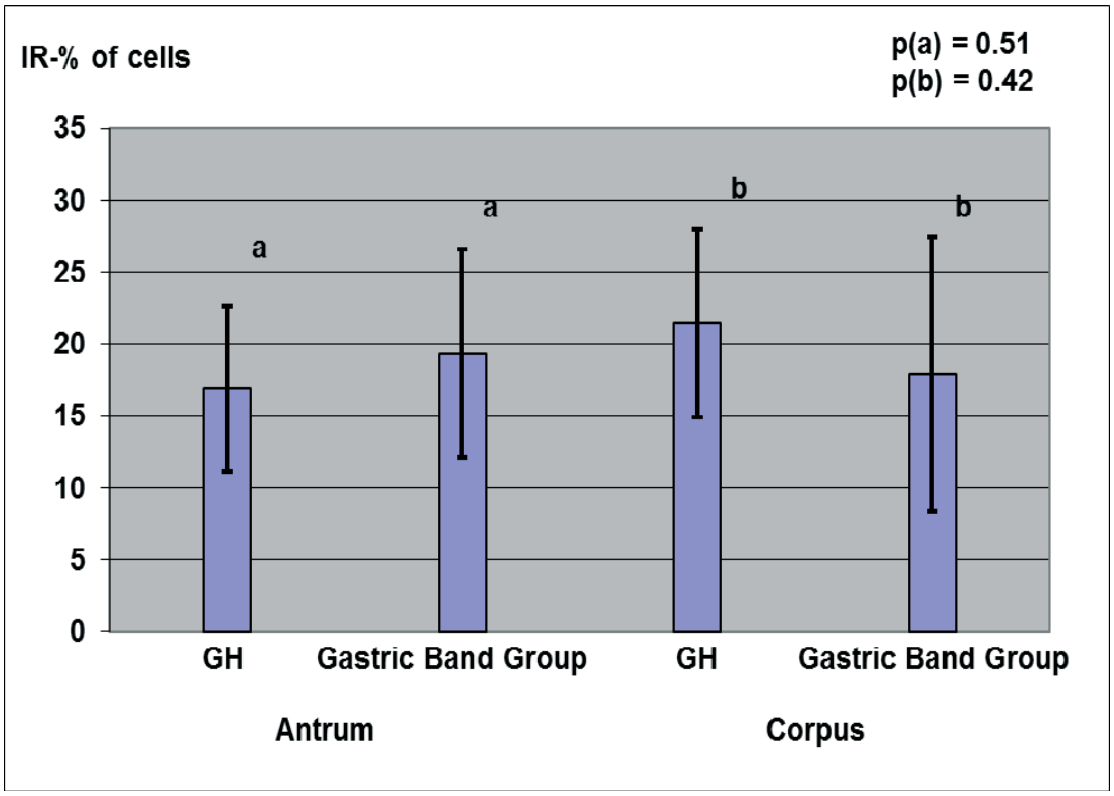

Figure 35: There are no significant differences between both groups regarding the percentage of apoptotic cells. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s) ($\bar{x} \pm s$).

5.8.4 Comparison of the results between the German Holstein cows with abomasal displacement and the experimentally induced abomasal impaction group after placing a gastric band

Gastrin

There is no significant difference between the German Holstein cows with abomasal displacement and experimentally induced abomasal impaction group (p = 0.12) (Fig. 36). Similar to the comparison between healthy German Holstein cows and gastric band group (Fig. 32), the IR-positive area of gastrin peptide is smaller in the experimentally induced abomasal impaction group than German Holstein cows with abomasal displacement. High inter-individual variation or low number of animals in gastric band group could be an explanation for this tendency.

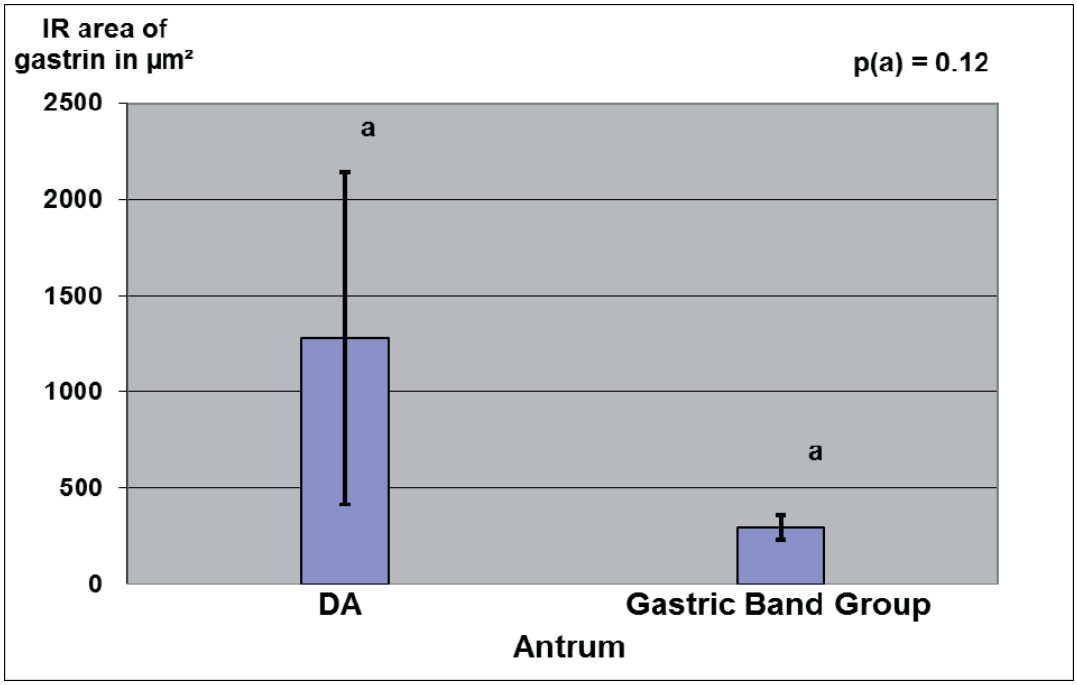

Figure 36: There is a tendency that the experimentally induced abomasal impaction group has smaller IR-positive areas of gastrin peptide than German Holstein cows with abomasal displacement (DA). However, this difference is not significant. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x}$ ± s).

Motilin receptor

The comparison between 20 German Holstein cows with abomasal displacement (DA) and 3 German Holstein cows sampled after placing the gastric band for 72 hours shows that the antrum of the gastric band group has a highly significant smaller IR area of motilin receptors ($p < 0.0001$ seperate). The corpus of the gastric band group shows also a smaller IR area compared to the corpus of German Holstein cows with abomasal displacement but this difference is not significant ($p = 0.09$), however the same tendency could also be observed (Fig. 37).

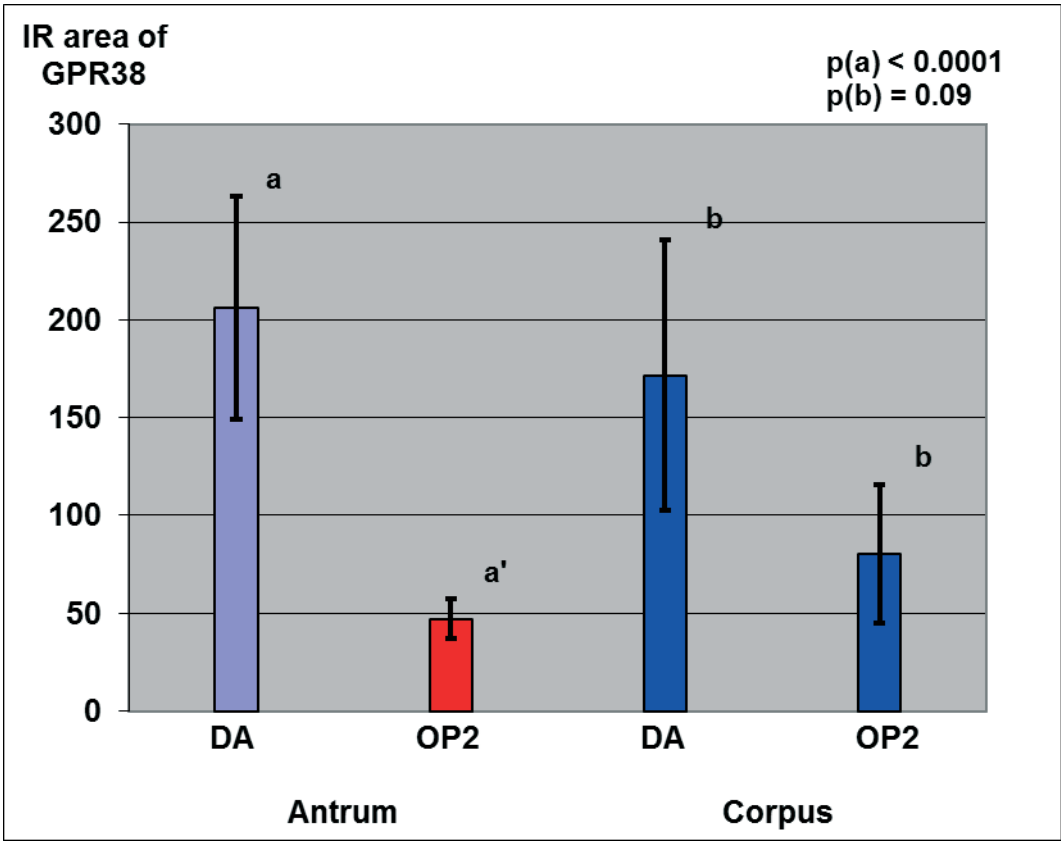

Figure 37: A decrease in IR-positive areas of motilin receptor (GPR38) is observed in experimentally induced abomasal impaction group (OP2) compared to German Holstein cows with abomasal displacement (DA) both in antrum and corpus abomasi. However, in the antrum, gastric band group has a significant smaller amount of motilin receptor ($p < 0.0001$), whereas in corpus there is no statistical significance but a tendency is observed ($p = 0.09$). The different letters show a significant difference between the DA and OP2 groups both in the antrum and the corpus. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

VPAC1 receptor

Regarding the IR-positive amount of VPAC1 (VIPR1) receptor, it is observed that the antrum of experimentally induced abomasal impaction group has a significant smaller amount of VPAC1 receptor compared to the German Holstein cows with AD (p = 0.04). However, this statistical significant decrease of VPAC1 receptors in the antrum of the experimentally induced abomasal impaction group can not be observed in the corpus abomasi between both groups (p = 0.36).

