Gastrointestinal motility and the role of gonadotropin-releasing hormone (GnRH)

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Abstract

This thesis explores the relation between gonadotropin-releasing hormone (GnRH) and gastrointestinal symptoms and dysmotility.

In primary Sjögren's syndrome (pSS), a patient group with high levels of GnRH antibodies, associations between objective signs and symptoms of autonomic dysfunction (AD), impaired gastric emptying (IGE), and inflammatory and serological features were studied. Forty-three percent of pSS patients had objective signs of IGE, which was associated with increased levels of inflammatory markers and was more common in rheumatoid factor seropositive patients. No associations between IGE and objective and subjective AD variables or gastrointestinal symptoms were found.

Gastrointestinal complaints in different patient populations with high levels of GnRH antibodies were investigated using the visual analog scale for irritable bowel syndrome (VAS-IBS) questionnaire. It was found that the VAS-IBS questionnaire can be used to assess gastrointestinal symptoms in individual patients, but does not aid clinicians in differentiating between different motility disorders.

Patients with severe dysmotility, having had full-thickness biopsy, were investigated with the aim of describing expression of GnRH in the enteric nervous system (ENS) and antibodies against GnRH in serum. In a control group GnRH was present in about 50% of human myenteric neurons. A subgroup of patients with severe dysmotility expressed antibodies against GnRH and had reduced expression of GnRH-containing neurons in the ENS.

Also, luteinizing hormone (LH) receptors were found in the gastrointestinal tract, in patients both with and without severe dysmotility, possibly providing a mechanism through which GnRH might affect the gastrointestinal tract.

Gastrointestinal symptoms and presence of antibodies against GnRH and its receptor in serum in women before and after in vitro fertilization (IVF) treatment with buserelin was investigated. Buserelin treatment caused gastrointestinal symptoms during treatment, and the effect on symptoms in a five-year perspective varied; no severe dysmotility or production of antibodies against GnRH or its receptor was detected.

Enteric neurodegeneration and titers of GnRH antibodies in rat in response to repeated administration of the GnRH analog buserelin was studied. Repeated administrations of buserelin were accompanied by up to 50% loss of enteric neurons in rat. However buserelin-treated rats do not display increased titers of GnRH antibodies in serum, nor do they lose weight compared to saline-treated control rats.

Taken together, GnRH and LH receptors were expressed in about half of human enteric neurons. GnRH seems to affect gastrointestinal motility and function. Some patients with motility disorders express antibodies against GnRH in serum and display lower levels of the peptide in the bowel. Repeated treatment with the peptide in rat causes loss of myenteric neurons.

Key words
Gonadotropin releasing hormone, intestinal motility, luteinizing hormone, Sjögrens syndrome, in vitro fertilization

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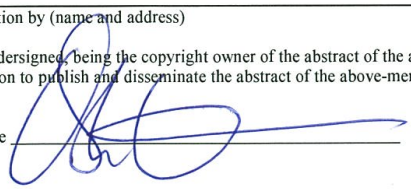
Signature 

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Elin Sand, Ulrike Voss, Oskar Hammar, Ragnar Alm, Gunilla Nordin Fredrikson, Bodil Ohlsson, and Eva Ekblad. Cell Tissue Res. 2012 Dec 20. [Epub ahead of print].

Abbreviations

ACh	Acetylcholine
AD	Autonomic dysfunction
AECC	American-European Classification Criteria
AI	Acceleration index
ANA	Antinuclear antibody
ANS	Autonomic nervous system
ASP	Autonomic Symptom Profile
BSA	Bovine serum albumin
CIPO	Chronic intestinal pseudo-obstruction
CMV	Cytomegalovirus
CNS	Central nervous system
ED	Enteric dysmotility
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
ESR	Erytrocyte sedimentation rate
FBD	Functional bowel disorder
FD	Functional dyspepsia
FSH	Follicle-stimulating hormone
GLP	Glucagon-like peptide
GnRH	Gonadotropin-releasing hormone
GnRH-R	Gonadotropin-releasing hormone receptor
hMG	Human menopausal gonadotropin
ICC	Interstitial cell of Cajal

IBS	Irritable bowel syndrome
IFN	Interferon
IGE	Impaired gastric emptying
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IR	Immunoreactive
IVF	In vitro fertilization
IQR	Interquartile range
KC/GRO	Keratinocyte-derived chemokine/growth-related oncogene
LH	Luteinizing hormone
MMC	Migrating motor complex
MRI	Magnetic resonance imaging
NO	Nitric oxide
PACAP	Pituitary adenylate cyclase-activating polypeptide
PCR	Polymerase chain reaction
PGP	Protein gene product
pSS	primary Sjögren's syndrome
RF	Rheumatoid factor
SF-36	36-item Short-Form questionnaire
TNF	Tumor necrosis factor
VAC	Vasoconstriction
VAS-IBS	Visual Analog Scale for Irritable Bowel Syndrome
VIP	Vasoactive intestinal peptide

Introduction

This thesis is based on the findings made in a patient who sought care at the Department of Medicine in Malmö, Sweden, about 10 years ago. She had just had her fourth in vitro fertilization (IVF). It was her experience that the treatment had a profound effect on her gut and that the last treatment resulted in symptoms of vomiting, abdominal pain, and constipation. Subsequent thorough multidisciplinary investigation revealed that she suffered from chronic intestinal pseudo-obstruction (CIPO), a severe gastrointestinal dysmotility condition. It was suspected that the dysmotility could be due to the gonadotropin-releasing hormone (GnRH) medication administered as part of the IVF treatment. As GnRH may play a part in gastrointestinal motility, it was hypothesized that antibodies against GnRH could have arisen as a sign of gastrointestinal dysmotility. Therefore, an enzyme-linked immunosorbent assay (ELISA) model was set up and was to show presence of antibodies against GnRH. The 30-year old woman lost weight and was in need of nutritional support. At this point she underwent a full-thickness biopsy of her bowel. Histological examination revealed that she, in contrast to healthy controls, had a bowel depleted of GnRH-containing neurons, in a pivotal part of the enteric nervous system (ENS), namely the myenteric plexus. The ENS controls gastrointestinal motility and is sometimes referred to as the "second brain" (1). These findings were the result of a veritable medical effort combining clinical, surgical, histopathological, and experimental skills in a quest to help a patient in need, conducted by colleagues at Malmö University Hospital just before I was introduced to this particular field of research. The findings were then used as a starting point for the hypothesis that GnRH plays a role in gastrointestinal motility, something that had been suggested previously (2). Within this thesis, possible connections between gastrointestinal motility and GnRH were further explored. In this quest, patients suffering from dysmotility-related diseases were investigated, animal trials were conducted, and other patients subjected to IVF or having gastrointestinal dysmotility problems were further investigated.

The gastrointestinal tract

The gastrointestinal tract has many complex tasks that can seem simple at first glance, for example, supplying the body with water, electrolytes, and nutrients. The alimentary tract is a canal in which food is moved through the body and represents the outside of the body, hence harboring foreign material and bacteria not accessible to the immune system. Food has to be digested through the action of digestive juices and mechanical mixing. Digested food and water are made available to the body through absorption into the blood stream. The entire alimentary tract represents a complex system where each part is adapted to specific functions. An elaborate control system is required to transform ingested food into energy and building material accessible to all cells in the body, and expel remnant matter that cannot be used and is potentially toxic. A wide array of transmitter substances and a nervous control system are in place to achieve this. I will elaborate briefly on the underlying embryology and anatomy of the gastrointestinal tract, and then focus on intestinal motor control, ways of studying intestinal motility, and different disorders involving intestinal motility relevant to this particular thesis.

Embryology

The anatomic formation of the gastrointestinal tract is achieved through a series of evaginations, elongations, and dilatations of the endodermal primary gut tube. Three distinct regions of the bowel give rise to specific portions of the gastrointestinal tract. The foregut is the precursor of the cranial portion of the gastrointestinal tract, and the midgut gives rise to the larger portion of the small bowel and half of the large bowel (3). The hindgut is the precursor of the distal colon. Neural structures are formed when neural crest cells migrate from the central nervous system (CNS) to colonize the gut (4). Having reached the gut, neural crest cells differentiate to form different types of neurons and glia to form the neural network that regulates the gut, namely, the ENS (5, 6).

Anatomy

The first section of the alimentary tract is the oral cavity, which apart from tongue and teeth contains salivary glands, all important in the processing of ingested food. The oral cavity opens into the esophagus, which in length measures about 20 cm. Approximately 5% of the upper esophagus, including the upper esophageal sphincter, is constituted of striated muscle. The 50%–60% of the distal part, including the lower esophageal sphincter, is smooth muscle, and the transition zone in between contains both muscle types (7). The esophagus opens into the

stomach, which plays several important roles in digestion, storing and breaking up ingested food. It also contains multiple glands releasing both acid and enzymes (8). The stomach in turn open into the small bowel, which is a long muscular tube extending from the pylorus of the stomach to the Bauhini valve before the cecal part of the large bowel. The small bowel is divided into three sections, the duodenum occupying the first 25 cm, and the jejunum and ileum occupying about 50% each of the remaining part of the small bowel. No anatomic distinction between these two parts is present. The transit for a solid meal through the small bowel is dependant on the subject, and the type of ingested food, but is estimated to be 2–12 hours (9). The colon in turn is divided into the ascending, transverse, descending, and sigmoid colons, and the rectum. Passage through the colon takes hours to days, and is shorter in males than in females (10, 11). The rectum ends in the anal canal, the end of the gastrointestinal canal.

Histology

The gastrointestinal tract consists of four major tunics; this plan is evident from the esophagus to the anus. These layers are subsequently described from the lumen outward (12).

1. The mucosa is the innermost layer surrounding the lumen, containing the mucus epithelium, which is wet with secretory and absorptive functions. It also contains the lamina propria, which is a layer of connective tissue containing glands and vessels. The muscularis mucosa is often also attributed to the mucosal tunic and is usually quite thin, responsible for the movement of the mucosa itself, and not in propulsion of food (12).

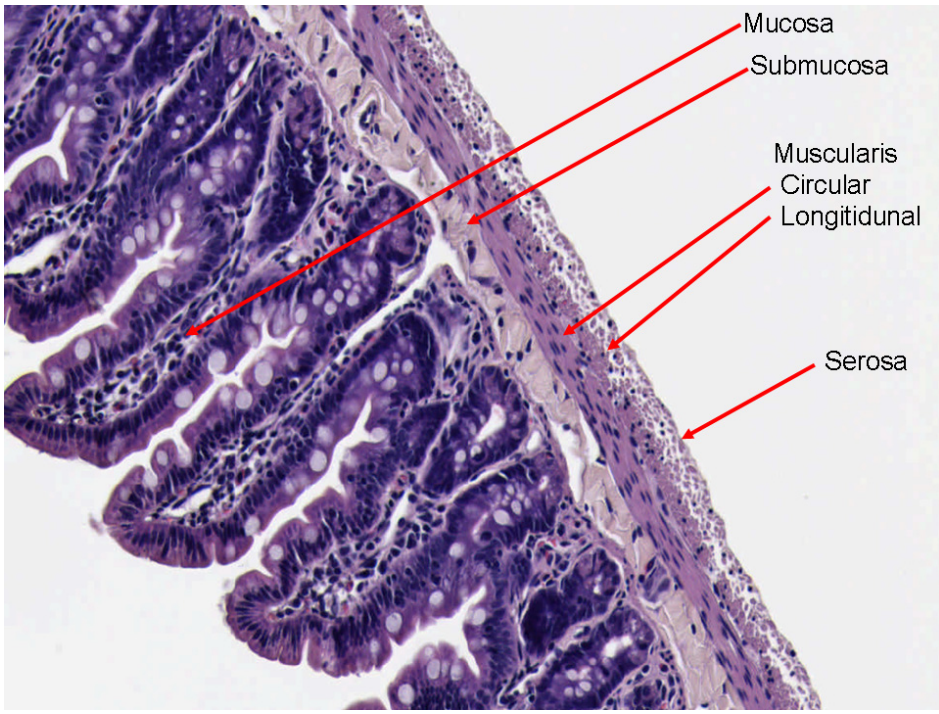
2. The submucosa is a thicker layer of connective tissue and the platform for the mucosa, containing nerves, vascular and lymphatic supply, and in some parts of the gastrointestinal tract also glands (12).

3. The muscularis contains the smooth muscle layers of the gastrointestinal tract, the inner circular and the outer longitudinal. The action of these two muscle layers creates an oral contraction and a simultaneous aboral or distal relaxation with the effect of moving the content of the gut in the anal direction. It is also capable of creating mixing movements. Vascular and nerve supply reside between the muscle layers and the ENS with its two main nervous plexus, namely the myenteric plexus (Meissner's plexus) and the submucosal plexus (Auerbach's plexus), situated apart from one other. The myenteric plexus is situated in between the muscle layers of the intestinal wall, and the submucosal just below the submucosa, as illustrated in Figure 1 (12). The ENS in turn contains more than 10^8 neurons with different electrophysiological properties, different targets or inputs, and different directions of axons, thus forming a complex

network controlling gastrointestinal motility. Further connections both to the sympathetic and the parasympathetic nervous system as well as to the CNS exist (13-16).

4. The adventitia or serosa is the outermost layer, covered by squamous epithelium, and mainly consists of connective tissue (12).

Figure 1
Gross intestinal histology illustrated in rat ileum hematoxylin-eosin coloring.



