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Expression of Neuroendocrine Markers in Normal and Neoplastic Tissue with an Emphasis on Ghrelin and Obestatin

MALIN GRÖNBERG



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Abstract

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The aim of this thesis was to characterize the expression of the peptides ghrelin and obestatin, as well as other neuroendocrine markers in human normal tissues, in invasive breast cancer and a wide panel of neuroendocrine tumors (NETs).

In normal tissues the expression of ghrelin and obestatin was mainly localized to the gastric mucosa, and in lesser extent in the remaining gastrointestinal tract, endocrine pancreas and mammary glands. Double immunofluorescence studies demonstrated that ghrelin and obestatin were co-localized in the same cells displaying the same cytoplasmic distribution.

In normal breast tissue, ghrelin, obestatin, adrenomedullin, apelin and vesicular monoamine transporter 2 were specifically demonstrated in the luminal epithelial cells. Consecutive sections indicated that mammary epithelial cells could express several of these peptides. Secretogranin II and III were also detected in breast tissue, but their presence was restricted to the outer layer of myoepithelial cells, whereas chromogranin B immunoreactivity was found in both the epithelial and myoepithelial cells.

Ghrelin and obestatin immunoreactivity was seen in invasive breast cancer, where the expression could be correlated to factors associated with prognosis. Furthermore, multivariate analysis indicated that ghrelin expression was a possible independent prognostic factor for prolonged recurrence-free and breast cancer-specific survival.

In a panel of NETs and endocrine-related disorders it was revealed that ghrelin and obestatin immunoreactivity was primarily found in tumors originating from the respective normal tissues. The two proteins were detected in only a few cases and only occasional tumor cells were immunoreactive.

In conclusion, ghrelin and obestatin are localized in the gastrointestinal tract, endocrine pancreas and mammary glands. This thesis has contributed to our understanding of the distribution of ghrelin and obestatin in both normal tissue and tumor cells. A potential role of ghrelin as a prognostic factor in invasive breast cancer has been identified and should be further explored.

Keywords: ghrelin, obestatin, neuroendocrine marker, immunohistochemistry, neuroendocrine tumors, breast cancer, mammary glands

Malin Grönberg, Department of Medical Sciences, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

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To My Family

List of Papers

This thesis is based on the following papers, which will be referred to by their roman numerals:

- I **Grönberg M**, Tsolakis AV, Magnusson L, Janson ET and Saras J.
Distribution of Obestatin and Ghrelin in Human Tissues: Immunoreactive Cells in the Gastrointestinal tract, Pancreas and Mammary Glands
J Histochem Cytochem. 2008;56(9):793-801*

- II **Grönberg M**, Amini R-M, Stridsberg M, Janson ET and Saras J.
Neuroendocrine Markers are Expressed in Human Mammary Glands
Regulatory Peptides 2010;160(1-3):68-74*

- III **Grönberg M**, Fjällskog M-L, Jirström K and Janson ET.
Expression of Ghrelin is Correlated to Good Outcome in Invasive Breast Cancer
Manuscript

- IV **Grönberg M**, Tsolakis AV and Janson ET.
Expression of Ghrelin and Obestatin in Neuroendocrine Tumors
Manuscript

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Additional Publications by the author

- Saras J, **Grönberg M**, Stridsberg M, Öberg KE and Janson ET.
Somatostatin Induces Rapid Contraction of Neuroendocrine Cells.
FEBS Lett. 2007 May 15;581(10):1957-62
- Lopez-Egido JR, Stålberg P, Wang Y, **Grönberg M**, Grimfård P, Wang S
and Skogseid B.
Differentially Regulated Genes in *MEN1*-transfected BON Cells Using
RT-differential Display and Oligonucleotide Microarrays
Anticancer Res. 2009 Jun;29(6):1859-66

Supervisors

Professor **Eva Tiensuu Janson**. Department of Medical Sciences, Uppsala University, Uppsala, Sweden

Dr. **Apostolos Tsolakis**. Department of Medical Sciences, Uppsala University, Uppsala, Sweden

PhD. **Jan Saras**. Department of Medical Sciences, Uppsala University, Uppsala, Sweden

Faculty opponent

Professor **Mauro Papotti**. Department of Clinical and Biological Sciences, University of Turin, Orbassano, Torino, Italy

Review board

Chair: Professor **Christian Berne**. Department of Medical Sciences, Uppsala University, Uppsala, Sweden

Associate Professor **Jan Zedenius**. Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

Professor **Gunilla Enblad**. Department of Oncology, Radiology and Clinical Immunology, Uppsala University, Uppsala, Sweden

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Abbreviations

BCSS	Breast cancer-specific survival
BM	Basement membrane
BSA	Bovine serum albumin
DAB	3,3' Diaminobenzidine
EC	Enterochromaffin
ECL	Enterochromaffin-like
EPOS	Enhanced polymer one step
GH	Growth hormone
GI	Gastrointestinal
HR	Hazard ratio
HRP	Horseradish peroxidase
IR	Immunoreactive
LDCV	Large dense-core vesicle
NE	Neuroendocrine
NET	Neuroendocrine tumor
MEN-1	Multiple endocrine neoplasia type 1
PBS	Phosphate buffered saline
PC	Prohormone convertase
PDEC	Poorly differentiated endocrine carcinoma
RFS	Recurrence-free survival
SLMV	Synaptic-like microvesicle
SMA	Smooth muscle actin
SV2	Synaptic vesicle protein 2
TDLU	Terminal ductal lobular unit
TMA	Tissue microarray
VMAT1	Vesicular monoamine transporter 1
VMAT2	Vesicular monoamine transporter 2
WHO	World health organization

Introduction

The Endocrine System

The regulation of body functions is carried out by the nervous system, the immune system and the endocrine system.