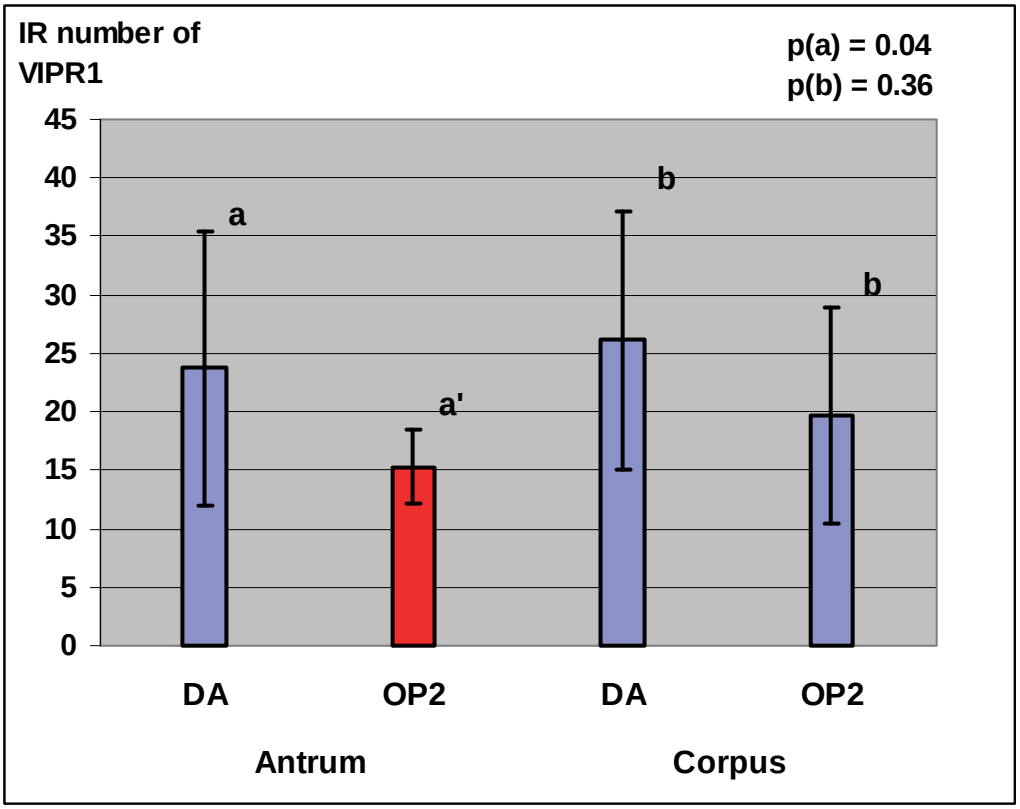

Figure 38: Antrum of the experimentally induced abomasal impaction group (OP2) has a significant smaller amount of VIP-1 receptor compared to German Holstein cows with abomasal displacement (DA) (p = 0.04). However, such a difference is not observed in the corpus (p = 0.36). The different letters show a significant difference between the DA and OP2 groups. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

Caspase-3

There is no significant difference between GH cows with AD and with experimentally induced abomasal impaction groups either in the corpus or in the antrum of the abomasal wall regarding the percentage of apoptotic cells (Antrum p = 0.42, Corpus p = 0.62) (Fig 39).

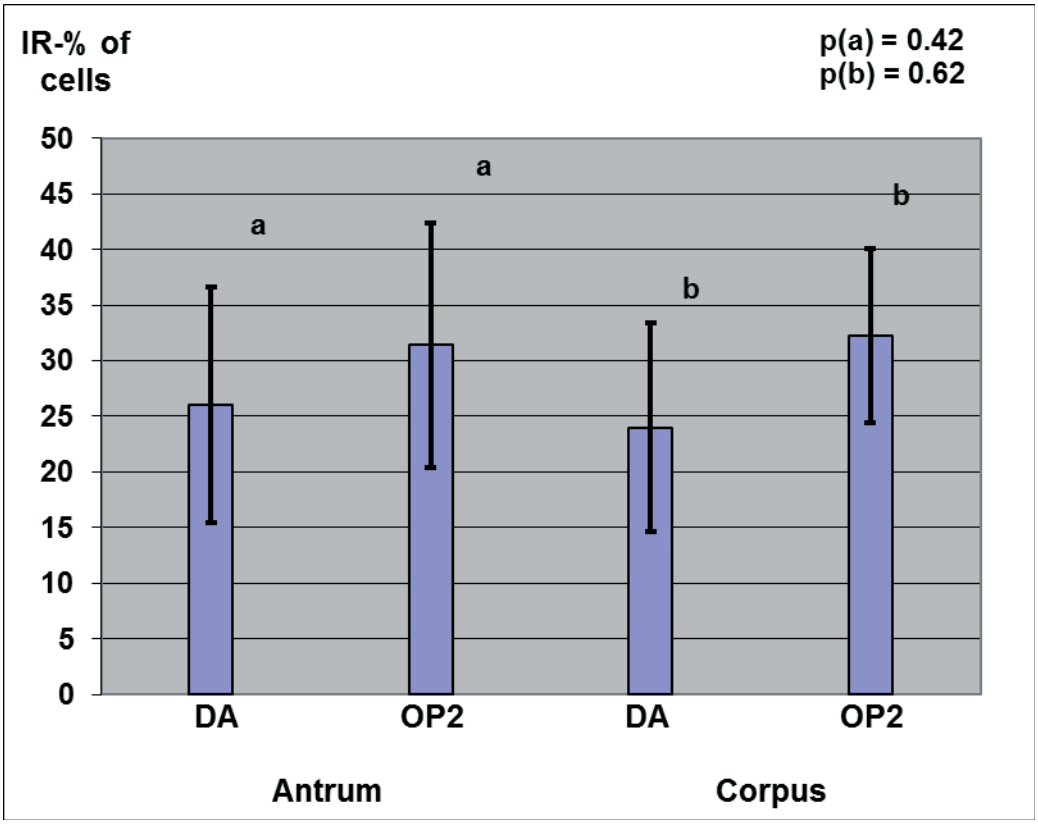

Figure 39: No significant differences are detected between the localizations and groups with regard to the % of caspase activity. The upper boundries of the boxes represent the mean value ($\bar{x}$) and the lines represent the standard deviation (s), ($\bar{x} \pm s$).

5.9 Relationship between the receptors and caspase activity

One of the purposes of this study is to evaluate the relationhip of these peptides and receptors with each other and with caspase activity in the abomasal wall. As the caspase activity is an indicator of apoptosis and cell death in tissues, it is reasonable to think that an increase in cell death of an organ can cause an effect on peptides and their receptors. It is mentioned in early chapters that some of these peptides and receptors may also have pro- or anti- apoptotic effects, or they may mediate the effects of other substances. It is also thought that these peptides and receptors induce stimulatory (motilin) or inhibitory effects (gastrin, VIP) on the abomasal motility. Therefore, a comparison of the relationship of these substances can give ideas about their roles in abomasal displacement.

5.9.1 Relationship between caspase activity and motilin receptor

As mentioned above, the antrum of both healthy German Holstein and German Fleckvieh cows has a significant larger IR area of motilin receptors than corpus abomasi ($p = 0.009$) (Fig. 18). This result is accompanied by a significant higher caspase activity in corpus abomasi of these both breeds compared to their antrum ($p < 0.0001$). Moreover, the comparison of caspase activity in the corpus abomasi of both breeds results in a significant higher caspase activity in the corpus abomasi of German Holstein cows than German Fleckvieh cows ($p = 0.0013$) (Fig. 25).

Another example to the relationship between caspase activity and motilin receptor abundance can be observed between healthy German Holstein cows and German Holstein cows with abomasal displacement (Fig. 19). The abundance of motilin receptors are significantly lower in GH cows with AD than healthy GH cows ($p = 0.0029$). However, the caspase activity is significantly higher in GH cows with AD than healthy GH cows ($p = 0.002$) (Fig. 26).