Gastrointestinal neural control

The neural control of the gastrointestinal tract is extremely organized and integrated, involving the CNS (brain and spinal cord), autonomic nervous system (ANS; sympathetic and parasympathetic), and ENS (14). The ENS is an intrinsic nervous system that can control intestinal function independently of the CNS. Optimal function of the ENS requires the involvement of the other parts in this integrated system, and the ENS is therefore not considered completely autonomous. (14).

Central nervous system

The CNS receives and processes information that it receives from, and coordinates the activity of, all parts of the body. It consists of the brain and the spinal cord. Together with the peripheral nervous system, it has a fundamental role in the control of the human body and of gastrointestinal function. Its precise role in presumed motility disorders is a subject for research and is incompletely understood (17, 18).

Autonomic nervous system

The peripheral components of the ANS can be classified into three divisions: sympathetic, parasympathetic, and enteric (13). The ANS plays a role in motility, but is most renowned as the nervous system that is not influenced by will. Apart from intestinal motility it controls, among other things, heart rate, blood pressure, vascular tone, and sexual function. Thus, the ANS innervates visceral organs of the thoracic, abdominal, and pelvic cavities. It also controls endocrine and exocrine glands throughout the body, and the blood vessels that supply all organs (13). The ANS is divided into two parts, namely, the parasympathetic and sympathetic. The sympathetic nervous system uses adrenaline and noradrenaline as its main transmitter substances with nerve fibers projecting from the spinal cord at Th1 to L2 level (the truncus symphaticus) (13, 19). The parasympathetic nervous system is the part most linked to intestinal function, and mainly uses acetylcholine (ACh), but also vasoactive intestinal peptide (VIP) and nitric oxide (NO), as transmitters (19).

Autonomic dysfunction (AD) is a complication or part of several chronic diseases such as diabetes mellitus, inflammatory bowel disease, and motility disorders (20-24). Autonomic testing in patients with gastrointestinal motility disorders is advocated in particular if an underlying neurologic disorder is suspected (21-24). Sjögren's syndrome, which constitutes one patient population investigated within this thesis, is a cohort in which autonomic dysfunction has previously been demonstrated (25, 26).

Enteric nervous system

The ENS consists of enteric ganglia, which in turn are made up by aggregation of nerve cells interconnected with axons, and also of nerve fibers reaching visceral effector tissues as well as CNS and sympathetic ganglia (14). It also innervates blood vessels, muscle cells, interstitial cells of Cajal (ICCs), immune cells, enteric glia, and endocrine cells within the gastrointestinal tract (27). The ENS contains functionally different types of neurons: sensory neurons, interneurons, and secretomotor neurons (27, 28). This network uses a wide array of transmitter

substances, of which the number of known substances increased dramatically during the 1990s (15).

The most commonly mentioned transmitter in ENS is Ach, which is present in motor and sensory neurons and interneurons, together with tachykinins like substance P and neurokinin A (NKA). Acetylcholine is regarded as the main excitatory transmitter, causing contraction (27). Inhibitory neurons contain NO, VIP, and pituitary adenylate cyclase-activating polypeptide (PACAP) (28).

The roles of other signaling substances may be more variable, depending on the region of the gastrointestinal tract or species and the receptors expressed (15).

Many different serotonin (5-hydroxytryptamine, 5-HT) receptors are expressed in the gastrointestinal tract, and some serotonin receptors initiate contractions, whereas others cause relaxation of the gut. It should be noted that neuronally released serotonin represents only 10% of the total concentration in the gut; the rest originates from epithelial endocrine cells (15, 16, 29).

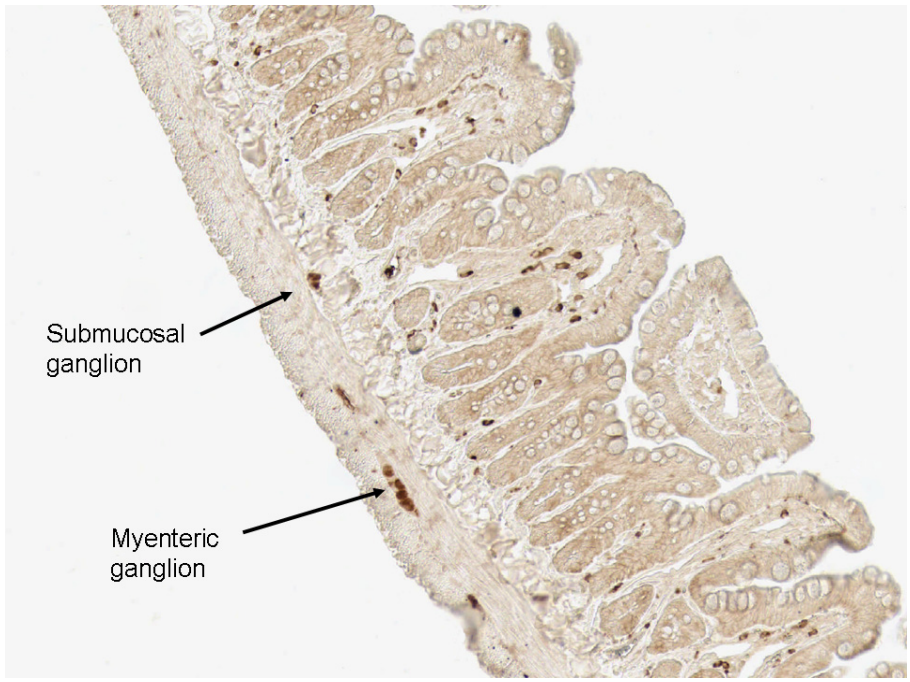
Serotonin is pivotal in gut motility, but even though thorough studies have been made, its precise role is not fully understood (16). Therapeutic advances using the knowledge of ENS and its neurochemistry have been less promising than might be expected. This is despite the fact that the market value of a drug targeting enteric dysmotility has been estimated to 10 billion dollars per year, underlining the complexity of gastrointestinal motor control (30-34). A brief overview of some transmitter substances is presented in Table 1.

Table 1
Transmitter substances: GRP, gastrin-releasing polypeptide; CCK, Cholecystokinin; NO, nitric oxide; PGE2, prostaglandin E2; TRH, thyrotropin-releasing hormone; CGRP, calcitonin gene-regulated peptide; GABA, gamma butyric acid; NA, noradrenaline; NPY, neuropeptide Y; PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal polypeptide. From Hansen 2003 and Olsson 2011 (15, 16).

Stimulatory	Inhibitory
Ach	Adrenaline/NA
GRP	CGRP
CCK	GABA
Adenosine	Galanin
Neurokinin A	NPY
Serotonin	Glucagon
Opioids	Opioids
Histamine	Neurotensin
Motilin	NO
Substance P	PACAP
TRH	Somatostatin
PGE2	VIP
	Secretin

Local reflex behavior is central in regulation of motor and secretory gastrointestinal behavior, and the ENS is intimately linked to both the ANS and the CNS through vagal and sacral afferents relaying information from the gastrointestinal tract to the CNS (35). Most CNS, or voluntary, control is exerted at the beginning and in the end of the gastrointestinal canal (14). ENS disturbances have been put forward as being of importance in motility disorders (28, 35).

Figure 2
Enteric ganglia illustrated in paraffin sections of ileum from rats immunostained with biotin-conjugated primary antibodies raised against the general neuronal marker neuronal protein (HuC/D).



Intestinal motility

Motility is not always propagating but also mixing, breaking down intestinal content, displaying it to enterocytes for absorption or to receptors lining the intestinal wall to optimize motor control taking the composition and amount of content into account (14, 15, 36). Gastrointestinal motility is influenced by various factors, among others, the type of ingested food. A fatty meal slows gastric emptying, as an example of the bowel's capacity of adapting to the content and amount of material within it (37). Simple things such as stretch of the intestinal wall and very complex systems such as intestinal muscle and ENS activity have profound effects on intestinal motility (36, 38, 39). Dysfunctions of the ENS and a neuroeffector mechanism behind intestinal dysmotility have been researched, but hormonal, inflammatory, and ANS activity are also highly relevant in this setting, as are CNS mechanisms and many others (35, 38, 40).

Motility is achieved through the coordination of the contraction of the circular and longitudinal muscle layers. Contractions of the smooth muscle syncytium are orchestrated by the ICCs, which have pacemaker activity resulting in so-called slow waves. These slow waves evoke influx of Ca^{2+} through voltage-dependent L-

type Ca^{2+} channels, ultimately causing the binding between actin filaments and myosin heads in intestinal smooth muscle (38, 40). The ICCs are interlinked between neurons and between neurons and myocytes, hence able to initiate and coordinate pacemaker function for the bowel, whereas frequency of contraction is regulated by a variety of physiological conditions, and shifts in pacemaker dominance can occur in response to both neural and non-neural inputs, as mentioned earlier (40).

Motility patterns are divided into fasting and postprandial patterns. Digestive local contractions and relaxations, which are often the predominant activity after food intake, may be initiated at any location along the gut and help mix the gut content (27, 36). After a while, propagating, propulsive contractile activity referred to as peristalsis, occurs. The stimuli are chemical or mechanical actions on the intestinal wall, as well as autonomic reflexes, release of hormones, and CNS input (16).

In the fasting, inter-digestive state, motility consists of cyclic activity called the migrating motor complex (MMC), a slowly propagating contraction traveling along the gastrointestinal tract (41, 42). In humans, there are four main types of patterns regarding MMCs. The most well known is MMC III, the housekeeping complex, propagating along the gastrointestinal tract to keep the gut free from indigestible particles, dead enterocytes, and unwanted bacteria. It occurs at a rate specific to that particular site in the bowel, with most frequent contractions in the oral parts of the bowel (15, 16, 42).

MMC I is almost silent, while phase II consists of irregular contractions. Phase IV in turn occurs after phase III and represents a short transition period back to phase I (41). In the most proximal and distal parts of the digestive tract, muscle under voluntary control complicates motor patterns. Many disorders of the gastrointestinal tract are caused by, or associated with, disordered motility (43).

A somewhat simplified summary of motor function would state that sensory neurons, sensitive to chemical and mechanical stimuli, propagate orally to synapse to excitatory motor neurons and aborally to synapse with inhibitory motor neurons (14), thus being able to orchestrate an appropriate response to whatever is detected by the sensory neuron.

The precise motor response of the bowel depends on which neurotransmitters and which receptors are present at the specific location and at the particular moment studied. These in turn depend on a multitude of factors, such as state of mind, stress levels, food content, amount of ingested food, blood glucose levels, possible medications, and so on. Some factors have previously been discussed, and several other, yet undiscovered factors probably also affect intestinal motility. Although some neurons act directly on intestinal muscle cells, ICCs have been shown to be

able to enhance and decrease the effect of different locally released transmitter substances or circulating hormones (40, 44, 45).

Changes in motility can be evoked by various conditions and diseases, such as diabetes mellitus and Parkinson's disease. Nervous axons themselves can also be affected by disease (46-48). Changes in intestinal motility affect all people at one stage or another, whether evoked by stress or bad prawns, and since 1899 when Bayliss (49) made his experiments on dog intestine to try to map the system for intestinal control, the matter has been subject to intense research. It is still, however, difficult to completely understand how the at least 10^8 neurons and numerous hormones work together in the quest to pass food from the mouth to the anus, extracting energy and building blocks from it (27). This thesis is merely a microscopic contribution in this quest to better understand the complex play at work in intestinal motility.

Evaluation of intestinal motility

The study of motility in different parts of the bowel is not always easily feasible. Intraluminal pressure monitoring, or manometry, is one method employed to study intestinal motility, and it is used to detect abnormal motor activity within the esophagus, stomach, small bowel, and large bowel. Reach of the instruments set the boundaries for this type of evaluation (43, 50). Different types of manometry equipment all share a common principle; pressure-sensitive gauges mounted on a tube are inserted into the gastrointestinal tract, and motor activity or MMCs are recorded as contractions which raise intraluminal pressure (43). Abnormalities in MMC III, as well as lack of activity, could suggest underlying CIPO or enteric dysmotility (ED) (48, 50-52). Other methods of studying intestinal motility include a capsule (smart pill) that monitors intestinal pressure and pH during its passage through the gastrointestinal tract (53).

Traditional radiography can be used to study esophageal motility, since the act of swallowing can be recorded on video (54). Plain radiography or computed tomography or passage examination is extensively used to try to rule out mechanical obstruction or perforation in the acute abdomen (48, 55, 56).

To study emptying of solids from the stomach, scintigraphy is considered the gold standard method, and scintigraphy can also be used to study colon emptying (43, 50). The method involves the ingestion of a standardized low fat meal, often an egg, where the yolk has been labeled with technetium, and subsequent detection of the isotope passage using a gamma camera (57, 58). Apart from standardization of the ingested meal, the method relies on standardization of the subject position, prior fasting period, scanning methods, and methods of calculation. Different

centers tend to have their own standardization and own control groups. The method is reproducible and is a useful screening tool for delayed gastric emptying and the results can be abnormal as a sign of underlying small bowel dysmotility. The method is non-invasive, but is associated with a small amount of radiation and necessitates the gamma camera on location (57).

Some difficulties can be overcome using another non-invasive method, namely the ^{13}C-octanoic acid breath test. The method involves the collection of breath samples after the ingestion of a solid meal, where again the marker, in this case octanoic acid, has been added to egg yolk. After disintegration in the duodenum, the octanoic acid is transported to the liver, where it is oxidized into CO_2 and thereafter exhaled. The main parameter determining the amount of CO_2 in breath is the rate of emptying from the stomach into the duodenum (59-61).