The endocrine system is composed of a number of different cells that produce and secrete their signaling substances, hormones, in order to influence the function of other cells. It is a communication system defined as cells present in endocrine organs (endocrine glands), including the hypothalamus, pituitary, thyroid, parathyroid, pancreas, adrenals, ovaries and testis. It also includes single cells or cell clusters that are not part of glands: the diffuse endocrine system, scattered throughout other organs [1]. The diffuse endocrine system is composed of specialized endocrine cells located in the digestive tract and, to a lesser extent, in the respiratory and urinary tract. There are also endocrine cells present in other tissues (e.g., Merkel cells that are found in the skin). The gastroenteropancreatic system is probably the largest endocrine organ in the body [2] where numerous different endocrine cell types have been described [3].

Modes of Communication

In the endocrine system the most common way to communicate a message from one cell to another is *endocrine signaling*, where the hormones are released into the circulation and transported to their respective targets. However, other modes of communication exist as well. *Paracrine signaling* occurs when the hormone is conveyed over short distances by diffusion to act on neighboring cells, whereas *autocrine signaling* is defined as when the cell signals itself through a hormone that it synthesizes and, in turn, stimulates a target on the cell surface or within the cell that the cell then responds to [4].

Neuroendocrine Cells

The neuroendocrine (NE) cells are specialized to produce, maintain and release secretory amines and peptides. When studied through a microscope, NE cells appear with uniform nuclei and abundant granular or faintly staining cytoplasm. If they are gland-forming, they are present as solid or small trabecular clusters. When they are dispersed among other cells, however, they are often difficult to recognize in sections routinely stained with hematoxylin and eosin. In these cases immunostaining with general and specific markers for NE cells enables their exact identification [5].

Markers of Neuroendocrine Cells

The NE cells express various specific peptides, which are located in different compartments of the cell. At the ultrastructural level they are characterized by cytoplasmic membrane-bound dense-cored secretory granules (large dense-core vesicle, LDCV). In these vesicles several markers of endocrine differentiation, including chromogranin A (CgA), are stored before release [6]. They also contain small vesicles (synaptic-like microvesicles, SLMVs) corresponding to the synaptic vesicles of neurons [7]. The general antigenic asset of NE cells includes molecules of the endocrine cell machinery found in the cytosol (e.g., neuron-specific enolase and protein gene product 9.5) [8] or in the secretory granules (e.g., CgA in LDCVs and synaptophysin in SLMVs) [6, 9].

In many endocrine cells cleavage of peptide hormone precursors is mediated by prohormone convertases (PCs) [10]. In NE cells the most frequently detected convertases are PC2 and PC1/3, which are located in the secretory granules [11, 12].

Immunohistochemical staining for general and specific NE markers is routinely used for diagnosis of NE differentiation and NE tumors (NETs). Immunoreactivity vs. CgA and synaptophysin is considered a golden standard for immunohistochemical identification of normal and neoplastic NE cells.

The specific markers include cell-type-specific regulatory peptides and amines (e.g., insulin, glucagon and serotonin) [13-16].

General markers

The chromogranins and secretogranins

The granins, CgA, CgB, secretogranin (Sg) II and the less well studied Sgs III-VII constitute a family of soluble, acidic proteins present in the secretory granules of a wide variety of NE cells. Cgs and Sgs can be distinguished by the presence of a disulfide-bonded loop at the N-terminus of the Cgs [13, 17-19]. Granin mRNA has been found in cells of various NE tissues that have a regulated secretory pathway [20-22]. Cgs are considered to play an important role in secretory granule formation and secretory protein sorting [13, 17].

Chromogranin A

CgA is a glycoprotein consisting of 439 amino acids, which is located in the secretory granules of NE cells and has been widely used as a general NE cell marker in histopathological diagnosis [6]. CgA was the first granin to be characterized as an acidic protein co-stored and co-released with catecholamines from the bovine adrenal medulla [23]. Examples of CgA-containing cells are enterochromaffin-like (ECL) cells [24], enterochromaffin (EC) cells [25] and endocrine cells of the pancreas [26]. Furthermore, CgA is the precursor of several functional peptides including vasostatin, pancreastatin, catestatin and parastatin [17].

Chromogranin B

CgB is a protein of 657 amino acids that is also produced and stored in NE cells. CgB is a major granin of the human adrenal medulla and may be a more sensitive marker for pheochromocytoma and related tumors than CgA or SgII [27, 28]. In addition, CgB (but not CgA) can localize to the nucleus and has been reported to modulate gene expression [29]. It can also be processed to small peptide fragments, including secretolytin, which has been shown to have antibacterial properties [30].

Secretogranin II

SgII is another member of the granin family. One important peptide fragment generated from SgII is secretoneurin. Secretoneurin is found in both nervous and NE tissue [31, 32]. It has been shown to have several biological functions including stimulation or inhibition of neuron transmitter release, chemotaxis of white blood cells and fibroblasts, proliferation of vascular smooth muscle cells and endothelial cells and activation of cell adherence [33-37].

Secretogranin III

SgIII is also an acidic secretory protein. Its expression has been demonstrated in three of the four major islet cell types in human pancreas as well as in various NETs [38, 39]. It has been found to interact with CgA in secretory granules where binding of CgA to SgIII is necessary for CgA targeting to secretory granules [40].

Secretogranin V

SgV is present in the secretory granules of NE cells. Its cellular function remained unknown for a long time but now it is thought to be involved in the transport of inactive PC2 from the endoplasmic reticulum to the secretory granules as well as in controlling its enzymatic activity [41].