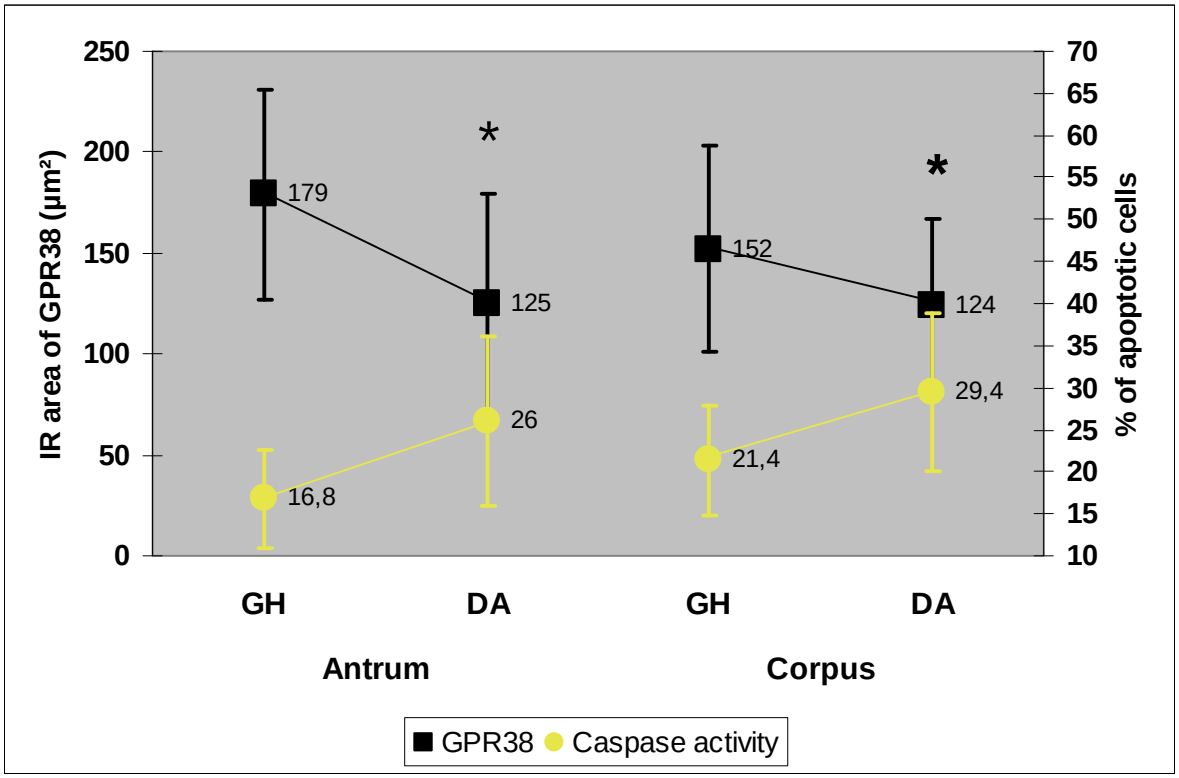

Figure 40: An increase in the percentage of apoptotic cells in German Holstein cows with AD (DA) is accompanied by a decrease in the IR area of motilin receptors (GPR38) both in antrum and corpus abomasi compared to the healthy German Holstein cows (GH). Data expressed in mean ($\bar{x}$) µm² ± standard deviation (s) for motilin receptor density and % ± s for caspase activity. (*) The mean value differs significantly between the GH and DA groups ($p < 0.05$).

5.9.2 Relationship between caspase activity and VPAC1/VIP-1 receptor

A significant increase in caspase activity in the German Holstein cows with abomasal displacement is accompanied also by a significant increase in VPAC1 receptor. An overview of the relationship between the caspase activity and the abundance of VPAC1/VIP-1 receptor in healthy German Holstein cows and those with AD is shown in the figure 41.

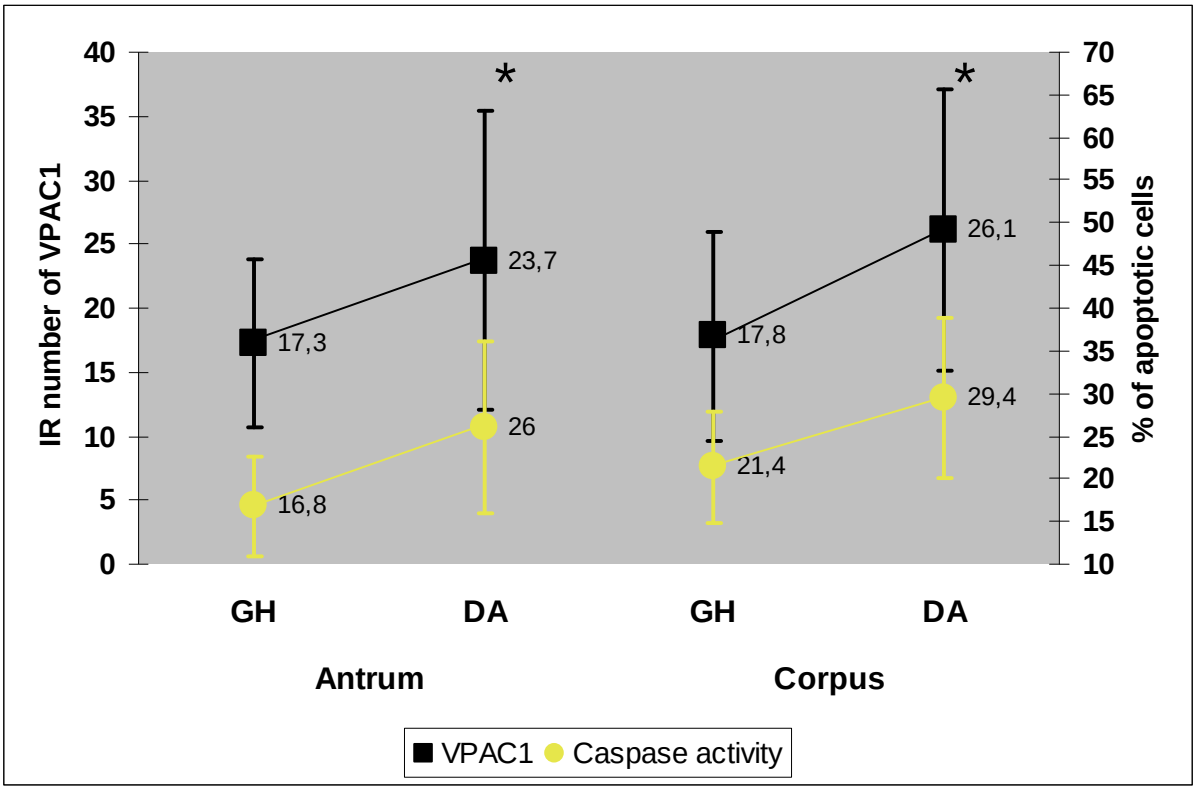

Figure 41: The percentage of the apoptotic cells increase in German Holstein cows with AD (DA) both in antrum and corpus abomasi compared to healthy cows (GH). In addition, an increase in amount of VPAC1/VIP-1 receptors is also observed. Data expressed in mean ($\bar{x}$) number ± s for the VPAC1 receptor and mean % ± s for the caspase activity. (*) The mean value differs significantly between the GH and DA groups ($p < 0.05$).

5.9.3 Relationship between VPAC1/VIP-1 receptor and motilin receptor

According to the results of this study, it is observed that the abundance of motilin receptors is significantly lower and the abundance of VPAC1 is significantly higher in GH cows with AD than in healthy GH cows. An overview of the relationship between the motilin receptor and the abundance of VPAC1/VIP-1 receptor in healthy German Holstein cows and those with AD is shown in the figure 42.

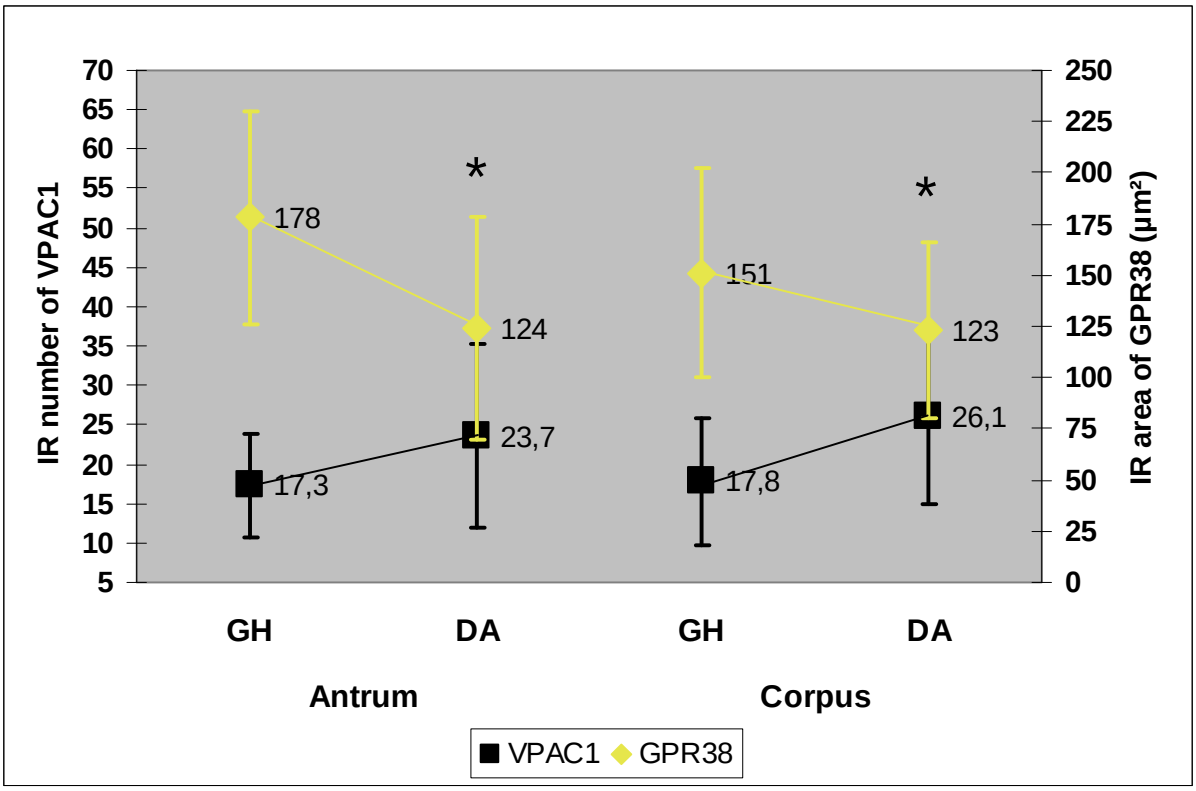

Figure 42: The relationship between the motilin receptor (GPR38) and the VPAC1/VIP-1 receptor is opposite between the German Holstein cows with abomasal displacement (DA) and healthy German Holstein cows (GH). Data expressed in mean ($\bar{x}$) number ± s for the VPAC1 receptor and mean µm² ± s for the motilin receptor. (*) The mean value differs significantly between the GH and DA groups ($p < 0.05$).