Magnetic resonance imaging (MRI) can be used to assess gastric emptying and small-intestinal motility, but is not to date a widely used tool, perhaps because of its relatively high cost and limited availability (62, 63). There seems to be potential for the method, though; in new dynamic cine-MRI it is possible to study motility in a completely new way. The limited availability of MRI machines as well as the horizontal position of the subject make the method impractical, and it is still inferior to manometry today (64).

The progress of radio opaque markers through the gastrointestinal tract using radiographs is another method of studying motility (10, 11, 43, 65-67). It is relatively inexpensive and readily available, requiring only equipment for fluoroscopy. The method does not necessitate preparations. Modified versions of the technique can even be used to assess transit times in all segments of the bowel, gastric and small intestinal as well as colonic (11).

Full-thickness biopsy

Intestinal histology, studied via full-thickness biopsies, can reveal signs of denervation, absence of muscle cells, and other signs, explaining a disturbed motility detected by the methods described above. A full-thickness biopsy can be a powerful complement in the evaluation of gastrointestinal motility disorders and is obtained via minimally invasive surgery (68). Laparoscopically, the abdomen is inspected and the small bowel is exteriorized and a diamond-shaped biopsy measuring about 1 cm × 1 cm is obtained (69). In Malmö, a section 1 m proximal to Bauhin's valve is selected (70, 71). There is evolving evidence that histopathological analysis of full-thickness biopsies in severe gastrointestinal motor disorder can contribute to accurate diagnosis and determine outcome, and may also contribute to changes in patient management in some cases (71).

Evaluation of the autonomic nervous system

Several methods are available for autonomic testing, and most tests measure various cardiovascular autonomic reflexes. For example, the deep-breathing test is used to measure the degree of sinus arrhythmia during deep breaths and is considered a parasympathetic test. The orthostatic test evaluates the heart rate and blood pressure reaction in response to tilting of the body, evaluating both the parasympathetic and sympathetic nervous system. In the case of blood pressure reaction, it mainly reflects sympathetic function. A Valsalva test evaluates parasympathetic function by monitoring heart rate in response to the Valsalva maneuver. The cold-pressor test in turn evaluates sympathetic function in response to contralateral cooling of the hand (72, 73). When studying populations with different ages and a female predominance, which has been done within this thesis, age and sex has to be taken into consideration, as autonomic function deteriorates with advancing age and some parameters differ between sexes (74, 75).

Functional and motility disorders of the gastrointestinal tract

Gastrointestinal complaints that could be attributed to an underlying gastrointestinal dysfunction or disorder, without visible organic explanation in routine examinations, tend to have a female predominance, and are very common in the population, causing considerable morbidity in the community (76). Some defined functional and motility disorders relevant to this thesis will be discussed further.

Irritable bowel syndrome

Many people consult doctors with gastrointestinal complaints, and as many as 10%–20% have problems so severe that they are considered to suffer from the illness “irritable bowel syndrome” (IBS), a disease that affects women 1.5 to 3 times more often than men. The reason/reasons underlying the condition are not completely known (77). IBS is the most commonly diagnosed gastrointestinal condition and accounts for approximately 30% of all referrals to gastroenterologists and 3% of all visits to general practitioners (78). IBS is a functional bowel disorder (FBD) characterized by chronic abdominal pain and altered bowel habits identified by its symptoms. The pathophysiology of IBS remains uncertain. It is viewed as a disorder resulting from an interaction among a number of factors (76). Previous diagnostic criterion (ROME II) presumed the absence of a structural or biochemical explanation (79). However, the assumption

that research will reveal that IBS or subgroups within the IBS group will demonstrate such structural or biochemical features have led to the presumption having been omitted in the current criteria (69, 76).

IBS is therefore diagnosed using the ROME III criteria, which states the following:

*Diagnostic criterion**

Recurrent abdominal pain or discomfort** at least 3 days/month in the last 3 months associated with two or more of the following:

1. Improvement with defecation

2. Onset associated with a change in frequency of stool

3. Onset associated with a change in form (appearance) of stool

*Criterion fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis

**"Discomfort" means an uncomfortable sensation not described as pain.

In pathophysiology research and clinical trials, a pain/discomfort frequency of at least 2 days a week during screening evaluation is recommended for subject eligibility (76).

Despite multiple investigations, data have been conflicting. No abnormality has been found to be consistent with IBS, and demonstrable pathological abnormalities or reliable biomarkers are lacking (76).

The symptoms listed above leading up to diagnosis are present also in healthy controls, making it challenging to identify a precise underlying pathology. Several mechanisms behind the symptom-based diagnosis IBS have been suggested and further investigated. Disturbed motility has been demonstrated using antroduodenal manometry (80). Visceral hypersensitivity (increased sensation in response to stimuli), particularly in the rectum, is a frequent finding in IBS patients (81). CNS modulation or modulation of the so-called brain-gut axis has been reported, using, among other techniques, MRI (18). Psychosocial factors and comorbidity with anxiety disorders have been demonstrated, and coping strategies seem very important in IBS (79, 82-84). IBS has also been shown to coexist with fibromyalgia in many patients, with ensuing low quality-of-life scores (85, 86). A comorbid triad of IBS, chronic fatigue, and musculoskeletal pain has recently been pointed out in a Norwegian population (87). Also recently, in a randomized control trial, it has been shown that physical activity improves IBS symptoms (88).

Infection is another suggested cause, and the odds of developing IBS are increased six-fold after an acute gastrointestinal infection. Risk factors for postinfectious

IBS included young age, severity of initial illness, female gender, anxiety, and depression (89, 90). An inflammatory pathogenesis has been suggested and lymphocytes have been demonstrated in the gastrointestinal tract in full-thickness samples of IBS patients (69), and mast cells have been reported in vicinity of enteric neurons (91). Altered serotonin signaling (82), autonomic disturbances (92), food allergy based on IgG antibodies against food itself (93), and genetic polymorphism, where TNFSF15 is a susceptibility gene for IBS (83), have been reported.

An IBS diagnosis does not negate another concurrent disease that can affect gastrointestinal motility. The condition is sometimes considered under-diagnosed, and some state that only roughly a third of subjects have been formally diagnosed (77). Many conditions might give rise to symptoms that could be interpreted as IBS; therefore, diagnosis depends on careful interpretation of the temporal relationships of pain/discomfort and bowel habits (76). Even if the diagnostic criterion is carefully used, the group of patients sharing a common IBS diagnosis is very heterogeneous. Possibly groups within the group could be extracted if an underlying clear pathology were to be found (69). Of particular relevance to this thesis is that IBS patients have recently been shown to express antibodies against GnRH to a larger extent and in higher levels than controls (94), perhaps representing a subgroup among IBS patients. The female predominance, the relation to a possible disturbance in autonomic function, and a high prevalence of depression and anxiety in the IBS group have been important in the selection of one of the other groups to be studied within the thesis, namely, the patients with primary Sjögren's syndrome (pSS), who share the same characteristics.

Chronic intestinal pseudo-obstruction and enteric dysmotility

Patients diagnosed with the most severe forms of IBS may have symptoms that resemble those of CIPO and ED. However, in contrast to IBS, the CIPO diagnosis necessitates the symptom of mechanical obstruction and objective signs of obstruction (95-97). Hence, abnormal small bowel contractile activity in combination with episodic or chronic signs mimicking mechanical obstruction of the small bowel is the defining feature of CIPO (48, 52). The condition is highly morbid and outcome is generally poor, with increased mortality (96, 98).

CIPO is a rare condition with uncertain or unknown prevalence and incidence; prevalence in Sweden has been estimated to be 3–5 per 100,000 (99, 100).

The diagnosis of ED requires documented abnormal contractile activity, but no past history of episodes, or current signs, mimicking mechanical obstruction. Nor should any medication that could potentially give rise to the changes in contractile activity be present (48). Both CIPO and ED are considerably more severe disorders compared to IBS, since the conditions need extensive medical and

nursing therapy, and many patients diagnosed with these conditions need nutritional support and regular analgesic treatment (101). Patients struck by CIPO are believed to represent a population that is more ill and has poorer prognosis as opposed to those with ED, even though both these conditions are linked to poor prognosis (101, 102). The two conditions share many features, both being highly morbid and presumed to be linked to neuromuscular gastrointestinal diseases. Symptoms cannot distinguish between the groups. The distinction is whether there are subocclusive events with radiological signs of mechanical obstruction, without its real presence (air/fluid levels on x-ray) (48, 103). Therefore, ED falls in between IBS and CIPO, with the lower limit being documented abnormal motor activity, and the upper being absence of radiological signs of subocclusive events. It has been speculated that ED may represent a subgroup of functional bowel disorders with a more advanced stage of a disease that can be detected by small bowel manometry, and that it is characterized by enteric ganglioneuritis leading to neurodegeneration and progressively impaired function (103).

Since the diagnoses CIPO and ED are set using radiological and manometric findings in combination with symptoms, the pathogenetic mechanisms may vary between different patients. However, the diseases are associated with disturbances in one or a combination of the following areas (55, 104, 105):

- abnormalities in the ENS
- abnormalities in the extrinsic nervous supply
- abnormalities in gastrointestinal smooth muscle
- abnormalities in ICCs

Advances in histopathology in recent years have led to the consensus that in some cases of severe CIPO and ED, where the etiology remains unknown, full-thickness biopsy may be beneficial (71). Studies of such biopsies have made possible a classification of neuromuscular pathology into the main groups of visceral myopathy, visceral neuropathy, and combined visceral neuromyopathy (103, 105). Some of the abnormalities are shared with other diagnostic entities and may be secondary to a known cause or idiopathic (105). In some rare cases, specific genetic mutations have been linked to CIPO, such as in the case of Waardenburgh-Shah syndrome (deafness and pigmentary anomalies in association with megacolon) (106). CIPO is most often referred to as a sporadic form, which is in contrast with the finding that some genes are associated with CIPO (99, 106, 107). Viruses (108); gynecological cancer (109); neuromuscular diseases, including amyloidosis; diabetes mellitus; Ehler-Danlos; and systemic sclerosis are also associated with CIPO (99, 107, 110). Diseases affecting the CNS or ENS can naturally also cause CIPO, and the most renowned examples are Hirschsprung's disease, neurofibromatosis, von Recklinghausen, or even stroke affecting the ANS

(99). Not to be forgotten in this context is the patient described earlier, who developed CIPO after repeated buserelin injections (70). Paraneoplastic CIPO has been reported in some cases related to antibodies against neural structures, such as antineuronal nuclear Hu antibodies (111-113).

Although great advances have been made in pathology in recent years, the need of consensus and similar treatment of biopsies is pivotal for future histopathological research. Today, differences in staining techniques and preparation techniques make it very difficult to compare results, and much of the interpretation relies on skilled individual pathologists (71, 114). Access to such a pathologist (Béla Veress) has been crucial to the thesis.

Gastroparesis

Gastroparesis or delayed gastric emptying is a common cause of nausea, vomiting, and other upper gut symptoms. The true prevalence of gastroparesis is unknown (115). In the US population, the incidence per 100,000 individuals has been estimated to be 37.8 and 9.6 for women and men, respectively. The condition was associated with significantly lower overall survival as compared to age- and gender-matched controls, hence, a rare condition, but associated with poor outcome (116). It is more common in patients with diabetes mellitus, where 11%–18% report symptoms, in particular, those with long-standing disease (46, 117). The etiology of gastroparesis can vary. The main categories are considered to be diabetic and idiopathic, which account for about one third each. Postsurgical, secondary to neurological and collagen vascular, constitute the major part of the remaining third of the categories of suspected causes (117, 118). There are several abnormalities that may result in motor dysfunction of the stomach, including autonomic neuropathy, enteric neuropathy, abnormalities of ICC, sudden fluctuations in blood glucose, and psychosomatic factors (117, 118). Histopathology shows that myopathic disorders are uncommon. In gastric biopsies, the most common intrinsic defects are recognized in the ICCs. Gastroparesis has also been reported to be associated with immune infiltration and neuronal changes, as in the case of CIPO (118).

In the case of Sjögren's syndrome, only one study has reported objective signs of impaired gastric emptying (IGE) (119). Symptoms of IGE and other gastrointestinal symptoms are, however, frequently encountered in pSS patients (25, 120, 121).

Sjögren's syndrome

Primary Sjögren's syndrome (pSS) is a chronic inflammatory disorder affecting mainly exocrine glands, resulting in dryness of primarily the eyes and mouth (122). pSS is diagnosed according to the American-European Classification Criteria (AECC), which requires fulfillment of at least 4 of 6 criteria, including some sign of autoimmunity (focal sialadenitis or presence of anti-SS-A and/or anti-SS-B antibodies) (123). The prevalence of pSS has been estimated to be 0.1%–0.6% (124). Apart from affecting exocrine glands, pSS has been reported to affect multiple non-exocrine organs, such as the nervous system and the gastrointestinal tract (122, 125). Previous studies have also shown that it can affect the ANS, and through this has the potential of affecting function in various organs, including the gastrointestinal tract (25, 126). Dry mouth is the most common complaint. Taking this into account, lack of saliva has been put forward as one explanation for swallowing difficulties, the most predominant symptom from the gastrointestinal tract (122, 127). However, many have demonstrated a lack of association between the symptom of dysphagia, salivary flow, and manometric abnormalities (128, 129). In one previous study, delayed gastric emptying was detected in 70% of pSS patients (119). It has been reported that many patients suffering from pSS also suffer from IBS and functional dyspepsia (FD) (120). It has also been reported that pSS patients express antibodies directed against GnRH to a greater degree in comparison to control patients with systemic sclerosis, another disease known for profound intestinal involvement (120).