Other general markers

Synaptic vesicle protein 2

Synaptic vesicle protein 2 (SV2), is an integral glycoprotein of the vesicular membrane that was initially identified in the central and peripheral nervous systems, where it serves as a vesicular marker of small synaptic vesicles [42]. The exact function is still under debate but in SV2 knockout mice it seems to be important for normal function of the nervous system [43]. An involvement in the regulation of calcium-stimulated exocytosis has also been suggested [44]. It has been identified in a vast majority of NE cell types and NETs and has been shown to be an important general NE cell marker [45].

Synaptophysin

Synaptophysin is a glycoprotein that initially was found in small vesicular membranes of neurons and of chromaffin cells in the adrenal medulla [46]. The actual function of this protein remains elusive but for example, an interaction between synaptophysin and cholesterol has been proposed to be essential for the biogenesis of SLMVs in NE cells [47]. Because synaptophysin can be detected in various NE cells and NETs, it has been routinely used as a general marker for normal and neoplastic NE cells [48, 49].

Vesicle monoamine transporters (VMATs)

Monoamines (catecholamines, serotonin and histamine) are small molecules that play an important role in the central and peripheral nervous system as well as in the endocrine and immune system. They can act as neurotransmitters, hormones or autocrine and paracrine factors. Their function depends on the location of the cells that synthesize, store and release them.

The uptake of monoamines from the cytoplasm into secretory vesicles is mediated by VMATs along an ATPase-generated proton gradient [50-53]. Two isoforms, VMAT1 and VMAT2, have been isolated from rat and human tissue [54-56]. VMAT1 is expressed in EC cells and adrenal chromaffin cells, whereas VMAT2 is expressed in gastric ECL cells, beta-cells in pancreas and adrenal chromaffin cells; however, VMAT2 is also expressed in a population of central, peripheral, and enteric neurons [51, 57, 58]. VMAT1 has been suggested to be specific for ileal NETs [59], whereas VMAT2 is suggested as a marker for ECL cell tumors [60-62].

Specific markers

Serotonin

Serotonin (5-hydroxytryptamine, 5-HT) is not only an important neurotransmitter but is also a peripheral hormone. It is synthesized in the EC cells of the gastrointestinal (GI) tract [63] and the serotonergic neurons of the central nervous system [64]. Serotonin is implicated in a variety of physiological processes (e.g., vascular tone regulation, smooth muscle contraction, smooth muscle proliferation and hemostasis) [65]. Approximately 80–90% of serotonin in the body is found in the EC cells and enteroendocrine cells of the digestive system [66] where it influences GI motility [67]. Furthermore, serotonin is synthesized and secreted from NETs of the small intestine and contributes to the carcinoid syndrome [68, 69].

Other peptides

Adrenomedullin

Adrenomedullin is a 52 amino acid peptide, belonging to the calcitonin gene-related peptide family. It has been implicated in the modulation of various physiological functions, ranging from vasodilatation, neurotransmission/modulation to regulation of cell growth [70-73].

Adrenomedullin is widely distributed in mammalian tissues (e.g., lung, skin, GI tract, adrenal glands and brain) and is expressed in cardiac myocytes and vascular smooth muscle cells, as well as in NE cells of the GI tract and neural tissues [74, 75]. It has been found in human mammary gland [76] and breast milk, and antimicrobial or growth factor effects have been postulated for the peptide [77, 78]. There is data suggesting that adrenomedullin might be a prognostic marker for patients with NETs [79].

Apelin

Apelin plays an important role in cardiovascular regulation and fluid homeostasis. The peptide has a widespread distribution in the body, expressed in brain, lung, spleen, placenta and GI tract [80, 81]. Wang et al. demonstrated the presence of apelin in human mammary gland as well as in breast carcinoma, but its function here is unknown [82]. In addition, it has been detected in human breast milk [83].

Ghrelin and Obestatin

During the past decade, many peptides derived from the GI system have been associated with effects on food intake and GI motility [84, 85]. There are more than 30 peptides currently identified as being expressed within the digestive tract. The regulatory peptides synthesized by cells throughout the GI system include hormones, peptide and amine neurotransmitters and growth factors. Several hormones and neurotransmitters first identified in the central nervous system and other endocrine organs have subsequently been found in endocrine cells or neurons of the gut [86]. Two of the more recently identified peptide hormones are ghrelin and obestatin.

Ghrelin

In 1999, ghrelin was discovered in rat stomach as the endogenous ligand for the growth hormone (GH) secretagogue receptor. It was demonstrated to stimulate GH release via the 1a receptor subtype. The name is based on its role as a GH-releasing peptide, with reference to the Proto-Indo-European root *ghre*, meaning “to grow” [87, 88]. Ghrelin exists mainly in two forms: one that is *n*-octanoylated at serine 3 and the other that is unacylated (des-acyl) ghrelin [89].

Ghrelin has been attributed a multiplicity of physiological functions such as affecting GH release, food intake, energy and glucose homeostasis, GI motility, cardiovascular, pulmonary and immune function, cell proliferation and differentiation and bone physiology [90].

Circulating ghrelin is produced predominantly in the oxyntic mucosa of the stomach but has also been detected in other organs, including the small intestine, endocrine pancreas, hypothalamus and pituitary [91-93]. Furthermore, ghrelin mRNA and protein have been demonstrated in human breast tissue [91, 94]. Ghrelin has also been identified in breast milk in several studies [95, 96]. Considering that ghrelin stimulates appetite, its presence in breast milk could influence infant feeding behavior and body composition.