6 Discussion

6.1 Discussion of the immunohistochemical ABC method

The present study utilized immunohistochemistry to detect the cellular location and presence of motilin, gastrin and their receptors, VIP receptors and caspase-3 activity in the abomasal wall of cattle using appropriate antibodies. This technique is based on binding of antibodies to a specific antigen and a visualisation of the occurring signal. Immunohistochemistry is an important method to detect the localization of tissue proteins and can also be used to demonstrate the presence of material that is specific for an infectious organism in animal tissues (Mulisch 2010). Complete preparation of the sample is critical to maintain cell morphology, tissue architecture, and the antigenicity of target epitopes. Authors have described many intrinsic and extrinsic factors which influence immunostaining. Intrinsic factors include fixation, duration, type of fixative, temperature, pH of fixative variables in tissue processing, antigen loss resulting from delays in fixation, tissue necrosis, and levels of antigen expression, whereas extrinsic factors include reagent dilution, detection system and chromogen, the method of antigen retrieval, retrieval solution pH, and molarity (Leong 2004, Taylor and Levenson 2006). Selection of appropriate antibodies is also one of the most important steps of immunohistochemical diagnostic process. Inappropriate antibodies may detect non-specific signals from cross-reacting proteins, such as if similar epitopes of different antigens are present in the sample (Mulisch 2010). Some of the antibodies used in this study were not tested on bovine tissues. Therefore, a positive control tissue staining and blocking of these antibodies with corresponding peptides were required. Furthermore, the protein sequences of these antibodies were compared with the bovine protein sequences. The antibodies which show the highest similarity to bovine sequences were chosen and used in immunohistochemical detection. It has been shown that, the antibodies produced for use in human tissues for immunohistochemical detection might also be applied in tissues of other species (Ruiz et al. 2005). In addition, using these antibodies with negative control sections and the absence of positive signals in these sections is also an indicator of specifity.

Another complication lies in the interpretative aspects and the subjective characteristics of immunohistochemistry. Interpretation and cut-off values are variables that greatly influence results (Leong et al. 2010). The lack of well defined standards of what constitutes a positive result and whether there are grades or degrees of positivity are some of the problems

encountered in this area. Zlobec et al. (2006) examined the intraobserver reliability among four pathologists of immunohistological scores and they found a poor agreement between the observers concerning the scoring of different factors. Such findings attest to the poor reproducibility when immunohistochemistry is used in a quantitative manner. Despite these shortfalls in the formulation of histological diagnoses, there is still a good reproducibility between observers for the recognition of many common diagnostic entities (Leong 2010). It has been suggested that objectivity can be attained by using image analysis methods. Tadrous (2010) proposed that such automated analytical methods are useful for repetitive processes such as counting, but repeatability and automaticity must not be confused with objectivity. However a lack of objectivity does not imply a lack of utility. Another important aspect of image analysis is that, it is achieved through the subjective selection and separation of stainings from the background (Leong et al. 2010). Even though enormous innovations in the capabilities of image analysis systems have been made, the evaluation of immunohistochemical methods can not produce accurate results if the background stain suffers from non-specific binding. As the purpose of this study is a quantitative comparison of the substances mentioned above, a visual control of the immunoreactive staining in the abomasal wall was required.

In this study, the positive immunoreactive areas of gastrin peptide and motilin receptors are encircled with the help of the AnalySIS® software program. The immunoreactive area of these substances is measured in five visual fields chosen at random in a section for each sample, as it is performed by Sickinger (2007). The positive cell staining of caspase-3 is counted and compared to the whole amount of cells from five randomly taken image areas as performed by Bodie (2006) whereas the mean number of VIP1 receptor staining is counted in a total visual area of approximately 0.28 mm^2 as carried out by Calingasan et al. (1984).

6.2 Discussion of immunohistochemical staining results

This study examines the role of the peptides gastrin, motilin, their receptors and VIP receptors as potential causes of abomasal motility impairment. The abundance of each of these peptides and their receptors was measured using the immunohistochemical ABC method. Following Sickinger's research, which has shown evidence of different neuropeptide contents (Substance P, VIP) as well as neurofilament 200 in the abomasal wall of German Holstein (GH) and German Fleckvieh (GFV) cows, an inter-breed comparison was made between the breeds German Holstein and German Fleckvieh; and an intra-breed comparison was made between healthy German Holstein cows and German Holstein cows with abomasal displacement (AD).

The role of these examined peptides and receptors in the gastrointestinal motility of ruminant species has been studied by various authors (Huang et al. 1999, Mcleay and Wong 1989, Reid et al. 1988). Motilin and its receptors are shown to be stimulating factors of gastrointestinal motility, whereas VIP, gastrin and their receptors play an inhibiting role. A hypo- or atony of the abomasum prior to the abomasal impaction and displacement is hypothesized to be the cause of the abomasal displacement. Therefore, it is analyzed if a low abundance of motilin and motilin receptors as gastrointestinal motility stimulating factors and/or a high abundance of VIP receptors, gastrin and gastrin receptors as gastrointestinal motility inhibiting factors, could be a cause of this motility impairment of the abomasum prior to the abomasal displacement.

Up to the present, immunohistochemical studies have largely been used to show the presence, distribution, and co-localization of the peptides in gastrointestinal tissues of non-ruminant species, however the number of the immunohistochemical studies in ruminants are limited (Balemba et al. 1999, Baltazar et al. 1998, Calingasan et al. 1984, Guilloteau et al. 1997, Kitamura et al. 1985, Myojin et al. 2000, Pfannkuche 2002a, 2002b, 2003, Sickinger 2007, Soehartono et al. 2002). Recently, researchers are focusing on the quantitative comparison of diverse gastrointestinal hormones and neuropeptides regarding inter- and intra-breed abundance of these substances (Geishauser et al. 1998, Ontsouka et al. 2007b, Sickinger 2007). Sickinger et al. (2008) published a breed comparison study for the first time and found that German Fleckvieh cows have significant higher amounts of substance P neuropeptide with lower amounts of VIP in the abomasal wall than German Holstein cows. Geishauser et al. (1998) and Ontsouka et al. (2007b) performed a quantitative comparison of gastrointestinal

motility-mediating substances and receptors between healthy cows and cows with AD and found that the cows with abomasal displacement exhibit lower amounts of motility stimulating substances accompanied by higher amounts of motility inhibiting substances.

The first aim of this study is to find out if the examined substances could play a role in the breed predisposition regarding the occurrence of AD between German Holstein and German Fleckvieh breeds, and to examine the difference in the abundance of these substances in healthy GH cows and GH cows with AD. For this purpose, gastrin, gastrin/CCKB receptor, motilin, motilin receptor, VIP receptors and caspase-3 activity are analyzed in the abomasal wall of these two breeds in order to examine if these substances could be a factor in the occurrence of AD.

Based on the results of this study, the role of gastrin in the occurrence of AD seems to be questionable. There are very few studies which examine the amounts of gastrin peptide between healthy GH cows and GH cows with AD. An inter-breed comparison has not yet been published. Some studies examined serum gastrin levels between healthy cows and cows with AD. Sen et al. (2002) found significantly higher levels of gastrin in cows with AD than in healthy cows, whereas Vlaminck (1986) found no significant difference between these two groups. In this study, no significant differences are found between healthy cows and cows with AD regarding the amounts of gastrin. This result might indicate that gastrin is not secreted from antral glands significantly more or less in cows with AD and seems not to be involved in the regulation of abomasal motility. Another explanation for the insignificant results could be that the variation of the results varies among the individuals greatly. This finding supports the results from Vlaminck (1986). Furthermore, concerning the breed comparison, no significant differences are found between healthy GH and GFV cows. According to this result, it can be suggested that gastrin does not play a role in this breed predisposition.

Furthermore, there were no gastrin/CCKB receptors found on the tunica muscularis of the abomasal wall using the immunohistochemical ABC method. The published work reviewed in this study shows no record of cases examining the immunohistochemical expression of gastrin/CCKB receptor in the abomasal wall of cattle. However, Yonekura et al. (2002) showed mRNA expression of gastrin/CCKB receptors in young calves and to a very slight extent also in adult cows (1.5- 2 years), suggesting that the age and weaning could have an

effect on the occurence of these receptors in cows. On the other hand, a very slight amount of this receptor was detected in the abomasal wall only after employing 60 cyclic amplitudes of PCR. Moreover, the samples taken from the abomasal wall are full thick specimens in Yonekura's study which also includes tunica serosa of the abomasal wall. It is possible that they have detected these receptors on the tunica serosa of the abomasum but not on the tunica muscularis. Another possibility is that the immunohistochemical ABC method is not able to detect very small amounts of this receptor in the abomasal wall. Nevertheless, the absence of gastrin receptors in the smooth muscle of the abomasum suggests that gastrin does not exert its motility effect directly by reacting with its receptors on the smooth muscle cells of the abomasal wall or it has no effect on the abomasal motility. This hypothesis is supported by the gastrin polypeptide comparison results of this study, which show no significant differences in the amounts of this polypeptide either in inter- or intra-breed comparison.

Motilin peptide immunoreactivity is not detected in any layer of the abomasal wall both in healthy cows and cows with AD. This finding is compatible with other immunohistochemical studies which were performed in bovine gastrointestinal tract (Kitamura et al. 1985, Calingasan et al. 1984). Duodenal biopsy samples from German Holstein cows after slaughter were used as positive control tissues for motilin peptide immunoreactivity in this study. Motilin immunoreactivity is detected in the duodenal mucosa, but not in the tunica muscularis of the duodenum. This result might indicate that the motilin peptide is secreted from duodenal glands of the bovine gastrointestinal tract and is transported through the blood circulation to its receptors in the abomasal wall, where it binds to its receptors and exerts its motility stimulating effects (Walsh 1994).