In vitro fertilization

In 2008 in Sweden, 13,408 complete IVF treatments were conducted, resulting in 3438 live births (130). An IVF is a procedure intended to overcome infertility and produce pregnancy. Generally, it means the hormonal stimulation of female ovaries to produce oocytes that are subsequently aspirated and later fertilized in a laboratory before being reinserted into the female uterus (130). Such a procedure usually spans two weeks and is referred to as an IVF cycle. The first successful IVF treatment led to a tubal pregnancy and was first described by Steptoe and Edwards in 1976, a pregnancy that had to be terminated due to its location. Two years later, the woman in question delivered a girl weighing 2700 g (131, 132). The Nobel Assembly at Karolinska Institutet awarded the Nobel Prize in Physiology or Medicine in 2010 to Robert G. Edwards for the development of human IVF (133).

In the first successful IVF treatment, a single oocyte was recovered in a natural ovulatory cycle. Nowadays, this has changed, and many different protocols are used in the IVF setting, aimed at creating multiple mature oocytes to be harvested and reinserted (134). Protocols are referred to as short or long protocols. In short protocols, sometimes GnRH antagonists are preferred over GnRH agonists. However, long protocols, in a meta-analysis seem to be more successful than short ones (134). A commonly used protocol starts with the administration of a GnRH agonist for two weeks to down-regulate luteinizing hormone (LH), preventing an LH surge and subsequent ovulation, destroying the possibility of harvesting oocytes. Subcutaneous or nasally administered GnRH can be used. The initial stimulatory effect of GnRH can be cancelled using oral contraceptives or by choosing the luteal phase of the menstrual cycle to begin treatment. The woman is then stimulated using human menopausal gonadotropin (hMG), follicle-stimulating hormone (FSH), or both, all under the continuation of GnRH to prevent an LH surge (135). Oocytes are recovered after injection of human chorionic gonadotropin (hCG), which has LH activity and can be used to imitate the LH surge. Recovery is achieved using vaginal ultrasound and an aspiration technique. Retrieved oocytes are fertilized in the laboratory dish. If sperm quality is poor, sperm can be injected into the oocyte (136). The fertilized oocytes are then placed in a medium and cultured, trying to create an environment similar to that of the uterus. About 48 hours later, the best embryo or embryos are selected and reinserted into the uterus via a catheter (137).

The risks of IVF treatment are mainly related to multiple gestations, and most risks have been related to the child, with increased risk for preterm birth, cancer, or neuropsychiatric disorders (138). Risk seems small and is considered to be declining with advancing technique (138). For the mother, a possible relation to ovarian cancer risk has been discussed. One possible reason is that ovarian pathology can cause both infertility and cancer risk (139). Other risks have been related to the ovarian stimulation treatment and ovarian hyperstimulation syndrome (140).

Gonadotropin-releasing hormone

The reproductive axis is controlled by GnRH, which is produced in hypothalamic neurons and secreted in a pulsatile fashion (141). GnRH reaches the anterior pituitary via the portal circulation and stimulates secretion of FSH and LH through GnRH receptor (GnRH-R) activation (142). FSH and LH target the gonads and regulate secretion of steroid hormones, like estrogen and progesterone (19, 143). In vertebrates, 23 native forms of GnRH exist. They are all decapeptides, and changes in amino acids in molecular positions 5 to 8 make them different from one

another (144, 145). This, through evolution, highly conserved peptide, can be expressed in more than one form in each vertebrate, some expressing up to three forms (143, 145, 146). In mammals, 2 types of GnRH have been deemed relevant. Known functions of these different GnRH types include

- GnRH I, which takes care of regulation of the hypothalamic-pituitary axis and gonadotropin production, and

- GnRH II, which is distributed in the brain, in particular the hind brain and spinal cord, thus being extra-hypothalamic. It is believed to participate in reproduction and sexual behavior through a neuromodulator role (145, 147).

Different receptors for GnRH have been described in mammals, although one (GnRH-RII) is not expressed as a fully functional receptor, due to a genomic stop sequence/frame shift (143, 148). However, GnRH II seems to be able to signal through the type I receptor (145, 149). GnRH was first isolated and characterized by Nobel Prize winners Roger Guillemin and Andrew Schally (1977). In vivo, its short half-life of 2–4 minutes, in combination with secretion into the portal circulation of the pituitary, which is anatomically inaccessible, has rendered sampling very complicated (141). Instead, pulses of the downstream-secreted LH are studied.

A variety of GnRH analogs are available on the market, derived from the native GnRH with substitutions in positions 6 and 10 of the native decapeptide, resulting in longer half-life and stronger binding to the receptor (142). Buserelin, for instance, is deemed to be 20 times more potent than the native analog (142).

The GnRH-R is a G-protein-coupled receptor with seven transmembrane domains, through which GnRH is believed to exert different roles (149). GnRH-Rs have been found in hypothalamus, brain, placenta, endometrium, myometrium, decidua, ovary, breast/mammary glands, testis, sperm, prostate, lymphocytes, T cells, mononuclear blood cells, spleen, liver, pancreas, kidney, adrenal glands, heart, skeletal muscle, submaxillary glands, gastric parietal cells, spinal chord, retina, and various cancers and cancer cell lines (143).

There are two main ways of using GnRH analogs in the clinical setting, one being the administration in a stimulating, pulsatile fashion and the other being continuous administration with down-regulating effects. Pulsatile, intravenous administration can be used to restore normal function in the pituitary-gonad axis and restore fertility (141). At first, GnRH analogs stimulate the release of LH and FSH, but after about 10 days of chronic treatment, they result in desensitization of gonadotropin secretion (150). This ultimately means chemical castration, which means that GnRH agonists are useful in several different clinical settings, such as in

- sex-hormone-dependent neoplasms such as prostate, ovary, and breast cancers;
- settings where sex hormones are considered to exacerbate medical conditions, for example, endometriosis, uterine fibroids, and polycystic ovarian syndrome;
- precocious puberty; and
- cases where pituitary hormones can interfere with the clinical goal, as in the case of IVF treatment (141).

The way in which the down-regulation of receptor sensitivity is achieved is not completely known, and many possible mechanisms are presented (143).

Since hypothalamic GnRH is not considered to reach the systemic circulation, speculation has been made regarding possible autocrine/paracrine actions in addition to its hormonal effects (151). The way in which GnRH interacts with the GnRH-R, evoking different reactions in different tissues, is a matter of intense research; at one end it causes the pituitary synthesis and release of LH and FSH, and at the other extreme it has a potential role in apoptosis and inhibition of cell proliferation of cancer cells and other cells in vivo and in vitro (an effect not linked to sex hormones) (143, 149). Several variables are thought to influence the GnRH effect: the pulse, speed, and amount of GnRH; the setting in which the cell that is exposed to GnRH finds itself; complex intracellular signaling cascades, involving mitogen-activated protein (MAP) kinase; calcium; the cells' cytoskeleton; protein kinase C; and many more, all taking part in the complex play that determines the cells' reaction to GnRH (143, 145, 149, 151).

Not all effects of GnRH have been completely explored. It seems to be a player in different stages of mammal development and to influence reproductive health; it may influence neural networks, potentially can interfere with biology in cancerous cells, and perhaps even evoke cell cycle arrest and apoptosis (143, 145, 149). In most target cells, the biological role and the response evoked by GnRH are not known and need further research and elucidation (143).

Gonadotropin-releasing hormone in the bowel

It is presumed that GnRH release is linked to the availability of nutrients and that onset of puberty and mammalian reproduction are linked to GnRH secretion at a hypothalamic level, establishing one connection between GnRH and the gastrointestinal tract (152). Other studies implicate a more direct role for the reproductive peptide hormones in the gastrointestinal tract (144).

In rat, GnRH and GnRH-R mRNA and/or peptide has been found in ganglion cells of the myenteric plexus (153), on gastric smooth muscle cells (154), and on parietal cells in the epithelium (155). The role of GnRH in the gut is not completely elucidated, but GnRH analogs have been shown to inhibit gastric secretion and gastrin release in rat and dog (156, 157), to inhibit cell proliferation in gastric epithelium (158), and to protect enteric rat neurons in culture when continuously stimulated (159), whereas shorter stimulation inhibits cell proliferation in gastric smooth muscle cells (154).

The analog leuprolide has been reported to restore motor function in the gastrointestinal tract in female, ovariectomized rats (160). In 1989 Mathias et al. (161) made an informal initial study attempting to treat 4 female patients with FBD, using the GnRH analog leuprolide and estrogen add back. In 1992 a patient who had had a heart-lung transplant and developed a cytomegalovirus (CMV) infection, developed CIPO and was successfully treated with the GnRH analog leuprolide, reducing the delay of gastric emptying by about a third, although she still had about 3 times the normal value of t(half) as compared to healthy controls (162). In 1994 Mathias et al. (163) completed a placebo-controlled study of 30 women with FBD, using leuprolide, having significant effect on a composite score evaluating the perception of abdominal pain, nausea, vomiting, bloating, early satiety, and anorexia compared to baseline.

In 1998 a multicenter study by the same group was made involving 100 premenopausal women with FBD, again using leuprolide and a composite score; significant effect over placebo was achieved for 2 of the domains, namely abdominal pain and nausea (2).

In 2005 another investigator, Palomba (164) used leuprolide at 2 centers in Italy, in a double-blind, controlled study of 120 patients suffering from IBS. Measured with 2 quality-of-life scores, leuprolide achieved significant improvement over placebo in GnRH-treated women. Additional significant effect was achieved using hormone add-back therapy.

GnRH-immunoreactive (IR) neurons were found in human gastrointestinal tract in 2007, in the patient that is the origin of this thesis (70). In another case report of 2010, a patient suffering from intestinal dysmotility and gastroparesis, along with a high titer of GnRH antibodies and reduced numbers of GnRH-IR enteric neurons, was described by Ohlsson et al. (159). GnRH immunoglobulin M (IgM) antibodies have, then, been found in higher prevalence and at higher levels in IBS and dysmotility patients (94).

The mechanism of action of GnRH is poorly understood, and speculation on action through activation of GnRH receptors located on myenteric neurons has been made, proposing GnRH analogs as neuromodulators (144). The involvement

of other reproductive axis hormones has also been put forward, and the finding that LH alters myoelectric activity in rat small bowel has further underlined them (165). In 2009 it was suggested that GnRH interacts with glucagon-like peptide (GLP)-1 and GLP-2 through paracrine and autocrine ways, taking part in glucose metabolism and insulin secretion (166).

Aims of the thesis

The overall aim of this thesis was to explore the relation between GnRH and gastrointestinal symptoms and dysmotility. The aims of the individual studies were as follow:

Paper I
To assess the prevalence of IGE in patients with pSS, a group with high levels of GnRH antibodies, using the octanoate breath test, and to study associations between objective signs and symptoms of AD, IGE, and inflammatory and serological features of pSS.

Paper II
To compare the degree of gastrointestinal complaints in different patient populations in which high levels of GnRH antibodies had earlier been described, using the Visual Analog Scale for Irritable Bowel Syndrome (VAS-IBS) questionnaire.

Paper III
To retrospectively scrutinize patients with gastrointestinal dysmotility so severe that they had had full-thickness biopsy, for information on coexisting diseases and etiologic factors, and to describe expression of GnRH in the ENS and antibodies against GnRH in serum, in order to investigate whether GnRH depletion is a widespread problem in this patient group.

Paper IV
To study the presence of LH receptors in the gastrointestinal tract, and if present, compare receptor expression in patients with or without severe gastrointestinal dysmotility, to establish one possible mode of action for the reported effect of GnRH analogs on gastrointestinal symptoms and motility.

Paper V
To assess gastrointestinal symptoms and the presence of antibodies against GnRH and its receptor in serum in women before and after IVF treatment with buserelin.

Paper VI

To investigate possible enteric neurodegeneration and titers of GnRH antibodies in rat, in response to repeated administration of the GnRH analog buserelin.

Materials and Methods

Subjects

Paper I

Patients with pSS: Twenty-eight consecutive patients (26 females) with pSS according to the AECC (123), from the outpatient clinic at the Department of Rheumatology, Skåne University Hospital, Malmö, who had previously been included in a prospective study on AD (25), were included in the current study. Their median age was 62 years, range 29–65 years.

Control population: The octanoate breath test controls consisted of 50 healthy controls recruited among laboratory staff and their relatives and friends (median age 43 years, range 25–59 years, 25 females). The control group for the deep-breathing test and the orthostatic heart rate test consisted of 56 healthy individuals (median age 40 years, range 16–59 years, 22 females), all of whom had passed a health examination without signs of cardiovascular disease, respiratory disorders, or diabetes mellitus (167). The controls for the orthostatic blood pressure reaction test consisted of 238 healthy non-diabetic individuals (median age 60 years, range 16–96 years, 106 females) (168). The finger skin blood flow test controls consisted of 80 healthy subjects (median age 43 years, range 19–81 years, 37 females), all of whom were non-smokers without any history of vascular disease, and were not taking any medication (74).