Ghrelin has been identified not only in normal tissues but also in various NETs (e.g., in tumors of the stomach and intestine) [97]. There have been some reports on patients with GI and pancreatic NETs displaying ghrelin immunoreactivity and elevated circulating levels of the peptide [98-100]. Further studies on gastric NETs have revealed that ECLomas type I and II frequently express ghrelin in the tumor cells [101]. Another study comprising a panel of NETs demonstrated the expression of ghrelin in a minority of the tumors included (e.g., tumors from the GI tract and pancreas) [102].

The obestatin peptide has mainly been localized to the stomach and pancreas. Co-localization of obestatin and ghrelin has been demonstrated as well [102, 108]. In accordance with ghrelin, obestatin has been identified in breast milk [109].

Obestatin has been demonstrated in various tumors, both in NETs of the stomach [110] and in other NETs (e.g., thyroid, parathyroid, GI and pancreatic tumors) [102].

The peptide was initially reported to have effects contrary to those of ghrelin, including reducing food intake and thereby resulting in body weight loss, as well as gastric emptying and suppressing intestinal motility. The biological activity of obestatin was suggested to depend on an amidation of its carboxyl terminus, and the effects were proposed to be through an interaction with the orphan receptor GPR39 [111].

However, a number of reports were unable to reproduce the suggested effects of obestatin on food intake and GI motility [112-116] and its interaction with the GPR39 receptor could not be confirmed [114, 117, 118]. All findings thus far suggest that obestatin is unlikely to be the ligand for GPR39 as initially claimed. Therefore, the native receptor for this peptide is still unknown.

Recent Revision of the Ghrelin Gene

The genomic structure of the ghrelin gene was thought to be relatively simple, consisting of a short first exon and four coding exons [119]. The proghrelin signal peptide is encoded by exon 1; and the ghrelin peptide is encoded by parts of exons 1 and 2 while exon 3 codes for obestatin. Another C-terminal peptide of ghrelin, C-ghrelin is encoded by exons 2, 3 and 4 and contains the obestatin peptide within its sequence [120].

However, a recent revision of the structure of the human ghrelin gene has revealed the presence of an additional exon, exon -1, and 5' extensions to exon 0 and 1, demonstrating that the gene consists of six rather than five exons, suggesting that the gene structure is more complex than initially thought.

Furthermore, splice variants containing a putative signal peptide in exon -1 would include transcripts that do not code for ghrelin, but instead may code for C-ghrelin, the obestatin peptide alone, and a novel C-terminal peptide (exon 3-deleted proghrelin) [121].

The Mammary Gland

Anatomy

The normal human mammary gland has tree-like structures in a branching ductal-lobular system embedded within irregular connective tissue (Figures 2 and 3). The basic components of the mammary gland are the acini (also referred to as alveoli) that form the functional secretory units of the mammary gland.

The acini structures, together with the smallest ducts (the intralobular ducts) form groups known as lobules. In the resting gland the terminal lobules are referred to as the terminal ductal lobular unit (TDLU). The lobules, which are the ultimate milk producing functional units of the breast, are organized into 15-20 lobes that are drained by collecting ducts [122, 123].

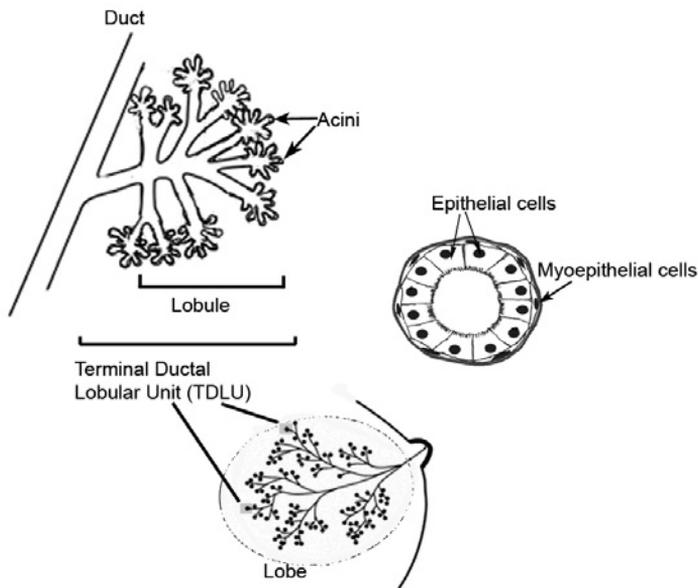


Figure 2. The breast consists of 15-20 lobes, each of which is drained by a collecting duct terminating in the nipple. The collecting duct has several branches that end in a terminal ductal-lobular unit. This unit is composed of a terminal duct and a cluster of acini. Each lobule is composed of several acini that drain into a common duct. The acini have a central lumen and are lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. The functional structures are surrounded by fat and collagenous tissue.

The acini and ducts have a central lumen and are lined by two cell layers, an inner layer of polarized luminal epithelial (milk-secreting) cells that line both the ducts and acini, and an outer layer composed of myoepithelial cells. During lactation, the luminal cells within the distal ducts and alveoli initiate milk production.

The myoepithelial cells can contract, similar to muscle cells, and thereby forcing the milk from the alveoli through the ducts toward the nipple. In the ducts myoepithelial cells form a continuous sheath. In the alveoli their distribution is sparser, creating a looser, basket-like structure that allows some of the luminal cells to be in contact with the basement membrane (BM) [124, 125].

Breast Cancer

Breast cancer is the most common cancer among women worldwide and the second leading cause of cancer-related deaths. According to the Swedish Cancer Registry, 7311 women were diagnosed with breast cancer in Sweden in 2008, representing 29% of all diagnosed cancer cases among women. The average annual increase of breast cancer has been 1.2% during the past two decades [126].