The motility inhibitory effect of VIP in the gastrointestinal tract of ruminants is already known (Reid et al. 1988). This neuropeptide reacts with both VPAC1 and VPAC2 receptors with similar affinities (Laburthe and Couvineau 2002, Schulz et al. 2004). The literature shows that the VPAC2 receptors are found to be present in the smooth muscle of the stomach of many different species (Harmar et al. 2004, Murthy et al. 1997, Schulz et al. 2004) and it is made responsible for the direct inhibitory effects of VIP in the smooth muscle. However, in this study, these receptors could not be detected in any layer of the abomasal wall using immunohistochemical ABC method and interestingly, VPAC1 receptors are detected in small amounts in the smooth muscle of abomasum. This result suggests a possible species difference between bovine and other species regarding the existence of VIP receptor subtypes

in the abomasum of cows and the stomach of human, mouse, and rabbit. Such a species difference was also observed between human and mouse lung tissues concerning the expression of VIP receptors. Mouse lung expresses predominantly VPAC2 receptors whereas human lung expresses predominantly VPAC1 receptors (Busto et al. 2000, Harmar et al. 2004, Reubi et al. 2000). Moreover, VPAC1 receptors have also been shown on the smooth muscle of different tissues such as cerebral arteries and arterioles, pulmonary artery and aortic smooth muscle of rat (Fahrenkrug et al. 2000, Hilaire et al. 2010).

It has already been shown that these VPAC1 receptors are widely distributed in human carcinoma cells which suggests that these receptors can upregulate themselves in certain circumstances (Schulz et al. 2004). The antiapoptotic action of VIP has also been shown in different species and this action is mediated by VPAC1 receptors (Delgado et al. 2003, Guitterez-Canas et al. 2003). As the apoptotic cell number of the abomasum increases with the abomasal displacement, the number of these receptors could also increase in order to exert its antiapoptotic actions. This could also be an explanation for the increase in VPAC1 receptors in this study. Sickinger (2007) found that the abundance of VIP neuropeptide in cows with abomasal displacement is lower than in the healthy cows. An overexpansion of the abomasal wall could cause impairment on the neuronal structures which could induce a disturbance of the transportation of VIP to its receptors on the cell walls. In order to make the cells more sensitive to VIP, these receptors may increase their numbers. Valette et al. (1995) examined receptor densities in dog hearts after surgical denervation and found an up-regulation of β-adrenergic receptors following heart denervation.

Studies on the gastrointestinal disturbances of ruminants also suggested the involvement of other neuropeptides and receptors such as adrenergic and muscarinic receptors in the gastrointestinal motility (Meylan et al. 2004, Ontsouka et al. 2010, Stoffel et al. 2006).
Ontsouka et al. (2007) found a significantly higher abundance of β-2 adrenergic receptors, which cause smooth muscle relaxation, in the duodenum of cows with abomasal displacement compared to healthy cows. This result is in accordance with the VPAC1 results of this study and suggests an increase of the receptors which mediate impairment of gastrointestinal motility in cows with abomasal displacement. However, Ontsouka et al. (2010) also detected an increase of M2 receptors (not significantly), which mediate the cholinergic contractile signals of ACh, in the abomasal wall of cows with abomasal displacement in comparison to the healthy cows. This result is conflicting with the results of this study, since the number of

motility stimulating motilin receptors is significantly lower in cows with AD in our study, whereas the number of motility inhibiting VPAC1 receptors is higher.

Considering the inter-breed comparison results of this study, the tendency that German Fleckvieh cows have more motilin receptors in the abomasal wall than German Holstein cows could explain why the incidence of abomasal displacement is lower in these cows than in German Holstein cows. Motilin has a gastrointestinal motility stimulating effect and the rareness of its receptors in German Holstein cows could be a cause of the decrement in the effectiveness of this polypeptide to exert its stimulating effect properly and directly on the smooth muscle of the abomasum. VPAC1 receptors show no significant difference between both breeds which suggests that stimulatory factors play a major role in this breed difference according to the results of this study. These results conform to the results from Sickinger (2007), who found a significantly higher motility stimulating neuropeptide SP and a lower amount of inhibiting neuropeptide VIP in German Fleckvieh cows, compared to German Holstein cows. Between these breeds, no difference is detected in caspase activity which shows that there is no difference in impairment levels in the healthy abomasi of these breeds. However, both breeds have a marked significantly higher caspase activity in their corpus abomasi than in antrum, which is accompanied by significantly higher abundance of motilin receptors in antrum of both GH and GFV cows compared to the corpus abomasi. This result indicates a correlation between the caspase activity and motilin receptors. If the impairment of the abomasal wall increases, which is detected by caspase-3 antibody, the abundance of motilin receptors decreases in the abomasal wall of cows.

Regarding the intra-breed comparison, German Holstein cows with abomasal displacement show a significant decrease in motilin receptors compared to the healthy cows and this result is accompanied by a significant increase in VPAC1 receptors in GH cows with AD. This result coincides with the results from Ontsouka's study who found significant lower motilin receptor densities in the duodenum of abomasal displaced cows compared to healthy cows (Ontsouka et al. 2007b). A decrease in motilin receptors accompanied by an increase in VIP receptors could be a cause of the abomasal motility impairment and ensuing abomasal displacement. It is reasonable to hypothesize that, if there are not enough motilin receptors for motilin peptides to bind on in the abomasal wall, the motility activating effect of motilin could be impaired. However, Wittek et al. (2008) could induce a higher abomasal motility rate after abomasal surgery for 4 hours with preoperative (1 hour) administration of 10 mg/kg i.m.

erythromycin, which is a motilin receptor agonist. It is possible that the secretion of motilin polypeptides from the duodenal endocrine M cells could also decrease in accordance with motilin receptors in the abomasal wall and that the administration of 10 mg/kg erythromycin can still cover all the expressed motilin receptors and induce its stimulating effect. The motilin polypeptide secretion from the duodenal mucosa should be further examined.

Furthermore, a significant increase in caspase activity in cows with displaced abomasi could be detected. The already mentioned decrease in the amount of motilin receptors might be explained by the increased caspase activity of the abomasal wall in cows with AD. As mentioned before, a hypoxia or mechanical stretching of the abomasal wall can trigger apoptosis cascades (Rowe et al. 2003). It has already been shown that the oxygen saturation of the abomasal wall is lower in cows with abomasal displacement than healthy cows (Wittek and Fürll 2003). It is probable that this hypoxia in the displaced abomasal wall is a result of the compression of the blood vessels due to the distention of the abomasal wall and the torsion of the abomasum itself.

High numbers of apoptotic cells in the displaced abomasum may also be a result of ischemic cell injury and subsequent inflammation. Rowe et al. (2003) found in horses with small intestine or large colon strangulation, obstruction or distention higher numbers of apoptotic cells than healthy horses. The distention of the colon in horses can be compared with the abomasal displacement of cows, in which the abomasum also distends. In vitro studies on rat lungs also showed that, distention of the rat lung (30% increase in the membrane surface area) leads to a significant increase in the percentage of apoptotic and necrotic cells (Hammerschmidt et al. 2004, Raaz et al. 2010). Therefore, it can be assumed that the distension of the abomasum leads to an increase in caspase activity, which in turn could influence the cells stability and the abundance of receptors in the abomasal wall.

Another aim of this study is to clarify, if the observed differences regarding the abundance of these gastrin and motilin peptides, their receptors and VIP receptors are a cause or a consequence of abomasal displacement. The evaluation of hitherto existing results suggests that the changes in the amounts of the examined substances are a consequence of the abomasal displacement rather than being a cause of it. Although the occurence of the examined substances show no significant differences before and after placing a gastric band, the tendency of the amounts of gastrin, motilin receptors, VIP receptors, and caspase-3

changed in the same manner as seen between the healthy cows and cows with AD. This indicates that, a decrease or an increase in the amounts of these substances could be caused artificially by placing a gastric band. Therefore, these changes in the amounts of these substances appear to be a consequence of abomasal displacement.

The low number of cows with experimentally induced abomasal impaction and the variation of the results among the cows of this group could be a cause for the insignificant results. In addition, if the existence of the gastric band and the passage disturbance in the abomasum would have been prolonged, this could potentially cause marked differences in the existing results as the gastric band induces a mechanical stress and pressure on the abomasal wall. Therefore, it could be concluded that these changes in the abundance of these substances are a consequence of AD.

It is known that after the abdominal surgery and mechanical stimulation of the gastrointestinal system, a panenteric atony takes place which is called a postoperative ileus (Bauer 2010). The involvement of different receptors, peptides and immune components were made responsible for causing this disorder. De Winter et al. (1998) suggested the involvement of VPAC1 receptors, NO and VIP in the pathogenesis of postoperative ileus in rats. For that reason, it is possible that the changes in the abundance of examined substances in this study could be a result of the abdominal surgery itself. To clarify this effect, further investigations will be necessary.

In this study, the sampling technique (slaughter vs. OP) does not have any influence on the amounts of apoptotic cells, gastrin peptide and VPAC1 receptor, whereas the amounts of motilin receptors are significantly lower in the antral wall of the abomasum in cows operated to place a gastric band. The same tendency could also be seen in the corpus but this result is not significant. The comparison of cows with AD and cows with experimentally induced abomasal impaction shows that there is a significant effect of the gastric band on the amounts of motilin receptors. This result is in accordance with the results from studies which show that the use of erythromycin in human patients with postoperative ileus does not resolve post operative ileus in these patients (Bonacini et al. 1993, Smith et al. 2000).

This finding suggests that motilin receptors might be more sensitive to mechanical stress factors such as pressure, hypoxic or ischemic stress. The presence of a gastric band could probably cause a higher mechanical pressure and hypoxia than the torsion of abomasum itself. It should also be considered that these results come only from 3 cows. Compared to the healthy cows, a significant decrease in the amount of motilin receptors and an increase in the amount of VIP receptors in cows with abomasal displacement is most probably a consequence of the abomasal displacement itself.

6.3 Conclusions

The results from recent studies indicate that the neurotransmitters and gastrointestinal peptides could play a role in the occurrence of AD. Varying densities of motilin receptors, muscarinic, and adrenergic receptors were found in the abomasal and duodenal wall of cows with AD in comparison to healthy cows (Ontsouka et al. 2007, 2010). Sickinger (2007) performed an inter- and intra-breed analysis regarding substance P and vasoactive intestinal polypeptide densities in the abomasal wall of German Holstein and German Fleckvieh cows. This study showed for the first time that the stimulating factor substance P is lower in GH cows than in GFV cows.