Paper II

Patients with IBS: Thirty-nine consecutive female patients (median age 37 years, range 18–69 years) visiting the out-patient clinic at the Department of Gastroenterology during a two-year period, suffering from abdominal pain and altered bowel habits, lacking objective abnormal findings, and who fulfilled the Rome III criteria, were classified as having IBS and included (76). None had nutritional support or opioid analgesics.

Patients with motility disorders: Twenty-one consecutive female patients (median age 43 years, range 26–84 years) referred for laparoscopic full-thickness biopsy because of symptoms or signs of severe dysmotility between 1998 and 2009, or patients with a severe motility disorder having had a bowel resection

within the time frame were identified. Sixteen patients fulfilled the diagnostic criteria for ED and 5 for CIPO (48, 55, 97, 169). Ten of the patients had peroral nutrition, whereas 11 had supplements of enteral or intravenous nutrition. Seventeen used opioid analgesics.

Patients with pSS: The 26 female pSS patients from Paper I (median age 62 years, range 29–65 years) were willing to be included in the study. None of the patients had previously undergone abdominal surgery, and none had nutritional support or opioid analgesics.

Control population: The control group was recruited among hospital staff and consisted of 52 healthy female volunteers (median age 44 years, range 22–77 years) who had not undergone prior abdominal surgery.

Papers III and IV

Patients with motility disorders: Twenty-two patients (19 females), having had full-thickness biopsy, mainly the same as in Paper II, with remaining, representative material containing sufficient amount of ganglia for GnRH and LH receptor staining, were included in a retrospective manner (median age 44 years, range 18–96 years). Regarding GnRH staining, 14 patients were diagnosed with ED and 8 patients with CIPO. In total, 19 small bowel specimens and 8 large bowel specimens were available, reflecting material from resections, with both small and large bowel specimens present in 5 patients. Fifteen patients (13 females) had available biopsies for LH receptor staining, out of these 10 patients were diagnosed with ED and 5 patients with CIPO.

Histological control group: As controls for GnRH and LH receptor +/- neurons in small bowel, sections from 6 cases (3 females) of bowel resection due to non-obliterating adenocarcinoma of the jejunum and ileum, and 2 cases of colonic carcinoma were used (median age 69 years, range 53–85 years). Regarding large bowel, the control group was 8 cases (5 females) with large bowel resection due to diverticulosis (median age 74 years, range 60–87 years). All samples were taken from areas with normal macro- and microscopic appearance, 10 cm above the tumor in the small bowel and from diverticulum-free normal parts of the colonic specimen.

Antibody control group: A cohort of 456 healthy blood donors were analyzed for the expression of GnRH antibodies in serum (94). From this cohort, 2 age- and gender-matched controls were randomly extracted for each patient sample and served as controls.

Paper V

Patients with IVF: One hundred and twenty-four consecutive patients at a fertility clinic in Malmö, using buserelin, were included. The mean age was 34 (range 24–41) years. Patients had been subjected to IVF treatment from 1 to 9 times, for 39.5% this treatment was the first.

Control population: Sixty-five age- and gender-matched controls, median age 37 (range 24–61) years, were recruited and answered questionnaires. A cohort of 69 healthy, female blood donors, median age 47 (range 23–64) years, served as controls for antibody tests.

Paper VI

Animals: Female Sprague-Dawley rats (n = 33, 170–180 g) were used. Twenty rats were given 20 µg (1 mg/ml) of the GnRH analog buserelin subcutaneously, whereas 11 received saline injections and were treated and sacrificed in a similar fashion. Animals were given injection treatment for 5 days. Three weeks later, a portion of them were deeply anaesthetized and euthanized. This process was repeated up to 4 times, rendering 4 different groups, whereby 3 (buserelin) + 2 (controls) had had treatment once (B1), 3 + 2 had had treatment twice (B2), 8 + 4 had had treatment 3 times (B3) and the remaining 6 + 3 had had treatment 4 times (B4). Two naïve rats were euthanized and used for examination of the presence and cellular localization of LH receptors.

Methods

All studies were performed according to the Helsinki declaration and approved by the Ethics Committee of Lund University (ethic committee approval numbers 472/2006, suppl 2011/44, 2008/563, 2009/209 and LU 735-02). All patients gave their informed consent before entering the studies. The animal trials of Paper VI were approved by the animal ethics committee, Lund and Malmö, Sweden. Animals were used in accordance with the European Communities Council Directive (86/609/EEC and 2010/63/EU) and the Swedish Animal Welfare Act (SFS 1988:534).

Paper I

In Paper I, the octanoate breath test was used. An omelet was ingested under standardized conditions. End-tidal breath samples were obtained before the meal and subsequently every 15 minutes. The samples were sent to Linköping for analysis. The half time ($t_{½}$) and lag time (t_{lag}) for gastric emptying were calculated

as previously described in detail by Ghoos et al. (59). The $t_{½}$ is defined as the time from ingestion of a bolus until 50% of the bolus has been cleared from the stomach. The t_{lag} is the time from ingestion until the bolus is beginning to be cleared from the stomach.

Laboratory tests

Blood samples were taken to assess signs of disease activity and to rule out other possible causes of dysmotility. They included, among others, erythrocyte sedimentation rate (ESR), rheumatoid factor (RF), and immunoglobulin G (IgG). They were performed as routine analyses.

Autonomic nerve function tests

Deep-breathing test

After supine rest, heart rate was monitored, and once constant, maximal expirations and inspirations were performed. An expiration/inspiration (E/I) heart rate ratio was calculated. The E/I ratio mainly reflects parasympathetic nervous function (170, 171).

Orthostatic heart rate and blood pressure test

Strapped on a tilt table, the subject was tilted to erect position, while heart rate and systolic and diastolic blood pressures were monitored. An acceleration index was calculated from R-R intervals before and after tilt, an index that measures both parasympathetic and sympathetic nervous function, while the blood pressure reaction is attributed to sympathetic nervous function (172, 173).

Finger skin blood flow test

The subject was seated with a finger of one hand on a holder. The temperature of the aluminum holder was kept stable, and finger skin blood flow was monitored with a laser Doppler device. The subject then immersed the contralateral hand and forearm into a cold-water bath and blood flow was monitored. A vasoconstriction (VAC) index could be calculated. This has been shown to be a sensitive test for sympathetic nervous function in the skin (74).

Questionnaire

The self-completed Autonomic Symptom Profile (ASP), assessing AD symptoms, was filled in during the octanoate breath test. The ASP evaluates presence and severity of AD symptoms (25, 174-177). Furthermore, patients were assessed for the presence of symptoms of IBS and FD, according to the Rome III criteria (76). This assessment was based on the answers from the ASP.

Paper II

Self-estimation of gastrointestinal symptoms was performed using the VAS-IBS questionnaire.

Patients estimated 7 different entities on a VAS scale from 0 to 100 mm, where 0 represents very severe problems and 100 represents absence of problems. The 7 entities were abdominal pain, diarrhea, constipation, bloating and flatulence, vomiting and nausea, perception of psychological well-being, and the intestinal symptoms' influence on daily life. This questionnaire has formerly been developed and psychometrically tested for patients with gastrointestinal symptoms without organic causes (178). Since age differed between groups, scores were standardized for age.

Paper III and IV

Histological analysis

Full-thickness slices perpendicular to each other were cut and embedded in paraffin for conventional transversal sections. The remaining part was tangentially cut. Serial sections from all the blocks were stained according to a protocol for CIPO analysis. Findings were classified and diagnosis was based upon international criteria (71, 103). Sections were also stained for GnRH (70, 159).

The number of GnRH and LH receptor +/- neurons per mm length of myenteric ganglia in transversal sections was counted, and the amount of GnRH + neurons was expressed as percentage of the total number of neurons. Method accuracy was verified using protein gene product (PGP) 9.5-labeled neurons.

ELISA

Analysis of anti-GnRH antibodies was carried out by an ELISA method that has been improved during the work with this thesis; its latest version is presented below and is also used in Paper V. The ELISA plates were coated with human GnRH and blocked with bovine serum albumin (BSA). Appropriately diluted serum was added, and antihuman biotinylated antibody was used to develop a color reaction measured in a spectrophotometer. Antibody levels were then presented as relative units (RU). Regarding Paper V, cut-off value in the control group was defined as levels > 97.5th percentile. In Paper III, 2 matched controls per patient were used.

Paper V

Patients underwent IVF according to clinical routines. Nasal inhalations of the GnRH analog buserelin (Suprecur®, Sanofi-Aventis, Bromma, Stockholm) were

used. Dosage varied according to clinical response. As treatment-naïve samples for patients, blood samples taken during pre-IVF screening were used in accordance with the Swedish Biobanks in Medical Care Act (SFS 2002:297). Blood samples after treatment were collected after the last inhalation. ELISA assays as described above were performed on this serum, concerning GnRH and GnRH-R IgM, IgG, and IgA. The VAS-IBS questionnaire from Paper II was used and completed before the start of the treatment and after the first 3 weeks of treatment with buserelin. Five years after the initial treatment, the VAS-IBS questionnaire and the 36-item Short-Form questionnaire (SF-36) were sent to patients at home. The quality-of-life form SF-36 is divided into 8 subscales, for physical functioning, role functioning-physical, bodily pain, general health, vitality, social functioning, role functioning-emotional, and mental health (179). Additionally, 2 dimensions can be calculated, physical and emotional health, according to weighting of the 8 subscales.

Paper VI

The animals were weighed prior to, and weekly during, the study. Blood sampling was performed by heart puncture before euthanizing the animals. The stomach, ileum, and transverse colon were used for analysis, as well as tissue samples from the distal part of the uterine horn, the hypothalamus, and the pituitary. Cryo- and paraffin-embedded material was processed for immunocytochemistry and histochemistry.

The thickness of mucosa, and circular and longitudinal muscle layers, was measured.

Antibodies against human neuronal protein HuC/D (HuC/D) and PGP 9.5 were used as general neuronal markers. Neurons were counted in submucous and myenteric ganglia on longitudinally cut sections; a total length of 30 mm, cut at 6–9 different depths per region and rat was used. Subpopulations were studied with regard to GnRH, GnRH receptor, LH receptor, VIP, or nNOS. Glial cells were studied using S100. Apoptotic neurons were demonstrated using antibodies against activated caspase-3. For ICC detection, c-kit receptor was used. T-lymphocytes were studied using CD3. Mast cells were examined using toluidine blue staining and eosinophils using eosinophilic peroxidase.

Serum analyses

The inflammatory markers interleukin 4 (IL-4), interleukin 5 (IL-5), interleukin 13 (IL-13), interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and keratinocyte-derived chemokine/growth-related oncogene (KC/GRO) were measured in sera. Antibodies against GnRH were also studied, as has been described for Paper III.

Statistical analyses

Statistical analyses were performed with SPSS 17-20 (Statistical Package for the Socal Sciences). Parameters affected by age and gender have been standardized using a linear regression model in which these parameters were added as covariates. Parameters were then expressed as z-scores within Paper I and Paper II. When in doubt regarding skewness, variables were analyzed for normal distribution by Kolmogorov-Smirnov test. Skewed distribution of several variables within the thesis has led to the Kruskal-Wallis test or Mann-Whitney U-test being used for comparisons between groups, and the Spearman rank correlation test for correlations. Fisher's exact test has been used for categorical variables. Results have been presented as median (interquartile range (IQR) limits) unless otherwise stated. In Paper VI, Dunn's multiple comparison test (all comparisons against control) or one-way analysis of variance test (ANOVA) was followed by Bonferroni's post hoc test, which was also employed. P-values <0.05 were considered statistically significant.

Results

Paper I

In patients with pSS, the age- and gender-standardized $t_{½}$ and t_{lag} were significantly prolonged in comparison to controls. Twenty-nine percent and 43% of patients with pSS had a pathologically (>2 standard deviations) prolonged $t_{½}$ and t_{lag}, respectively (Table 2). Although 82% of patients reported various non-exocrine symptoms, these were not associated with signs of IGE (data not shown).

T_{lag} was found to significantly correlate with both ESR (r_s = 0.51; p = 0.01) and IgG (r_s = 0.43; p = 0.02). Accordingly, the ESR and IgG were found to be significantly increased in patients with pathological t_{lag} compared to patients with normal t_{lag} (24 mm (range 17–34) vs. 9 mm (range 7–16), p = 0.03, and 19.6 mm (range 14.1–30.8) vs. 15.2 mm (range 10.9–18.8), p = 0.03, respectively).

Rheumatoid factor seropositive patients had significantly prolonged standardized times as compared to RF seronegative patients for $t_{½}$ (1.61 (0.09, 2.86) vs. -0.75 (-1.24, 0.25), p = 0.02, respectively), and t_{lag} (2.39 (1.18, 4.22) vs. -0.44 (-0.91, 1.05), p = 0.02, respectively). In addition, antinuclear antibody (ANA) seropositive patients had a non-significant tendency towards a prolonged $t_{½}$ and t_{lag} (p = 0.06).

Patients with pSS were found to have both parasympathetic and sympathetic dysfunction. Furthermore, patients reported significantly more AD symptoms in comparison to controls, mirrored by significantly increased ASP scores (Table 2).

Only one autonomic test variable, namely, the lowest diastolic blood pressure ratio, was found to be significantly correlated with t_{lag} (r_s = -0.47; p = 0.01).

Thirteen patients (46%) were found to suffer from IBS and 25 patients (89%) from FD according to the Rome III criteria (76). However, no significant associations between presence of symptoms of IBS or FD and signs of IGE were found (data not shown).