Diagnosis

Early breast cancer does not usually cause pain, but when the cancer grows, it may cause changes in the size or shape of the breast with a lump or thickening being noticed. In advanced cases large lymph nodes metastases may be present. The “triple diagnosis” includes clinical examination, mammography, ultrasonography or both, as well as fine-needle aspiration for cytology or biopsy for histopathological examination [127].

Invasive Breast Cancer

Breast cancers are generally characterized by the loss of epithelial polarity and tissue organization. When tumor cells remain within the BM of the ductal-lobular system, the tumor is classified as cancer *in situ*, but once the tumor cells rupture the BM and enter the adjacent stroma, the tumor has become invasive (Figure 3). Loss of myoepithelial cells, increase in myofibroblasts and immune cells in the stroma together with enhanced vascularization are the most prominent changes in the cellular composition when the mammary gland progresses from normal tissue to invasive cancer [123, 128-132].

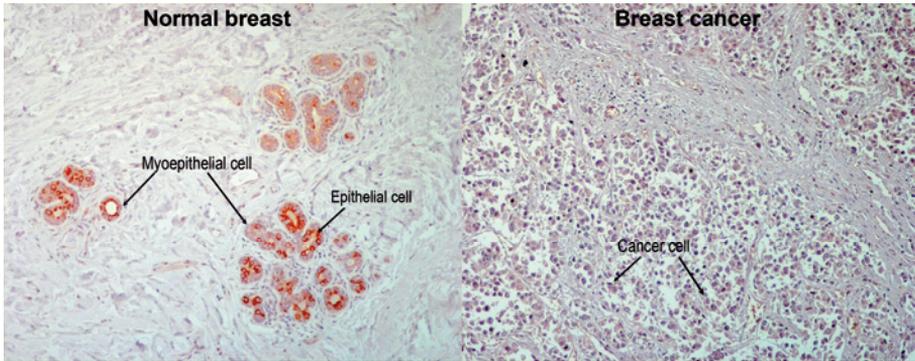


Figure 3. The normal and malignant breast. Normal breast immunostained with obestatin (left) and an invasive cancer (right). The normal mammary gland is a highly organized structure. The acini have a central lumen and are lined by an inner layer of luminal epithelial cells and an outer layer of myoepithelial cells. Breast cancers have lost the organized tissue architecture.

Histologically, invasive breast cancers are categorized into different subtypes based on growth patterns and cytological characteristics of the tumor cells. The two most common histologic types of infiltrating breast cancer are invasive ductal and lobular cancer [133].

Prognosis and Prediction

Prognostic and predictive factors are both important for therapeutic decisions.

A *prognostic factor* is a measurement associated with clinical outcome in the absence of therapy or with the application of a standard therapy that patients are likely to receive. It can be thought of as a measure of the natural history of the disease.

A *predictive factor* gives information as to whether a tumor is likely to respond to a specific treatment.

The most common prognostic factors used today include age, lymph node status, tumor size, histologic grade and human epidermal growth factor receptor 2 (HER2). The estrogen and progesterone receptors are considered as predictive factors for response to endocrine therapy [134, 135].

Neuroendocrine Tumors

NETs are characterized by the production of peptide hormones that may give rise to different endocrine symptoms. They represent a group of tumors that can occur wherever NE cells are found throughout the body. NETs are generally slow-growing tumors.

NETs are classified as functioning or non-functioning. Functioning tumors are characterized by inappropriate hormone secretion that gives rise to a clinical syndrome. An example of functioning tumor is insulinoma, where the tumor produces insulin that results in hypoglycemia. Non-functioning tumors produce hormones that do not induce specific endocrine symptoms. These tumors may continue to grow until other symptoms (such as pain or intestinal obstruction) leads to the diagnosis [136].

The incidence of GI NETs is about 3-5/100 000 inhabitants per year. However, the prevalence is much higher (up to 35/100 000 inhabitants per year) because of the long survival of these patients [137].

Diagnosis

The diagnosis of a NET is made by the measurement of different hormones in blood or urine. Other investigations that may be performed include GI endoscopy, chest x-ray, computed tomography scan, magnetic resonance imaging, somatostatin receptor scintigraphy or positron emission tomography with specific tracers. A biopsy of the tumor can be obtained by ultrasound guidance and is performed for histopathological diagnosis [136].

Classification of Neuroendocrine Tumors

The World Health Organization 2000 classification of NETs includes neoplasms originating from endocrine organs as well as organs containing NE cells (e.g. GI and respiratory tract) [138].

Techniques

Immunohistochemistry/Immunofluorescence

Immunohistochemistry (IHC) enables the visualization of the tissue distribution of specific antigens (or epitopes). In 1941, Coons et al. introduced this technique. Today, there are numerous immunohistochemical staining methods available. Coons and his colleagues used a direct immunostaining method, which involves a labeled antibody reacting directly with the antigen in tissue sections [139]. However, because of low signal amplification, making it quite insensitive, it is rarely used today. Over time, the method has been refined into methods that are more sensitive. One such method that increases the signal amplification is the enhanced polymer one-step staining (EPOS) method, where primary antibody molecules and peroxidase enzymes are attached to a dextran polymer called “backbone” [140].

A relatively new detection system, EnVision, has been described as a very sensitive detection method for routine IHC [141]. This two-step polymer method, which permits binding of a large number of enzyme molecules to a secondary antibody via the dextran backbone, is based on the polymer technology in the EPOS labeling system (Figure 4).

3,3' Diaminobenzidine (DAB) is a widely used chromogen for immunohistochemical staining. In the presence of the peroxidase enzyme, DAB produces a brown precipitate which is easily recognized under the light microscope [142].