The lower amounts of motilin receptors in the abomasal wall of GH cows found in this study could be a factor why GH cows are more prone to AD than GFV cows. However, the difference between these two breeds is not significant. Other gastrointestinal neuropeptides or hormone disturbances could also play a role in this disorder. This result coincides with the results from Sickinger (2007), who suggested that a lower amount of the motility stimulating factor SP in GH cows might be responsible for the more frequently occurring AD in this breed, compared to GFV cows. Assumably, stimulating factors play a more important role in this inter-breed difference between GH and GFV cows, compared to inhibiting factors.

Concerning the intra-breed comparison, the abomasal wall of GH cows with AD has a significantly lower density of motilin receptors, accompanied by an increased number of VPAC1 receptors, compared to healthy GH cows. This could be the cause of the predisposition for the occurrence of AD. Furthermore, a significant increase in apoptotic cells is found in the abomasal wall of GH cows with AD in comparison to healthy GH cows. This result could explain the decrease in the amount of motilin receptors in cows with AD. It is also possible that, an increase in apoptotic cells in the abomasal wall of cows with AD not only affects the amount of motilin receptors, but also hormones, neuropeptides, cell walls, and neuronal structures, which suggests the involvement of other possible factors and substances in the occurrence of AD.

The findings of this study indicate that the differences in the amounts of motility inhibiting and stimulating factors are a consequence of the abomasal displacement itself, rather than being a cause of it.

7 Summary

Objective

The aim of this study was to examine the possible effects of gastrin, motilin peptides, their receptors and vasoactive intestinal polypeptide (VIP) receptors on the occurrence of abomasal displacement (AD). A decreased amount of stimulating factors (motilin, motilin receptors) accompanied by an increased amount of inhibiting factors (gastrin, gastrin receptors, VIP receptors) in the abomasal wall could be a cause of the hypo- or atony of the abomasum prior to the abomasal displacement. The caspase-3 activity of the abomasal wall was also examined as apoptosis marker in order to investigate the degree of impairment of the abomasal wall between healthy cows and cows with AD.

Material and methods

Biopsies of the corpus abomasi and antrum pylori were available from 20 slaughtered German Holstein cows and from 20 German Fleckvieh cows. Furthermore, material from 20 other German Holstein cows with abomasal displacement as well as biopsies from another three cows of this breed were available. In the latter, an abomasal impaction was experimentally induced by placing a variable gastric band. The paraffin embedded biopsy samples were cut 6µm thick with a microtome for the immunohistochemical investigations. The positive signals were quantified by measuring the stained areas using semiautomatic software program AnalySIS® (Olympus). In this study gastrin and motilin peptides, their receptors, VIP receptors and caspase-3 were stained immunohistochemically (ABC-/AEC-technique).

Results

Gastrin was detected only in the tunica mucosa of the antrum. No significant breed difference was found between German Holstein (GH) and German Fleckvieh (GFV) regarding the immunoreactive (IR) area of this gastrointestinal peptide (GFV: 1174 ± 655 µm², GH: 1105 ± 930 µm²). The comparison between GH with abomasal displacement (AD) and without AD, GH cows with AD revealed a larger IR gastrin area (GH with AD: 1277 + 863 µm²). However, this difference was not significant.

Gastrin receptors (CCKBR) were detected neither in GH nor in GFV cows in the tunica muscularis of the abomasal wall.

Motilin also could not be detected in any layer of the abomasal wall of both breeds contrary to the motilin receptors. In both localizations of the abomasal wall of GFV cows, the IR area for these receptors tended to be larger than in GH cows without AD (antrum: 206 ± 57 µm² vs. 179 ± 53 µm²; corpus: 172 ± 69 vs. 152 ± 52 µm²). No significant differences were found in this regard ($p = 0.11$).

The intra-breed comparison observed significant larger motilin receptor IR areas in GH cows without AD compared to GH cows with AD in both localizations (antrum: 125 ± 55 µm²; corpus: 124 ± 43 µm²; $p = 0.003$). In GH cows with experimentally induced abomasal impaction, a decrease of the IR area of motilin receptors was detected after placing the gastric band for 72 hours (antrum, 1st OP: 95 ± 46 µm², 2nd OP: 47 ± 8 µm²; corpus, 1st OP: 101 ± 19 µm²; 2nd OP: 80 ± 28 µm²), however this decrease was not significant ($p = 0.27$).

Regarding the number of VPAC1 receptor in the abomasal wall, no inter-breed difference could be detected (antrum: GFV 19 ± 10, GH 18 ± 6; corpus: GFV 19 ± 10, GH 18 ± 8). However, GH cows with AD have a significant higher number of these receptors compared to the healthy cows of this breed (GH with AD antrum: 24 ± 11, corpus: 26 ± 11; $p = 0.005$). It is also observed that, experimental induction of an abomasal impaction has also increased the number of VPAC1 receptors (antrum, 1st OP: 13 ± 5, 2nd OP: 15 ± 4; corpus, 1st OP: 16 ± 6, 2nd OP: 20 ± 10), however this difference was not significant.

Caspase activity, as a criterion for the impairment of the abomasal wall, was significantly higher in GH cows with abomasal displacement compared to healthy GH cows (GH cows with AD antrum: 26 ± 11%, corpus: 29 ± 9%, GH cows without AD antrum: 17 ± 6%, corpus: 21 ± 7%, $p = 0.002$). The same tendency was also observed in GH cows with experimentally induced AD, however this difference was not significant ($p = 0.12$). Also no significant difference was detected in the ratio of apoptotic cells between both breeds.

Conclusions

The motilin receptor seems to have an important role in the abomasal motility. Motilin receptors are detected in the tunica muscularis of abomasal wall of all subjects. The tendency that GFV cows have higher amounts of motilin receptors in their abomasal wall, might explain why the cows of this breed are less susceptible to AD. The reduced amounts of motilin receptors in the GH cows with AD and experimentally induced abomasal impaction, in comparison to the healthy GH cows, indicates that AD could cause mechanical destruction of these receptors due to the mechanical stretching or hypoxia of the abomasal wall, to which the increase of the caspase activity could provide evidence.

Gastrin seems not to be involved in the occurrence of AD. The absence of the gastrin receptors in the abomasal wall found in this study supports this finding.

A lower amount of motilin receptors and a higher amount of VPAC1 receptors in GH cows with AD, compared to healthy GH cows, could be an explanation for the occurrence of AD in these cows. However, according to the results gathered from the subjects with artificially induced abomasal impaction indicates that these changes were likely a consequence of AD itself, rather than being a cause of it.

8 Zusammenfassung

Zielsetzung der Studie

Das Ziel dieser Studie war es, die Bedeutung von Gastrin und Motilin, sowie deren Rezeptoren und Vasoaktives-Intestinales-Polypeptid Rezeptoren beim Auftreten von Labmagenverlagerungen (LMV) zu untersuchen. Eine reduzierte Zahl stimulierender Faktoren und eine erhöhte Zahl inhibitorischer Faktoren in der Labmagenwand könnte eine Ursache für die Hypo- oder Atonie des Labmagens vor einer LMV sein. Caspase 3 -als Apoptose-Marker- wurde zur Einschätzung des Schädigungsgrades der Labmagenwand bestimmt und zwischen den gesunden Kühen und solchen mit LMV verglichen.

Material und Methode

Zur Untersuchung standen Gewebeproben von jeweils 20 gesunden Kühen der Rassen Deutsches Fleckvieh und Deutsche Holsteins zur Verfügung. Ebenso konnte auf Material von 20 Deutschen Holstein-Kühen mit Labmagenverlagerung sowie auf Gewebeproben von drei weiteren Kühen dieser Rasse zurückgegriffen werden, bei denen experimentell – durch Anlegen eines Magenbandes - eine Labmagendilatation induziert wurde. Die in Paraffin eingebetteten Gewebeproben wurden für die immunhistologischen Untersuchungen in 6 µm dicke Schnitte aufgearbeitet. Die dargestellten Peptide wurden quantifiziert, indem die angefärbten Flächen und Zellen unter Verwendung des halbautomatischen Softwareprogramms AnalySIS® (Olympus) ausgemessen wurden. Berücksichtigt wurden in dieser Studie die Peptide Gastrin und Motilin und deren Rezeptoren sowie zusätzlich der Vasoaktives Intestinales Polypeptid Rezeptor-1 (VIPR1) und die Caspase Aktivität. Für die immunhistochemischen Untersuchungen kam ein indirektes Verfahren mit der ABC-/ AEC-Methode zur Anwendung.

Ergebnisse

Gastrin konnte nur in der Mukosa des Antrumbereichs dargestellt werden. Bezüglich der immunreaktiven Fläche für diesen Neurotransmitter ergab sich kein signifikanter Unterschied zwischen den Rassen Deutsches Fleckvieh (DFV) und Deutsche Holsteins (DH) (DFV: 1174 ± 655 µm², DH: 1105 ± 930 µm²). Im Vergleich zwischen Deutschen Holsteins mit Labmagenverlagerung (LMV) und solchen ohne diese Erkrankung hatten erstere eine größere IR-Gastrin-Fläche (DH mit LMV 1277 ± 863 µm²). Dieser Unterschied war allerdings nicht signifikant.

Gastrin-Rezeptoren (CCKBR) konnten weder beim DFV noch bei den DH in der glatten Muskulatur der Labmagenwand nachgewiesen werden.

Motilin konnte bei keiner Kuh in irgendeinem Bereich des Labmagens dargestellt werden – im Gegensatz zu den Motilin-Rezeptoren. Hierbei erwies sich die IR-Fläche für diese Rezeptoren bei den DFV-Kühen in beiden Lokalisationen des Labmagens tendenziell größer als bei den Deutschen Holsteins ohne LMV (Antrum: 206 ± 57 µm² vs. 179 ± 53 µm²; Corpus: 172 ± 69 vs. 152 ± 52 µm²); signifikante Unterschiede konnten diesbezüglich jedoch nicht nachgewiesen werden ($p = 0,11$).