Table 2
Results of the octanoate breath test in 28 patients with primary Sjögren's syndrome (pSS) and 50 controls, and results of the objective autonomic nervous function tests (ART) and the Autonomic Symptom Profile (ASP) questionnaire in patients with primary Sjögren's syndrome (pSS)

	pSS patients	Controls	p-value
Half time ($t_{½}$)	1.18 (-0.71, 2.06)	-0.06 (-0.72, 0.74)	0.03*
Half time ($t_{½}$) pathological	29%	2%	0.00***
Lag time (t_{lag})	1.40 (-0.14, 3.11)	-0.03 (-0.57, 0.66)	0.00***
Lag time (t_{lag}) pathological	43%	4%	0.00***
ART variables			
E/I ratio	-0.82 (-1.47, 0.20)	-0.25 (-0.62, 0.60)	0.01**
AI	-0.17 (-0.90, 0.51)	0.03 (-0.67, 0.65)	0.57
VAC index	0.31 (-0.43, 1.60)	0.09 (-0.67, 0.62)	0.07
lSBP ratio	-0.64 (-1.26, 0.27)	0.00 (-0.61, 0.70)	0.00**
lDBP ratio	-1.66 (-2.80, -0.29)	0.00 (-0.47, 0.54)	0.00***
ASP variables			
Orthostatic intolerance	1.35 (-0.31, 2.45)	-0.39 (-0.78, 0.79)	0.00***
Urinary dysfunction	0.12 (-0.55, 1.98)	-0.51 (-0.71, 0.32)	0.02*
Gastroparesis	0.00 (0.00, 1.50)	0.00 (0.00, 0.00)	0.00***
Autonomic diarrhea	0.66 (-0.53, 2.10)	-0.42 (-0.60, 0.68)	0.02*
Constipation	1.00 (-0.56, 2.60)	-0.30 (-0.52, -0.18)	0.07
Secretomotor dysfunction	3.36 (2.13, 4.41)	-0.45 (-0.72, 0.52)	0.00***
Pupillomotor dysfunction	1.84 (0.87, 3.00)	-0.42 (-0.71, 0.55)	0.00***
Vasomotor dysfunction	0.89 (-0.45, 2.78)	-0.33 (-0.49, -0.20)	0.00**
Reflex syncope	0.00 (0.00, 0.00)	0.00 (0.00, 0.00)	0.61
Sleep disorder	0.39 (-0.11, 1.80)	-0.05 (-0.79, 0.35)	0.00***
Total score	2.35 (0.72, 3.30)	-0.21 (-0.82, 0.72)	0.00***

Results are presented as z-scores (median (IQR limits)) adjusted for age and gender as well as percentage with pathological increased time defined as a z-score ≥2. P-values were calculated using the Mann-Whitney U-test and Fisher's exact test, respectively. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. E/I = Expiration/Inspiration R-R intervals, AI = acceleration index, VAC = Vasoconstriction index in response to contralateral cooling, lSBP = lowest systolic blood pressure in tilt test, lDBP = lowest diastolic blood pressure in tilt test.

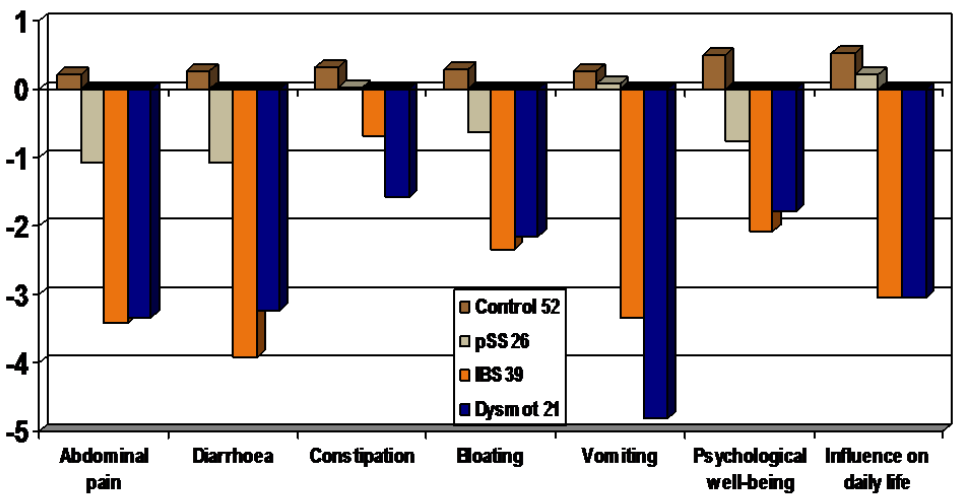
Paper II

Healthy subjects scored high values on the VAS-IBS scale (median values 95–100, interquartile ranges 78–100), except for bloating and flatulence that was present also in controls (86 (71–99)). Before comparison between groups, scores were standardized for age, since pSS patients were significantly older. Both patients with IBS and those with motility disorders rated their gastrointestinal symptoms as more severe compared to controls. Both groups differed significantly from controls in all variables. There was no statistical difference in any of the individual symptoms between these 2 groups, except for vomiting and nausea, which were found to be more common in dysmotility patients (Figure 3).

Although patients reported great impact of intestinal symptoms´ influence on daily life, their overall psychological well-being was not affected to the same extent (Figure 3).

All variables differed significantly between controls and patients with pSS (Figure 3). However, patients with pSS rated their gastrointestinal symptoms as less severe than patients with IBS and dysmotility. They had significantly less severe symptoms than IBS patients in all variables, except for constipation ($p = 0.186$). Compared to patients with motility disorders, they differed in all variables, except constipation ($p = 0.247$) and psychological well-being ($p = 0.252$) (Figure 3).

Figure 3
Visual Analog Scale for Irritable Bowel Syndrome (VAS-IBS) z-scores



Paper III

No complications were reported in relation to the laparoscopy-assisted procedures. A majority of the patients had undergone abdominal and/or gynecological surgery several times. It was difficult to evaluate whether these were performed prior to or as a consequence of dysmotility-related symptoms. Five out of the 19 female patients (26%) suffered from endometriosis. Three patients had received GnRH analogs in combination with IVF and had also received GnRH analogs for endometriosis.

Histopathological analysis revealed inflammatory neuropathy as an independent disease or in combinations with myopathy in 11 dysmotility patients and degenerative neuropathy or combined myoneuropathy in the remaining 11 dysmotility patients.

All diverticulosis patients and non-obliterating carcinoma patients, who served as controls, were found to have normal histology in the samples. GnRH was found in the cytoplasm of approximately 50% of the myenteric neurons, whereas all other cell types of the bowel wall were negative. A group of submucosal neurons were also labeled for GnRH.

When the dysmotility patients as a group were compared to diverticulosis or non-obliterating carcinoma patients, there was no significant difference regarding percentage of neurons labeled with GnRH in small or large bowel ($p = 0.31$ and $p = 0.96$, respectively). However, 5 dysmotility patients demonstrated a markedly lower percentage of labeled neurons as compared to diverticulosis and non-obliterating carcinoma patients. These 5 could be characterized as outliers, as shown in the box-plot in Figure 4.

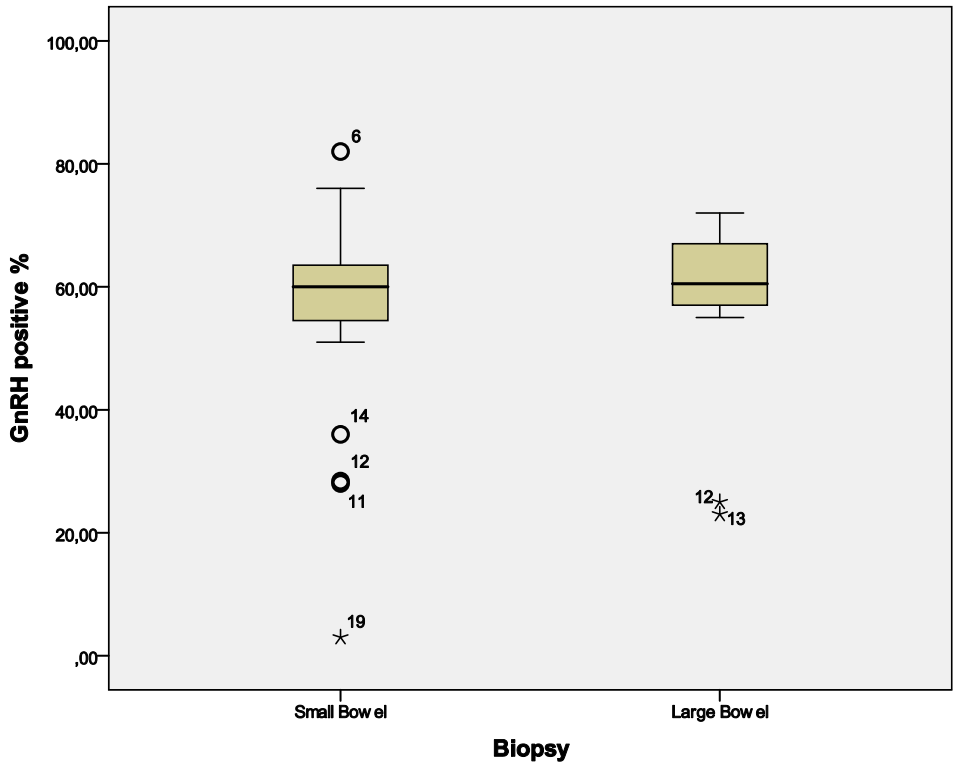


Figure 4
Percentage of GnRH-labeled neurons in 27 small bowel samples (8 controls, 19 dysmotility patients) and in 16 large bowel samples (8 controls, 8 dysmotility patients). The outlier number 12 represents a patient with both small and large bowel material.

Three out of these 5 patients had a history of treatment with GnRH analogs as part of IVF treatment prior to the onset of gastrointestinal symptoms, while the other 2 (one male) had no known history of such treatment.

When the dysmotility patients as a group were compared to controls, there was no significant difference ($p = 0.071$) regarding antibody levels in serum. In patients with reduced expression of enteric GnRH and available sera ($n = 4$), all had levels of antibodies above the range of age- and gender-matched controls. Two cases had been analyzed historically and were not reanalyzed; antibody levels were 400 μg compared to 0 μg (70), and 1.030 compared to reference value <0.800 for controls (159). In the presently analyzed patients, the antibody titer was 4.4 RU and 0.6 RU, respectively, as opposed to age- and gender-matched controls where all titers were found within the range 0.0–0.3 RU.

Paper IV

All specimens in both the dysmotility group and the control group displayed material positive for LH receptors. The LH receptor was positive in cytoplasm of approximately 50% of myenteric neurons and in glial cells, neutrophils, endothelial cells, and mast cells for both the dysmotility group and the controls. A group of submucosal neurons were also labeled for LH receptor. The percentage of labeled neurons in the dysmotility group was 42.50% (IQR 38.25–48.00, range 26.00–60.00) in the small bowel and 50.00% (IQR 23.00–51.00, range 12.00–59.00) in the large bowel. In controls, the median value was 47.14% (IQR 42.69–49.49, range 31.69–52.99) and 43.40% (IQR 42.14–46.48, range 32.53–47.44) in the small and large bowel, respectively, which was not significantly different between the groups ($p = 0.25$ and $p = 0.68$, respectively).

Paper V

The only significant difference in VAS-IBS between patients before treatment and the controls recruited five years later was with regard to increased nausea and vomiting in patients, 95 (IQR 87–98) compared to 98 (IQR 92–99), $p = 0.011$. Comparing VAS-IBS from before and after treatment shows that treatment in the IVF setting had significant negative effect on constipation, nausea and vomiting, psychological well-being, and the intestinal symptoms' influence on daily life. The amount of abdominal pain and bloating showed a non-significant tendency towards worse symptoms during treatment, $p = 0.052$ and $p = 0.079$, respectively, whereas diarrhea was not influenced in a major way ($p = 0.617$).

Abdominal pain had deteriorated at the five-year follow-up, from 92 (79–97) before treatment compared to 84 (71–97) at follow-up, $p = 0.041$, but psychological well-being had improved compared to the measurement before treatment, from 87 (69–96) to 93 (79–98), $p = 0.036$. Nine patients (out of 62 responders) had marked deviations in VAS-IBS compared to before treatment. These patients were contacted by phone, and the aggravation in symptoms seemed to be explained by development or exacerbation of IBS symptoms. No one had developed severe dysmotility. No correlations could be found between number of treatments and any of the VAS-IBS or SF-36 variables.

Comparing results between patients and controls, obtained in the questionnaires sent home to patients 5 years after treatment, the only significant difference across groups was in the role-emotional domain of SF-36, where patients scored norm-based scores median 56.2 (55.3–56.2) compared to 56.2 (49.2–56.2) in controls (p

= 0.012). No differences were detected regarding feeling of incomplete evacuation and need to defecate (data not shown).

IgM antibodies against GnRH in IVF patients before and after treatment did not occur more frequently over cut-off values than in non-treated blood donor controls. Regarding GnRH-R on the other hand, 2.9% of controls vs. 12% of IVF patients ($p = 0.075$) expressed antibodies, and hence showed a trend towards significance. However, when comparing patients before and after treatment, 12% expressed antibodies before treatment and 9.6% after treatment (not significant). Two patients had a major increase in antibody level against the receptor after treatment, but this was unrelated to symptoms.