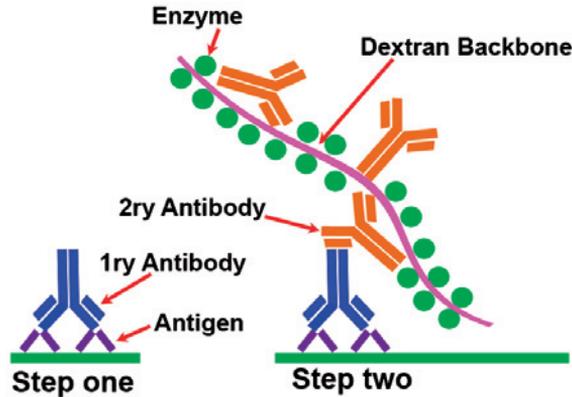


Figure 4. Two-step polymer method (EnVision).

Coons et al. were the first to label antibodies with a fluorescent dye, and use it to identify antigens in tissue sections [139]. The double or triple immunofluorescence technique allows the identification of two or three proteins at the same time in the same tissue specimen. There are several fluorescent dyes available that can be visualized at different wavelengths [139, 143].

Both polyclonal and monoclonal antibodies are used, where the polyclonal antibodies react with various epitopes of a certain amino acid sequence, whereas the monoclonal antibodies react with one specific epitope on the antigen against which they are raised.

The preservation of the tissue is an important part of the immunohistochemical process. The most frequently used fixative is a solution of formalin. During the fixation step, different reactions between the fixative and end group of proteins can occur, resulting in products that in turn may cross-link with other end groups.

If the fixative reacts with amino acids within the epitope to which the antibody is directed, the antibody will not be able to bind, resulting in a false-negative immunostaining. The demonstration of the antigens can be improved by pretreatment with an antigen retrieval solution that breaks the protein cross-links formed by the formalin fixation, e.g. by heating the sections in retrieval solution (usually in a microwave oven) the epitopes can become available to react with the antibody [142].

Western blotting

Western blot analysis combines the resolution of gel electrophoresis and the principles of immunological recognition of a protein by the appropriate antibody. The technique was named Western blot [144] and is a play on the name Southern blot, a technique for DNA detection developed by Edwin Southern.

Western blotting is a technique used to detect and determine the molecular weight of specific proteins from extracts made from cells or tissues. Using gel electrophoresis, the proteins are separated by size. The proteins are then transferred to a membrane, either within a buffer-filled tank (wet transfer) or between buffer-soaked filter papers (semi-dry transfer). This process is called blotting. Next, the membrane is blocked to prevent any non-specific binding. It can then be used to probe for proteins of interest with methods similar to IHC. Detection is usually performed using peroxidase-linked antibodies to catalyze a chemiluminescent reaction [145, 146].

Aims of the Current Investigation

The specific aims of this thesis were to:

- characterize a panel of normal human tissues with respect to the expression of obestatin in relation to ghrelin
- describe human mammary glands with respect to the expression of ghrelin and obestatin, as well as a set of NE markers
- analyze the distribution of ghrelin and obestatin in breast cancer and to evaluate their possible role as biomarkers
- to study the expression of ghrelin and obestatin in a comprehensive series of neuroendocrine tumors

Material and Methods

Non-Malignant Tissue Samples (Papers I and II)

Human tissues in Papers I and II were obtained from surgically removed material, except for pituitary tissue, which was obtained at autopsy, and third trimester placenta and umbilical cord, which were obtained at partus.

Mammary tissues in Paper II were represented by 28 cases, both women (all non-lactating) and men with a median age of 48 years (range: 15-67 years). Breast resection (benign breast tissue), fibroadenosis and hyperplasia were the established diagnosis for the women, whereas gynecomastia was the established diagnosis for the male population.

In order to detect rare IR cells only large specimens of high histological quality were used in the studies. All tissue samples were examined by a pathologist and assessed as macroscopically and microscopically normal (except the gynecomastia specimens obtained from men in Paper II). Specimens were fixed in 3.7% formaldehyde in phosphate buffered saline (PBS) and embedded in paraffin. Paraffin blocks were cut into approximately 4 μm sections and attached to positively charged glass slides (Superfrost Plus, Menzel Gläser, Braunschweig, Germany).

Tissue Microarrays (Paper III)

Formalin-fixed and paraffin-embedded human breast cancer tissue from 144 patients with invasive cancer of the breast was collected. Two 1-mm tissue cores were taken using a semi-automated arraying device (TMArrayer, Pathology Devices, MD, USA), mounted in recipient blocks, cut into 3-4 μm sections and transferred to glass slides.

Tumor Samples (Papers III and IV)

In Paper III, tissue samples from 144 patients with invasive breast cancer, diagnosed between 2001 and 2002 at the Department of Pathology, Malmö University Hospital, Sweden, were analyzed. All tumors were reclassified with reference to histologic subtype and Nottingham Histological Grade at Malmö University Hospital before tissue microarray (TMA) construction. Patient characteristics including age, tumor size, grade, expression of hormone receptors, Ki67-index, lymph node status and HER2 status, have been previously described and are summarized in Paper III, Table 1.

In Paper IV, tissue specimens from 131 cases were collected from the laboratory of Pathology at the University Hospital in Uppsala, Sweden. All patients had a verified diagnosis of a NET or an endocrine-related disorder according to the WHO criteria. The different diagnoses are listed in Paper IV, Table 1.

Antibody Production (Paper I)

A peptide, CFNAPFDVGIKLSGVQYQQHSQAL-amide, corresponding to human obestatin with an additional N-terminal cysteine residue, was synthesized. The peptide was coupled through the cysteine residue to maleimide-activated keyhole limpet hemocyanin. Free peptide was removed using dialysis. A rabbit was immunized with the peptide-carrier complex using a standard immunization protocol. The antiserum was used without further purification.