Innerhalb der Rasse Deutsche Holsteins hatten Kühe ohne LMV an beiden Lokalisationen eine signifikant größere IR-Fläche an Motilin-Rezeptoren, als dies bei den Tieren mit LMV der Fall war (Antrum: 125 ± 55 µm²; Corpus: 124 ± 43 µm²; $p = 0,003$). Bei den drei DH-Versuchskühen mit experimentell induzierter Labmagendilatation kam es in Zusammenhang mit der Dehnung der Labmagenwand innerhalb von 72 Stunden zu einer Abnahme der IR-Flächen für die Motilin-Rezeptoren (Antrum, 1. OP: 95 ± 46 µm², 2. OP: 47 ± 8 µm²; Corpus, 1. OP: 101 ± 19 µm²; 2.OP: 80 ± 28 µm²). Allerdings war dieser Unterschied nicht signifikant ($p = 0,27$).

Bezüglich der Anzahl der VIP1-Rezeptoren in der Labmagenwand ergaben sich keine Rassenunterschiede (Antrum: DFV 19 ± 10, DH 18 ± 6; Corpus: DFV 19 ± 10, DH 18 ± 8). Allerdings lag bei den DH-Kühen mit LMV deren Anzahl – im Vergleich zu den gesunden Probanden dieser Rasse, signifikant höher (DH mit LMV Antrum: 24 ± 11 Corpus: 26 ± 11; $p = 0,005$).

Es war auch festzustellen, dass die experimentelle Induktion einer Labmagenanschoppung mit einem Anstieg der Anzahl an VIP-Rezeptoren verbunden war (Antrum, 1. OP: 13 ± 5, 2. OP: 15 ± 4, Corpus, 1. OP: 16 ± 6, 2. OP: 20 ± 10). Allerdings war dieser Unterschied nicht signifikant.

Die Caspase-Aktivität, als Kriterium einer Schädigung der Labmagenwand, war bei den Kühen mit Labmagenverlagerung - im Vergleich zu den gesunden Kontrollkühen – signifikant erhöht (DH ohne LMV Antrum: 17 ± 6 %, Corpus: 21 ± 7 %; DH mit LMV Antrum: 26 ± 11 %, Corpus: 29 ± 9 %; $p = 0,002$). Bezüglich des Rassenvergleiches ergab sich kein signifikanter Unterschied zwischen den Rassen Deutsches Fleckvieh und Deutsche Holsteins.

Schlussfolgerungen

Dem Motilin-Rezeptor scheint bei der Motilität des Labmagens eine wichtige Bedeutung zuzukommen. So konnten auch in diesen Untersuchungen in allen Proben Motilin-Rezeptoren in der Labmagenwand nachgewiesen werden. Die tendenziell größere Dichte an Motilin-Rezeptoren bei den DFV-Kühen könnte erklären, warum Kühe dieser Rasse weniger häufig an Labmagenverlagerung erkranken. Die bei den Holstein-Kühen mit Labmagenverlagerung bzw. mit induzierter Labmagenanschoppung – im Vergleich zu den gesunden Probanden dieser Rasse - nochmals reduzierte Zahl an Motilin-Rezeptoren dürfte auf eine mechanische Zerstörung dieser Rezeptoren im Zuge des Krankheitsgeschehens zurückzuführen sein, wozu die Erhöhung der Caspase-Aktivität einen Hinweis liefern könnte.

Gastrin scheint keinen direkten Effekt auf die Labmagenverlagerung zu haben. Die Abwesenheit der Gastrin-Rezeptoren in der Labmagenwand ist im Einklang mit diesem Ergebnis.

Eine reduzierte Zahl an Motilin-Rezeptoren und erhöhte Zahl an VIP1-Rezeptoren bei den Holstein-Kühen mit LMV - im Vergleich zu den gesunden Probanden dieser Rasse könnte eine Erklärung für das Auftreten einer Labmagenverlagerung liefern. Die Ergebnisse der Untersuchungen an Probanden mit einer expeimentell induzierten Labmagenanschoppung deuten jedoch darauf hin, dass es sich bei den ermittelten Unterschieden zwischen gesunden Holstein-Kühen und solchen mit einer Labmagenverlagerung eher um eine Folge der Verlagerung als um deren Ursache handelt.

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Appendices

Used chemicals and tools

- Vector Vectastatin ABC-Elite-Kit PK-6100 Standard, Vector Laboratories USA
- Peroxidase substrate AEC-Kit, product no. AE002, Biologo Dr. H. Schultheiß e. K., Kronshagen
- Methanol for synthesis, 2.5 l, product no. 8.222283.2500, Merck Schuchardt OHG, Hohenbrunn
- PBS tablets, product no. P4417-50/100TAB, Sigma Aldrich Chemie GmbH, Taufkirchen
- Superfrost Plus microscopic slides, product no. 6776214, Thermo Fisher Scientific, Steingrund
- Goat serum, product no. C-37651, Promocell GmbH, Heidelberg
- Rabbit serum, product no. X090210-8, Dako Deutschland GmbH, Hamburg
- Horse serum, product no. BA-2000, VectorLaboratories Inc., Burlingame, USA
- Bovine serum albumin, albumin fraction V, product no. 8076.2, Carl Roth GmbH & Co. KG, Karlsruhe
- Triton X-100 Detergent, product no. 648462, Calbiochem, EMD Biosciences, Merck, Darmstadt
- Cover slips, 24 x 24 mm, Roth, Karlsruhe
- Gelatine, product no. 1.09242.0100, Merck, Darmstadt
- Hematoxylin, product no. 6765015, Anatomical Pathology Pittsburgh, USA
- Aqua dest
- Xylol, product no. UN 1307, Stockmeier Chemie Dillenburg GmbH & CoKG, Dillenburg
- Ethanol, product no. UN 1170, Stockmeier Chemie Dillenburg GmbH & CoKG, Dillenburg
- Microtome, HM 400 Microm, Leica Instruments GmbH, Nussloch
- Magnetic stir/hot plate, Heidolph MR 3001, MAGV Laborbedarf, Rabenau-Londorf
- Wacom intuos® 3 graphics tablet, Wacom Europe GmbH, Krefeld
- AnalySIS Pro 3.2 software; Soft Imaging System GmbH; Olympus Soft Imaging Solutions GmbH, Münster

Formulation for solutions and buffers

Citrat buffer:

Solution A: 0,1 m citric acid

4,2 g $C_6H_8O_7$ x H_20 in 200 ml aqua dest.

Solution B: 0.1 m sodium citrate

29,41 g $C_6H_5O_7Na_3$ x 2 H_2O

Usage: 7 ml solution A + 41 ml solution B + 452 ml aqua dest.

After mixing aqua dest and solution B, the pH of the buffer will be adjested to 6.0 using solution A

Blocking buffer:

21 µl triton X

1g BSA

Dissolve in 70 ml PBS

0,3 % H_2O_2 in icecold methanol:

0,5 ml 30 % H_2O_2

49,5 ml methanol (icecold)

PBS solution

1 Sigma Aldrich P 4417 PBS tablet + 200 ml aqua dest.

Table 9: Data for statistical analysis

1= with OP GH													
2= without OP GH				1=Antrum				2=Corpus					
3= without OP GFV													
							Localisation						
Groups		Animal No											
			Ga1	MR1	VIPR1	Casp1	Ga2	MR2	VIPR2	Casp2			
1		1	2573,18422	117,039914	43	9,17		157,773999	36	7,5			
1		2	1276,14644	171,809109	28	12,41		154,305552	33	15,2			
1		3	1598,47001	119,338769	29	15,07		106,473251	41	17,41			
1		4	1586,57243	213,470802	13	15,15		199,35503	12	20,17			
1		5	1930,75528	43,5169082	14	19,27		202,863807	22	24,67			
1		6	1726,80254	60,2541806	20	20,08		58,3586341	24	26,16			
1		7	1042,8329	190,562921	13	20,56		181,085188	43	26,16			
1		8	556,282405	145,836089	12	20,72		126,557978	42	26,7			
1		9	199,153376	89,6956477	21	20,91		67,9170282	22	27,09			
1		10	56,382426	153,055298	37	22,73		94,5353409	21	28,04			
1		11	1933,41711	91,3895403	52	24,5		110,06269	29	30,71			
1		12	118,693476	174,591933	17	24,94		112,724521	16	31,94			
1		13	1187,91694	181,004526	20	26,4		104,255058	24	32,66			
1		14	1252,55294	133,252887	36	30,05		153,781252	47	36,38			
1		15	2174,23218	60,6574883	14	31,51		126,759632	23	36,93			
1		16	3013,27366	58,9635957	10	39,48		89,453663	8	37,45			
1		17	2018,23273	162,734684	16	39,53		161,242446	25	37,5			
1		18	509,861681	190,441928	17	41,23		119,137115	12	39,89			
1		19	148,538251	37,6286148	28	41,88		77,5157531	19	42,25			
1		20	636,50032	99,8590034	34	44,84		68,9656284	22	42,67			
2		3	924,66372	119,016122	24	6,86		192,61979	38	6,88			
2		4	719,339735	170,437863	25	7,79		105,787628	17	12,75			
2		12	1365,27746	129,865101	9	8,03		149,707844	12	14			
2		13	975,440168	144,989143	12	10,1		186,570174	22	14,83			
2		14	1619,0387	159,951861	14	11,42		150,393467	13	15,81			
2		18	1839,04309	187,618774	24	13,72		164,146262	15	16,4			
2		19	1810,4889	219,197772	17	13,78		257,068371	32	20,02			
2		20	2390,98574	279,859944	15	15,79		238,395222	31	20,57			
2		23	172,777048	135,108102	8	17,72		112,966506	20	21,51			
2		24	2720,47222	152,12769	14	17,82		94,4546793	20	21,81			
2		25	562,009375	145,271458	17	18,79		98,2457723	14	22,4			
2		27	1411,29487	148,417259	8	18,9		132,204286	11	22,4			
2		28	2831,34153	196,24956	21	18,98		120,831007	19	23,23			
2		29	521,476944	135,269426	20	19,59		131,518663	8	24,68			
2		31	200,484292	134,180494	29	20,02		189,554651	22	24,74			
2		32	1891,67833	195,442944	21	20,72		227,344589	18	25,1			
2		43	24,8034278	158,943591	25	23		75,4185527	13	28,47			
2		44	119,94373	285,985538	22	24,23		139,181511	11	30,37			
2		48	0	288,728031	8	25,02		180,480226	12	30,86			
2		50	0	191,127551	12	25,26		88,2437397	8	31,9			
3		6	1030,69333	189,99829	23	5,82		166,324124	35	8,74			
3		9	751,322041	315,346344	25	6,08		269,893559	26	9,47			
3		10	1514,461	120,064723	9	7,88		142,24665	9	14,41			
3		11	639,968767	199,113045	7	8,77		144,766803	7	15,05			
3		15	915,387642	195,805921	29	11,5		98,0037877	33	15,09			
3		17	1015,28698	272,353736	28	11,86		135,471079	28	15,2			
3		21	752,047995	265,457173	24	14,6		125,025408	29	15,7			
3		22	1704,94326	176,568141	6	14,95		139,947796	9	16,68			
3		26	915,21697	122,202254	5	16,44		187,780097	4	18,26			
3		33	2581,85534	201,532892	25	16,91		82,7990848	33	19,29			
3		34	1295,2229	214,96304	10	18,63		256,745725	27	20,84			
3		37	2142,04822	287,155131	6	20,91		148,175274	28	21,4			
3		38	782,497732	202,581492	30	21,45		97,5601491	20	24,28			
3		39	1121,88122	255,737456	7	23,96		155,031506	3	24,86			
3		40	1704,70127	202,662154	27	24,1		112,11956	22	26,02			
3		41	971,205437	226,578304	26	24,97		349,829158	26	26,85			
3		42	1766,4007	134,140164	32	28,35		271,345466	8	27,49			
3		46	1860,49565	101,714219	29	28,43		179,512288	10	28,92			
3		52	25,6503741	216,132633	13	31,41		147,167005	20	34,05			
3		54	0,1	218,875126	19	32,2		222,948534	7	38,97			