Paper VI

All rats looked healthy and exhibited normal activity throughout the study period. No difference in weight gain between the saline- (control) and buserelin-treated (B1–4) rats was observed. At the end of the first 3 treatment sessions, buserelin-treated rats showed a transient increase in body weight compared to control rats ($p < 0.01$). Visceral organs appeared normal. Saline-treated rats were pooled into one control group.

Hematoxylin-eosin revealed a normal histology of the uterus and gastrointestinal tract in all rats. Repeated sessions of buserelin treatment (groups B3 and B4) induced a significant thickening of the uterine musculature compared to controls (medians: control rats 310 μm, B1 rats 370 μm, B2 rats 386 μm, B3 rats 446 μm ($p < 0.01$), and B4 rats 435 μm ($p < 0.05$)). Thickness of intestinal layers was measured, but displayed no significant pattern across groups.

In fundus, 4 sessions of buserelin treatment (B4) significantly reduced the number of submucous neurons ($p < 0.05$). In myenteric ganglia, the numbers of neurons were approximately 10/mm in control, B1, and B2 rats, while being markedly decreased in rats belonging to the B3 ($p < 0.05$) and B4 groups ($p < 0.001$).

In ileum from controls and after 1 to 3 sessions of buserelin treatment (B1–3), the numbers of submucous and myenteric neurons were approximately 4/mm and 10/mm, respectively. A significant reduction in the numbers of both submucous ($p < 0.05$) and myenteric neurons ($p < 0.01$) were noted in B4 rats.

In colon, 6 submucous neurons/mm were noted in controls and in buserelin-treated B1–3 rats. The number of submucous neurons was reduced in B4 rats ($p < 0.01$). Myenteric neurons were 17/mm in controls as well as in buserelin-treated B1–2 rats. A significant reduction in the numbers of myenteric neurons was detected in both B3 and B4 rats ($p < 0.001$).

No GnRH- or GnRH-R-IR nerve cell bodies or fibers were detected in rat gastrointestinal tract, irrespective of treatment. Rat hypothalamus, used as positive control, contained numerous intensely stained GnRH- and GnRH-R-IR nerve cell bodies and fibers.

Buserelin treatment did not affect the relative numbers of neurons IR for VIP, except in myenteric neurons in colon from B2 rats ($p < 0.05$).

Comparison of controls and B1–4 rats revealed no differences in the relative numbers of nNOS-IR submucous neurons in ileum, while in colon a significant increase of such neurons was noted in B4 rats ($p < 0.01$). In myenteric neurons, nNOS-IR increased in fundus of B3 rats ($p < 0.01$), and in colon of B1 ($p < 0.05$) and B4 rats ($p < 0.01$).

Intense LH receptor immunoreactivity was found in enteric neurons throughout the gastrointestinal tract, irrespective of treatment. In colon from control rats, approximately 10% of submucous and 20% of myenteric neurons displayed LH receptor immunoreactivity. In rats treated four sessions with buserelin (B4), markedly reduced numbers of both submucous and myenteric LH receptor-IR neurons were noted ($p < 0.05$). Approximately 3% of submucous and 12% of myenteric neurons displayed LH receptor immunoreactivity.

Evaluation revealed no differences in occurrence or topographic distribution of glia within the gastrointestinal tract between controls and buserelin-treated rats.

No signs of increased presence of inflammatory cells (eosinophilic leukocytes, T-cells, or mast cells) were noted in the gastrointestinal tract. Neither was there any increase in circulating levels of interleukins/cytokines noted after the buserelin treatment. GnRH antibody titers measured in sera were low or absent and did not differ in buserelin-treated as compared to control rats.

Due to the finding that buserelin-induced neuronal loss was particularly significant in colon, extended studies on the presence of apoptotic neurons, ICC, T-lymphocytes, and eosinophilic leukocytes were performed using this gastrointestinal region.

The major finding in this subgroup analysis was that in control, B1, B3, and B4 rats, extremely few (less then 1% of total) submucous neurons IR for activated caspase-3 were noted, but in B2 treated rats, the relative number of activated caspase-3-IR submucous neurons increased and comprised 6.5% ($p < 0.01$).

Discussion

IBS and other FBDs are widespread and affect approximately 10%–15% of a western population, affecting women 1.5–3 times more often than men (77). Trying to explain why women are affected to a larger extent has led to speculation regarding connections between sex hormones, particularly progesterone, and gastrointestinal function (180, 181). GnRH is the hypothalamic hormone in the sex hormone axis. The GnRH analog leuprolide acetate has previously been shown to stimulate cycling motor activity in rat gut, through a yet unknown neural mechanism (160). In addition, leuprolide significantly decreases nausea, abdominal pain, early satiety, anorexia, and abdominal distension in patients with FBD (2, 164). Huang et al. (153) have shown GnRH immunoreactivity in rat, in small and large bowel, and in parasympathetic ganglion. Within this thesis GnRH has also been shown to be present in the human ENS in Paper III (182), and antibodies against the peptide have been shown to be more common in IBS and dysmotility patients as compared to controls (94).

GnRH and its receptor have also been demonstrated in a wide array of organs, including rat submaxillary glands, where IR materials were colocalized in the epithelial cells of the serous acinus and glandular duct (143, 183). Furthermore, exocrine glands are the main target for inflammation in pSS (122). Antibodies against GnRH have been found in the pSS population studied in Paper I, in which the population that earlier had demonstrated high prevalence of GnRH antibodies also showed markedly impaired gastric emptying related to inflammatory and serological markers (120, 184). Although IGE, at least in diabetes mellitus, is thought to be related to AD (185), and despite our finding of AD in Paper I, signs of AD and IGE were not associated. Autonomic dysfunction in pSS has been attributed to various immunological mechanisms such as antimuscarinic-3 receptor (M3R) antibodies (186-188); cytokines interfering with nervous signaling (189, 190); and inflammation of autonomic nerves, nerve vessels, and ganglia (191, 192). Since the M3R have a role in regulating gastrointestinal motility (193), the anti-M3R antibodies also may play a role in delayed gastric emptying, as has been previously suggested by Kovacs et al. (119) in patients with pSS and by Goldblatt et al. (194) in patients with systemic sclerosis. The effects of these antibodies may not be detected by the cardiovascular autonomic reflex tests used within Paper I. The lack of association between signs of AD and objective signs of IGE could also

be due to differences in mechanisms behind cardiovascular AD and IGE, or to the small sample size. End-organ failure, the effects of which are difficult to distinguish from the effects of AD, may also obscure associations between AD signs and IGE. The lack of associations between gastroparesis symptoms and objective signs of IGE are in accordance with previous studies in diabetes mellitus. The discrepancy is well known and is under evaluation (195-197).

Comparing the different patient entities within this thesis, all linked to elevated levels of GnRH antibodies, Paper II would show another conflict seen in daily practice. Patients with functional diagnosis and severe motility disorder were hard to distinguish from one another using a symptom-based questionnaire. Health care professionals consider dysmotility a more morbid condition than IBS, while IBS patients seem to experience the same degree of symptoms. pSS patients were found to represent a group in-between healthy subjects and IBS and dysmotility patients, with regard to presence of gastrointestinal symptoms. The findings underlined that the very feasible, easy-to-use questionnaire has its greatest potential when following the same patient over time or separating people with gastrointestinal symptoms from healthy individuals. Distinguishing between groups with objective signs of dysmotility and those with IBS was, however, not possible using the VAS-IBS questionnaire (198).

The case of CIPO that was the origin for this thesis showed a marked reduction of GnRH-containing neurons in the ENS (70). This led to the scrutiny of full-thickness samples in 22 patients with severe gastrointestinal motility disorders, in Paper III, revealing 5 patients with decreased levels of enteric GnRH-containing neurons, underlining the possibility for a role as a neuropeptide, since it is not found in other cells. Three of these 5 had had repeated treatments with GnRH analogs in an IVF setting (182). Serum was available for antibody analysis in 4 out of the 5 patients, and these expressed antibodies against GnRH (182). The importance of antibodies against GnRH in the development of dysmotility is not known (70, 94, 159). Development of antibodies against GnRH after intermittent buserelin treatment has been described earlier, but in the setting of allergic reaction (199). Antibodies against GnRH may be involved in neurodegeneration, but did not affect neuron survival in vitro (159). Presence of autoimmune processes against the ENS in CIPO, especially when secondary to malignancy, has long been known (200, 201). In some cases, antibodies have been proposed as evoking the enteric neurodegeneration (113). However, the antibodies found in our patients may also be secondary to exposure of GnRH during a degenerative process started by other factors, as is the case in other autoimmune conditions when antibodies that serve as markers for the disease are innocent bystanders rather than being pathogenic (202). As GnRH and LH receptor content in the gastrointestinal tract did not differ between controls and patients when whole

groups were compared, depletion of these peptides is not responsible for the development of ED or CIPO in general.

In Paper V, the evaluation of 124 patients who underwent IVF treatment would reveal that treatment did not give rise to antibodies against GnRH or its receptor. However, the treatment led to several gastrointestinal symptoms when administered. In a five-year perspective, abdominal pain deteriorated, but psychological well-being improved. One reason for improved psychological well-being after 5 years compared to prior to IVF, could be explained by the great stress it means to go through IVF treatment. None of the investigated patients developed any severe gastrointestinal motility disorder. It is well known that treatment with buserelin is associated with gastrointestinal side effects (203), as well as that IVF is associated with many psychological aspects that might have an effect on gastrointestinal symptoms (204). No relation between gastrointestinal symptoms and expression or levels of antibodies in sera was present; suggesting that the dysmotility patients described earlier in Paper III are not representative of the majority of patients receiving GnRH as part of IVF treatment (70, 182). Genetic factors, concurrent infection, and other, yet unknown sensitivity to GnRH treatment might have been involved in the cases in Paper III, explaining their possibly GnRH-related severe dysmotility. That buserelin affects VAS-IBS parameters during treatment underlines prior speculation regarding GnRHs role as a player in gastrointestinal motor control (144, 166).

Applying what is known today, trying to explain the effects of GnRH on the bowel, it is possible to speculate on two main explanations.

Endogenous GnRH is secreted into the hypothalamic portal circulation in a pulsatile fashion. It is rapidly degraded and barely detectable in peripheral circulation. In contrast, systemically administered GnRH analogs have a longer half-life and cause greater exposure of the peripheral tissues (141, 143). GnRH analogs have been suggested to act directly on enteric neurons (144), and GnRH and its receptors have been reported to occur and play a role in the rat digestive tract (153, 154, 158, 160, 166). The effect evoked by GnRH through the GnRH-R on the individual cells in turn depends on a complex combination of pathways and factors (143). The specific role of GnRH in the gut is today not completely elucidated, but GnRH analogs have been shown to inhibit gastric secretion and gastrin release in rat and dog (156, 157), to inhibit cell proliferation in gastric epithelium (158), and to protect enteric rat neurons in culture when continuously stimulated (159), whereas shorter stimulation inhibits cell proliferation in gastric smooth muscle cells (154). It also induces apoptosis and inhibits cell proliferation in several cancer cells (149, 205). This, taken together, renders a theory wherein GnRH could exert a direct effect on the gastrointestinal tract. However, within Paper VI, we could not confirm that rat enteric neurons express GnRH or GnRH

receptors. These negative findings were unexpected, since the methods used were able to stain GnRH in hypothalamus and pituitary, but not bowel, as has been achieved historically by Chinese research groups using different techniques (153). The suggestion that rat ENS lacks GnRH receptors is, however, supported by disposition studies where 3H-labeled buserelin given to rats bound only to the pituitary after bolus, and only a trace amount was found in the gastrointestinal tract (206). The latter trial has been repeated by Heinrich et al. (207). For humans, however, the finding in Paper III strongly supports the presence of GnRH, at least, in human myenteric neurons. Preliminary polymerase chain reaction (PCR) studies also suggest that GnRH and its receptor are present in the gastrointestinal tract (unpublished).

A second explanation for the GnRH effect on the bowel might be that GnRH acts through the sex hormone axis, as it initially raises circulating levels of FSH and LH (141). Prolonged administration turns off the release of FSH and LH, the effect sought in the IVF setting (141). It is possible that GnRH exerts its effect on the gastrointestinal tract indirectly, through pituitary LH release or absence. LH has also been shown to influence motor activity in rat small bowel (165). Furthermore, in Paper IV, LH receptors were described on several different cell types of human gastrointestinal tract, including myenteric neurons (208). In Paper VI, rats treated repeatedly with the GnRH analog buserelin showed reduced numbers of submucous and myenteric neurons in fundus, ileum, and colon compared to saline-treated controls. Paper VI also showed that in rat, numerous submucous and myenteric neurons in fundus, ileum, and colon expressed LH receptors. The percentage of LH-positive neurons also declined in colon in response to GnRH treatment, advocating its importance in the intestinal effect of GnRH. The finding of enteric neuron death in rats was preceded by an increase in activated caspase-3 after two treatment sessions, which suggests increased apoptosis (209, 210). Of interest in this setting is the finding that some laboratory mice in lactation develop fatal CIPO, along with increased levels of activated caspase-3 in the gastrointestinal tract (211). The enteric neuron death in rats after repeated GnRH treatment is in line with a reduced number of GnRH-containing neurons in the human gastrointestinal tract in Paper III after repeated GnRH treatment. The effect exerted by GnRH is dependent on which cell type the GnRH receptor is situated on (143). In the pituitary, stimulation of the receptor leads to synthesis and release of FSH and LH (141). In some cancer cells, stimulation leads to increased apoptosis and inhibited cell proliferation (149, 205). Thus, the effect of GnRH on enteric neurons may be similar to the effect on cancer cells.