Primary Antibodies used for Immunohistochemistry (Papers I-IV)

Table 1. Primary antibodies used for immunohistochemistry (Papers I-IV)

<i>Antibody</i>	<i>Code</i>	<i>Host species</i>	<i>Source/reference</i>
Adrenomedullin	H-010-01	Rabbit	Phoenix Pharmaceuticals, Belmont, CA, USA
Apelin	H-057-15	Rabbit	Phoenix Pharmaceuticals
Chromogranin A	-	Rabbit	Portela-Gomes and Stridsberg, [26]
Chromogranin B	-	Rabbit	Portela-Gomes and Stridsberg, [147]
Ghrelin	H-031-30	Rabbit	Phoenix Pharmaceuticals
Obestatin	-	Rabbit	Grönberg et al. [148]
Secretogranin II	-	Rabbit	Stridsberg et al. [38]
Secretogranin III	-	Rabbit	Stridsberg et al. [38]
Secretogranin V	-	Rabbit	Portela-Gomes et al. [149]
Serotonin	M0758	Mouse	Dako, Glostrup, Denmark
Synaptic vesicle protein 2	NCL-SV2	Mouse	Novocastra Laboratories, Newcastle upon Tyne, United Kingdom
Synaptophysin	NCI-L-synap-299	Rabbit	Novocastra Laboratories
VMAT1	Sc-15313	Rabbit	Santa Cruz Biotechnology, Santa Cruz, CA, USA
VMAT2	Ab1767	Rabbit	Chemicon International, Temecula, CA, USA

Primary Antibodies used for Immunofluorescence (Papers I, II and IV)

Table 2. Primary antibodies used for immunofluorescence (Papers I, II and IV)

<i>Antibody</i>	<i>Code</i>	<i>Host species</i>	<i>Source/reference</i>
Chromogranin A	LK2H10	Mouse	Boehringer, Mannheim, Germany
Ghrelin	Y-031-44	Chicken	Phoenix Pharmaceuticals
Obestatin	-	Rabbit	Grönberg et al. [148]
Secretogranin II	-	Rabbit	Stridsberg et al. [38]
Secretogranin III	-	Rabbit	Stridsberg et al. [38]
Smooth muscle actin	M0851	Mouse	Dako

Antibodies used for Western Blots (Paper I)

The primary antibodies used for Western Blots were obestatin (Grönberg et al, [148]) and ghrelin (H-031-30, Phoenix Pharmaceuticals). The secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies (Amersham Biosciences, Buckinghamshire, UK).

Immunostaining (Papers I-IV)

All paraffin-embedded sections were deparaffinized before use. For antigen retrieval the sections were subjected to pre-treatment (microwave heating for 10 min at 750W, followed by 15 min at 380W in Tris-HCl buffered saline, pH 8.0). The sections were immunostained using the Dako EnVision Plus-HRP Detection Kit (Dako).

DAB was used as the chromogen. The sections were counterstained in Mayer's hematoxylin, mounted and evaluated under light microscope. Appropriate washing with PBS was performed between each step, and all incubations were performed in a moist chamber.

Double and Triple Immunofluorescence (Papers I, II and IV)

For double and triple immunofluorescence staining, the sections were deparaffinized and subjected to pre-treatment retrieval (microwave heating for 10 min at 700W in Tris-HCl buffered saline, pH 8.0). Sections were incubated for 30 min with blocking solution (donkey serum, Jackson Immunoresearch, Newmarket, UK, diluted 1:5 in PBS).

For triple immunofluorescence in Papers I and II, the sections were incubated for 1 h at room temperature with the appropriate primary antibodies (chicken, mouse, rabbit). The sections were thereafter washed in PBS with 0.05% Tween-20 and incubated for 1 h at room temperature with appropriate secondary antibodies (with specificity *vs.* chicken, mouse, or rabbit). Washing in PBS with 0.05% Tween-20 was performed between each step and incubation was performed in a dark moist chamber.

For double immunofluorescence staining in Paper I, II and IV the sections were prepared as described above and incubated for 1 h at room temperature or overnight with appropriate primary antibodies (chicken, mouse or rabbit).

In Papers II and IV, nuclei were counterstained with 4', 6-diaminidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA).

Fluorescein isothiocyanate (FITC, Dako), tetramethyl rhodamine isothiocyanate (TRITC, Dako), Alexa 594 (Invitrogen Cooperation, Carlsbad, CA,

USA), Alexa 488 (Invitrogen) and aminomethylcoumarin acetic acid (AMCA, Jackson Immunoresearch) were used as fluorescent chromogens.

Tissues were photographed by an Axiocam HRm camera employing the Axiovision imaging software using a 63x PlanApochromat objective and a Zeiss Axioplan 2 microscope (Carl Zeiss, Göttingen, Germany) or a Zeiss Observer Z1 microscope and Axiovision software (Carl Zeiss).

Antibody Controls (Papers I-IV)

The antibody specificity tests included (a) omission of the primary antibodies, (b) negative controls in which the antibodies were replaced by normal serum at the respective dilution and in the same diluent (Papers I and II), (c) neutralization tests conducted by preabsorption of the antibody with the homologous antigen (except VMAT2, adrenomedullin and apelin) before application to the sections (Papers I, II and IV) and (d) cross-reaction tests performed by incubation of the ghrelin antibody with the obestatin peptide and vice versa (Paper I).

Human gastric mucosa, pancreas and colon were used as positive controls in the papers.