Table 10: Data for statistical analysis including animals with gastric band operation

Data for statistical analysis				**1=Antrum**							
Gastric band				**2=Corpus**							
						1			2		
Groups											
1= Gastric band OP1				Ga1	MR1	VIPR1	Casp1	Ga2	MR2	VIPR2	Casp2
2= Gastric band OP2		1	MB1OP1	288,728031	124,823754	9	25,74		101,996535	13	12,39
3= GH with OP		1	MB2OP1	10,848979	42,1859926	12	20,77		81,3471769	22	28,94
4= GH without OP		1	MB3OP1	333,091886	118,693476	19	11,43		119,056453	12	12,45
		2	MB1OP2	245,735423	58,4796264	16	43,91		39,3628382	30	39,65
		2	MB2OP2	252,067355	43,0732697	12	27,14		101,028596	12	33,02
Abbreviations		2	MB3OP2	383,626349	39,2418459	18	23,16		99,9799958	17	24,1
Ga = Gastrin		3	OP1	2573,18422	117,039914	43	30,05		157,773999	36	17,41
MR = Motilin receptor		3	OP2	1276,14644	171,809109	28	20,91		154,305552	33	26,16
VIPR= Vip receptor 1		3	OP3	1598,47001	119,338769	29	20,08		106,473251	41	42,67
Casp = Caspase-3		3	OP4	1586,57243	213,470802	13	39,48		199,35503	12	20,17
MB = Gastric band		3	OP5	1930,75528	43,5169082	14	41,88		202,863807	22	27,09
		3	OP6	1726,80254	60,2541806	20	9,17		58,3586341	24	15,2
		3	OP7	1042,8329	190,562921	13	24,5		181,085188	43	30,71
		3	OP8	556,282405	145,836089	12	20,56		126,557978	42	24,67
		3	OP9	199,153376	89,6956477	21	26,4		67,9170282	22	28,04
		3	OP10	56,382426	153,055298	37	39,53		94,5353409	21	37,45
		3	OP11	1933,41711	91,3895403	52	15,15		110,06269	29	7,5
		3	OP12	118,693476	174,591933	17	22,73		112,724521	16	32,66
		3	OP13	1187,91694	181,004526	20	12,41		104,255058	24	36,38
		3	OP14	1252,55294	133,252887	36	44,84		153,781252	47	31,94
		3	OP15	2174,23218	60,6574883	14	15,07		126,759632	23	26,16
		3	OP16	3013,27366	58,9635957	10	24,94		89,453663	8	26,7
		3	OP17	2018,23273	162,734684	16	31,51		161,242446	25	36,93
		3	OP18	509,861681	190,441928	17	41,23		119,137115	12	42,25
		3	OP19	148,538251	37,6286148	28	19,27		77,5157531	19	39,89
		3	OP20	636,50032	99,8590034	34	20,72		68,9656284	22	37,5
		4	3	924,66372	119,016122	24	18,9		192,61979	38	14,83
		4	4	719,339735	170,437863	25	18,98		105,787628	17	16,4
		4	12	1365,27746	129,865101	9	24,23		149,707844	12	22,4
		4	13	975,440168	144,989143	12	6,87		186,570174	22	20,57
		4	14	1619,0387	159,951861	14	25,26		150,393467	13	15,81
		4	18	1839,04309	187,618774	24	17,82		164,146262	15	20,02
		4	19	1810,4889	219,197772	17	13,72		257,068371	32	21,51
		4	20	2390,98574	279,859944	15	8,03		238,395222	31	6,88
		4	23	172,777048	135,108102	8	23		112,966506	20	22,4
		4	24	2720,47222	152,12769	14	11,42		94,4546793	20	30,37
		4	25	562,009375	145,271458	17	20,02		98,2457723	14	24,68
		4	27	1411,29487	148,417259	8	13,78		132,204286	11	14
		4	28	2831,34153	196,24956	21	7,79		120,831007	19	23,23
		4	29	521,476944	135,269426	20	10,1		131,518663	8	21,81
		4	31	200,484292	134,180494	29	15,79		189,554651	22	12,75
		4	32	1891,67833	195,442944	21	25,02		227,344589	18	28,47
		4	43	24,8034278	158,943591	25	20,72		75,4185527	13	30,86
		4	44	119,94373	285,985538	22	19,59		139,181511	11	25,1
		4	48	0	288,728031	8	18,79		180,480226	12	31,9
		4	50	0	191,127551	12	17,72		88,2437397	8	24,74

Acknowledgement

I am very grateful to Prof. Dr. Doll for letting me perform this research study in the Clinic for Ruminants of the Justus Liebig University Giessen. This work would not have been possible without his support and guidance.

I would also like to thank Prof. Dr. Dr. Arnhold for all his help during the work up and immunohistochemical analysis of my samples.

Special thanks to Dr. Marlene Sickinger who supported me throughout the entire process of my doctorial work and was integral to the completion of my thesis. Thank you Marlene for always being available to my questions and the many hours you invested for the revisions of my paper.

I give my thanks to Dr. Failing for the statistical analysis of my results and for taking the time to explain these statistical findings to me.

I am most grateful to my family who always supported me and helped me to achieve all my aspirations. This doctorial thesis was only possible because of their hard work. *Anne, Baba bu doktora sizin sayenizde yazıldı.*

I would like to thank Paula and Zarah for the grammatical and linguistical revisions of this study. A special thanks to Paula for always being there for me.

Susanne (Lück) thanks a lot for being such a great office buddy. It was wonderful to work with you.

I would like to thank my colleagues, Ali Al-Bayati, Eva-Maria Spitzley, Marco Herr, Petrit Berisha, Uschi Schwarz, Lisa Wenzel, Verena Schmid, Filippo Fiore for the memorable times we shared.

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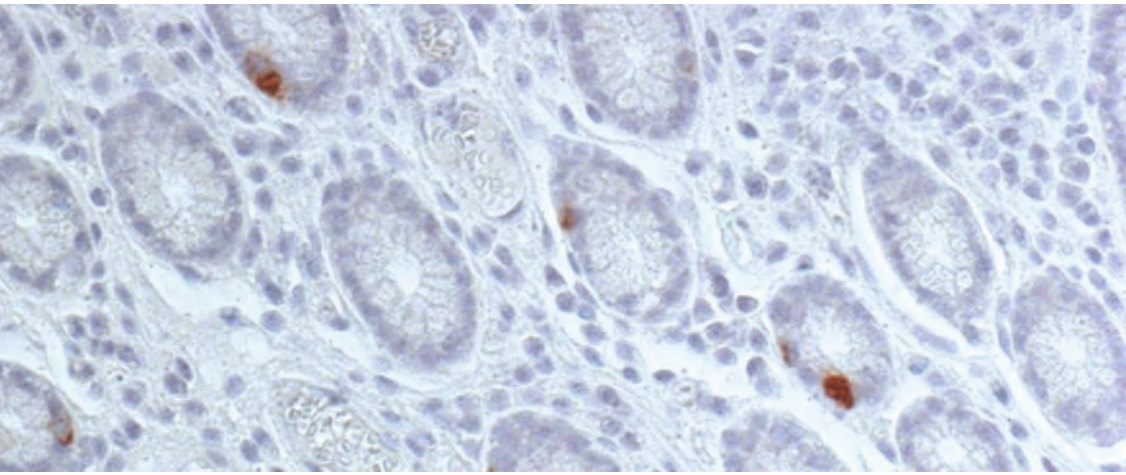

édition scientifique
VVB LAUFERSWEILER VERLAG

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STAUFENBERGRING 15
D-35396 GIESSEN

Tel: 0641-5599888 Fax: -5599890
redaktion@doktorverlag.de
www.doktorverlag.de

ISBN: 978-3-8359-5914-9