Taken together, the findings in this thesis support the idea that GnRH affects intestinal motility and symptoms, although the exact mechanism through which it acts remains to be further elucidated. The decrease in neuronal population in rat ENS related to GnRH administration is disturbing, but earlier epidemiological

studies (138) and Paper V provide reassuring data on the safety of IVF treatment for the majority of infertility patients. The findings within this thesis, and the fact that GnRH seems important in many very different settings, not least in cancer therapy, further underlines the fact that the role of GnRH is not confined solely to its effects within the reproductive axis, but also includes, among others, intestinal effects.

Strengths and Limitations

The strengths of this thesis are that the studies emanate from a clinical problem, found in a patient with severe gastrointestinal dysmotility after IVF treatment, and that it involves a comprehensive approach, including both retrospective studies of patients with gastrointestinal dysmotility and FBD, and a prospective study evaluating gastrointestinal symptoms in IVF-treated patients, as well as an animal trial.

There are several limitations of the studies included in the thesis. These include the small sample sizes in the studies. In addition, control groups would have benefited from being better matched as well as drawn from the general population. However, we have tried to address and compensate for these flaws.

Recruiting controls for Paper V was extremely challenging; 248 questionnaires were sent out to randomized controls in the general population, and after one reminder only 29 were returned. The reasons behind this are probably multiple. Women this age are likely to be working full time and also caring for a family, hence filling in even a short questionnaire might be difficult to prioritize. With only 12% answering the questionnaire, the risk of not getting a representative sample of the population was imminent, and the remainder of the control group was recruited among women of the same age working at Skåne University Hospital. In doing so, we hope to have avoided selection bias, but it may also be argued that in doing so we have introduced it.

Another flaw is the absence of objective examinations of intestinal motility in the IVF group, which would, of course, have been preferable. A five-year follow-up of antibody concentrations would also have been preferable, but only 6 out of 124 subjects consented to new blood samples, even though cinema tickets and compensation for travel expenses were offered.

The unexpected findings in Paper VI, where GnRH- or GnRH-R-IR neurons were not present in rat digestive tract, remain surprising. However, using the setup described within the study, they remain reliable. Immunohistochemical difficulties in staining bowel peptides or unspecific antibody properties might explain the

finding. No less, the finding needs to be further addressed. PCR methods or other techniques may be valuable in achieving this, since the antibody used by the Chinese group that demonstrated the peptide earlier is not readily available.

Furthermore, the commercial antibodies used in this thesis are developed against GnRH I. The cross-reactivity to GnRH II is not known by the manufacturer (personal communication). Theoretically, both GnRH I and GnRH II may be present as neurotransmitter and/or hormone in the gut, as in the CNS, but GnRH II is not guaranteed to be detected by our methods used.

In summary, all papers within the thesis, in some way or another, demonstrate a relation between GnRH and gut motility. At this stage of research on the topic, the ability to further underline this relation has to be viewed as a strength, although it would have been preferable to be able to more precisely state the role of GnRH and the mechanisms behind its actions.

Future perspectives

The thesis identifies GnRH as a player in the regulation of gastrointestinal function and motility. It also indicates that some patients, with previous GnRH treatment, have decreased amounts of the peptide in their ENS. Since few patients who suffer clinically have a history of treatment with GnRH analogs, it is important to identify the link between treatment and symptoms, and in doing so, identify the persons for whom treatment might be harmful.

The effect of repeated GnRH administration on the ENS in rat was established in Paper VI, where the number of neurons decreased. In the near future, further animal trials seem to be the best way of trying to identify connections between GnRH stimulation and loss of neurons in the gastrointestinal tract, and the mechanism through which GnRH exerts this effect. Two possible scenarios need to be pursued. In a first step, a direct GnRH effect on enteric neurons could be further investigated using, for instance, cell cultures. Cultured cells could be exposed to GnRH in different ways, and not only continuously stimulated, which seemed to have a protective effect on neurons (159). The second scenario is to further explore a possible indirect effect through other downstream hormones such as LH. This could be done by laboratory trials on cell cultures using this peptide.

The physiological effects of the neuronal loss within Paper VI were not studied in detail. Treated rats did not lose significantly in weight, but other possible physiological effects such as alterations in transit time in treated rats should be studied as well as other metabolic parameters. Perhaps other stressors should be

added into the model to try to provoke the development of dysmotility or neuronal loss.

To study the GnRH content in full-thickness biopsy material, genetic analyses and a possible history of GnRH treatment of more patients with severe dysmotility also seems an attractive way of trying to further map potential causes of GnRH depletion and dysmotility. Our findings need verification in other cohorts, since the number of subjects is small.

Further, the possible relation that is hinted at in Paper III, between infertility, endometriosis, gut dysmotility, and possibly GnRH depletion, needs further attention. This could be studied using full-thickness material from patients undergoing surgery for their endometriosis, and could be of particular interest, since endometriosis itself has been shown to affect the ENS locally (212), and possibly disturbed menstrual flow and hence tubal dysmotility (213, 214).

In conclusion, verifying our findings in larger patient cohorts and further animal trials as well as cell culture trials seem to be the most important next steps.

Conclusions

Forty-three percent of pSS patients have objective signs of IGE, which are associated with increased levels of ESR and IgG, and are more common in RF-seropositive patients. Impaired gastric emptying is, however, poorly associated with both objective and subjective AD variables as well as gastrointestinal symptoms.

The VAS-IBS questionnaire can be used to assess the level of gastrointestinal symptoms in individual patients. However, symptom-based VAS scores do not aid clinicians in differentiating between different FBDs and motility disorders.

Gonadotropin-releasing hormone is present in about 50% of human myenteric neurons, possibly in the role of a neuropeptide, since it is not found in other cells. In addition, there seems to be a subgroup of patients with severe dysmotility who express antibodies against GnRH and have a reduced expression of GnRH-containing neurons in the ENS.

Luteinizing hormone receptors are present in the gastrointestinal tract in patients both with and without severe dysmotility, thus possibly modulating gastrointestinal motility. In addition, their presence provides a possible mechanism through which GnRH may affect the gastrointestinal tract.

Buserelin treatment in the setting of IVF causes gastrointestinal symptoms during treatment, but within the study, it did not cause significant dysmotility or antibodies against GnRH or its receptor.

Repeated administrations of buserelin are accompanied by up to 50% loss of enteric neurons in rat. Buserelin-treated rats do not display high titers of GnRH antibodies in serum, nor do they lose weight as compared to saline-treated control rats.

Taken together, GnRH and LH receptors are expressed in about half of human enteric neurons. GnRH seems to affect gastrointestinal motility and function. Some patients with motility disorders express antibodies against GnRH in serum and display lower levels of the peptide in the bowel. Repeated treatment with the peptide in rat causes loss of myenteric neurons.

Populärvetenskaplig sammanfattning

Könshormon påverkar tarmrörelser

Många av kroppens funktioner styrs av hormoner. Att hormoner som vi i första hand förknippar med fortplantning också skulle kunna spela en avgörande roll för tarmfunktion är dock en relativt ny upptäckt.

Få saker är så viktigt för en människas välbefinnande som en välfungerande magtarmkanal. De flesta av oss tänker inte på att frukostfrallan vi precis svalt ner är på väg mot en mycket komplicerad utvinnings-, sönderdelnings- och sorteringsprocess som hade fått de flesta processingenjörer att rygga tillbaka. En normal tarmfunktion är ett komplext samspel mellan hormoner och nervsignaler som ingen ännu lyckats kartlägga i sin helhet. Det nätverk, nervsystem, som styr tarmmotoriken innehåller ungefär lika många nervceller som hjärnan som styr vårt själsliv, tänkande och alla våra rörelser.

En magtarmkanal i otakt upplever alla någon gång i livet, exempelvis i samband med en stressig situation eller en maginfluensa. Fler än var tionde människa har så påtagliga besvär från magtarmkanalen över tid att det klassas som sjukligt. Besvären bland dem som klassas som sjuka varierar mycket, men det finns personer som är så svårt sjuka att de inte klarar att äta den mat de behöver. Bakom detta döljer sig sannolikt flera olika för oss ännu okända sjukdomar eller orsaker till varför tarmen inte fungerar som den ska.

Kvinnor har i större utsträckning än män besvär med tarmen. En kvinna som hade genomgått upprepade provrörsbefruktningar utvecklade en gravt störd tarmfunktion. Hon saknade mottagare på tarmen för ett hormon, det så kallade GnRH-hormonet, som används i samband med provrörsbefruktning. Denna avhandling syftar till att vidare kartlägga det eventuella sambandet mellan detta hormon och magtarmkanalens rörelser.

Eftersom individer med den reumatiska sjukdomen Sjögrens syndrom med mycket tarmbesvär, tidigare har visat sig bilda antikroppar mot GnRH hormonet, undersöktes dessa med avseende på magsäckstömning samt funktion i det icke-viljestyrda nervsystemet. Undersökningen visade att många hade förlångsammad magsäckstömning och att detta var relaterat till inflammation hos dessa patienter. Dessa resultat är publicerade i delarbete 1.

Vidare har, inom ramen för delarbete 2 olika patientgrupper som uttrycker antikroppar mot GnRH-hormonet värderats avseende sina symptom från magen med hjälp av en visuell skala som ofta används i sjukvården. Det skulle visa sig att skalan fungerade bra för att kartlägga symptomen men tyvärr inte var till någon hjälp när det gällde att kartlägga den bakomliggande orsaken till symptomen.

För att ytterligare kartlägga hormonets relation till tarmfunktion undersöktes inom ramen för studie 3, 22 patienter med mycket ovanlig och gravt störd tarmfunktion. Studien visade att fem i gruppen hade sänkt antal nervceller i tarmens nervsystem innehållande GnRH-hormonet och att tre utav dessa också fått provrörsbefruktning. Detta var en viktig observation som förtjänar fortsatt uppföljning, men den innebär på intet sätt att vi etablerat ett orsak-verkan samband mellan provrörsbefruktning och gravt störd tarmfunktion. Det faktum att de studerade patienterna tillhör en mycket hårt drabbad grupp som är enormt sjuk i sin tarm gör nämligen att många andra saker hos dessa individer kan förväntas påverka både tarmen och förmågan att fortplanta sig.

GnRH-hormonet används inom provrörsbefruktning och därför undersöktes inom ramen för studie 5, 124 patienter som fått provrörsbefruktning. I studien skulle det visa sig att behandling med syntetiskt GnRH hormon gav upphov till magsymptom i samband med att det tillfördes och vid uppföljning fem år efter behandlingen kvarstod ingen svår motorikstörning. Att behandlas med hormon innebar inte heller att man utvecklade antikroppar.

Hos den kvinna som var upprinnelsen till avhandlingen hade man gett upprepad provrörsbefruktning och först efter den fjärde behandlingen blev symptomen från magen riktigt tydliga. Med utgångspunkt i detta sattes inom ramen för arbete 6 ett djurförsök upp där råttor fick upprepade behandlingar med syntetiskt GnRH hormon med pauser emellan. Det visade sig då att i nervsystemet i råttornas tarm sjönk antalet nervceller, dock utan att påverka råttornas vikt. Vi lyckades i denna studie inte hitta GnRH hormonet i råttans tarm och råttorna utvecklade heller inga antikroppar. Kanske var metoderna för att påvisa detsamma inom ramen för denna stuide otillräckliga men fyndet ledde till spekulationen att hormonet hos råttorna och kanske hos människa har effekt via andra könshormon. Man kunde nämligen också kartlägga ett annat könshormon, luteniserande hormon (LH) som frisätts av just GnRH, i råttans tarm. Inom ramen för studie 4 kunde dessa receptorer för detta hormon också påvisas i mänsklig tarm.

Sammantaget kan sägas att fynden i avhandlingen ytterliggare understryker att könshormonet GnRH förefaller inverka på tarmen, något som också andra forskargrupper tidigare gjort gällande.

Att behandling med syntetiskt GnRH hormon som är relativt vanlig, ges förutom till patienter i samband med provrörsbefruktning också till patienter som besväras

av chokladcystor, polycystiska äggstockar, vissa cancerformer, för tidig och försenad pubertet och flera ytterliggare tillstånd, vanligen skulle kunna ge upphov till rubbningar i tarmens rörlighet är osannolikt i ljuset av att fler fall inte uppmärksammats trots extensiv användning. Vidare visar studie 5 att problemet inte heller är utbrett i samband med provrörsbefruktning. Resultaten i framförallt studie 3 och 6 ger dock viss anledning till vaksamhet i samband med upprepade provrörsbefruktningar då uppkomst av nya magsymtom bör bevakas. Det eventuella sambandet måste dock ses i ljuset av sin vanlighet där befolkningsgruppen i studie 3 representerar uppskattat 0.003-0.005 promille av befolkningen. Sannolikt är det också så att, de 3 fall där vi hittat lägre halter GnRH i tarmen och samtidig provrörsbefruktning, har någon annan känslighet, medfödd eller förvärvad eller kanske båda som gjort att mängden hormon innehållande nervceller i tarm sjunkit. Icke desto mindre förtjänar hormonets påverkan på tarmen ytterliggare uppmärksamhet då avhandlingen visar ett samband mellan hormonet och tarm där sambandets ursprung och natur inte än är fullständigt känt.

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