Western Blot (Paper I)

Peptides were subjected to SDS-PAGE 16.5% tris-tricine gel (Bio-Rad, Hercules, CA, USA) and subsequently transferred to polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was blocked in PBS (pH 7.4) with 5% bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) and 0.5% Tween-20 (Sigma-Aldrich) for 1 h at room temperature. The membrane was incubated with the anti-obestatin antibody in PBS with 1% BSA and 0.1% Tween-20 overnight at 4°C and rinsed in PBS with 0.5% Tween-20. After incubation with HRP-conjugated anti-rabbit secondary antibody (Amersham Biosciences) in PBS with 5% BSA and 0.5% Tween-20 for 1 h, the membrane was washed in PBS with 0.5% Tween-20 three times, the bound antibodies were visualized using Lumi-Light reagent (Roche, Basel, Switzerland) and detected with the ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was stripped, blocked and incubated with the anti-ghrelin antibody and afterwards treated as described above.

Statistics (Paper III)

Spearman's rank correlation test was used to assess correlations between variables. Kaplan-Meier plots were applied for survival analysis and log-rank test was performed to compare curves separated according to ghrelin and obestatin expression. Recurrence-free survival (RFS) was defined as time from diagnosis until recurrence and breast cancer-specific survival (BCSS) was defined as the time from diagnosis until breast cancer-related death.

Univariate Cox analyses were performed to test how each of the parameters correlated to breast cancer survival and recurrence. Multivariate Cox proportional hazards regression model was used to estimate hazard ratio (HR).

A reproducibility study of the evaluation of staining was performed on the scoring data using kappa values [150].

All statistical tests were two-sided and P -values of <0.05 were considered statistically significant. Calculations were performed using IBM SPSS Statistics 18 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Ghrelin and Obestatin are Present in the Human Gastrointestinal Tract, Pancreas and Mammary Gland (Paper I)

The aim of Paper I was to characterize the expression of obestatin in relation to ghrelin in normal human tissues using IHC. For this, a commercial antibody *vs.* ghrelin was used, and a polyclonal antibody *vs.* obestatin was produced. Totally, 35 tissue blocks from different organs were included in the study. The specificity of the produced obestatin antibody and commercial ghrelin antibody was examined using Western blot. No cross-reactivity between the antibodies was detected (Paper I, Figure 1).

Initially, all tissues were screened using ghrelin and obestatin antibodies diluted 1:8000 and 1:2000; the latter was used to be sure not to miss possible cells with weaker immunoreactivity. Ghrelin and obestatin were demonstrated to be specifically expressed throughout the GI tract, pancreas and also in the mammary glands. Ghrelin/obestatin-IR cells were abundant in normal human gastric mucosa, where the number of IR cells decreased distally. The ghrelin and obestatin intensity of immunoreactivity in the GI tract was very strong as suggested by the fact that it could be clearly detected even at very low antibody concentrations (1:16000). In the pancreas, a few IR cells were found in the periphery of the pancreatic islets. In addition, a few scattered IR cells were also seen in connection to the pancreatic ducts.

Interestingly, ghrelin/obestatin-IR cells were found in the mammary gland (Paper I, Figure 4). Cytoplasmic immunoreactivity was observed in specific luminal epithelial cells, whereas the myoepithelial cells were non-IR. However, the amounts of protein in the mammary glands seemed to be less compared with those from the GI tract and pancreas, especially considering the fact that a higher antibody concentration (1:2000) was needed to detect IR cells.

Immunofluorescence staining demonstrated that ghrelin co-localized with obestatin in all IR tissues, and the two peptides showed the same cytoplasmic distribution pattern (Figure 5). All detected ghrelin-expressing cells were IR for obestatin and vice versa. The subcellular distribution of both peptides showed an intracellular granular pattern.

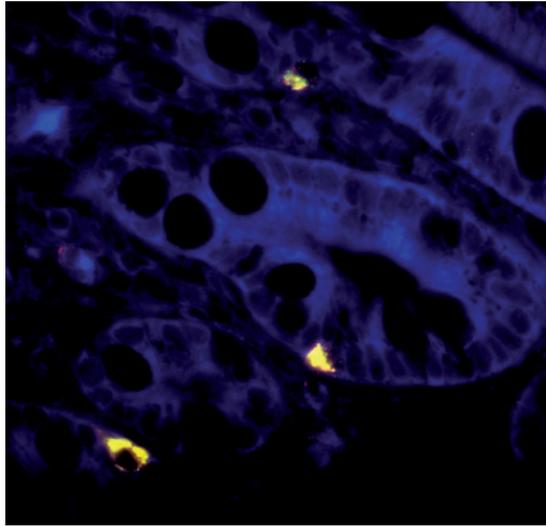


Figure 5. Human duodenum double immunostained for ghrelin (green) and obestatin (red). Co-localization (yellow) implies that ghrelin and obestatin are expressed in the same cell.

Furthermore, triple immunofluorescence staining with ghrelin, obestatin and CgA was performed. It has previously been reported that ghrelin-IR cells [151] and obestatin-IR cells [152] contain CgA in rat gastric mucosa. In this study only a minority of the ghrelin/obestatin-IR cells were IR for CgA. The intensity of CgA immunostaining in these cells was constantly weak; however most of the ghrelin/obestatin-IR cells were non-IR for CgA (Paper I, Figure 5), including mammary tissue. Because the monoclonal CgA antibody used in this study is widely used as a marker of NETs, the finding that ghrelin/obestatin-IR cells in human tissues appear to be non-IR for this antibody is of clinical importance. Thus, lack of CgA immunoreactivity does not rule out the presence of ghrelin and obestatin expressing cells.

The function of ghrelin and obestatin in the mammary gland is still unknown, but the abundance of IR cells found could indicate that these cells might influence circulating ghrelin and obestatin levels. Previously, both ghrelin and obestatin have been detected in human breast milk [95, 96, 109]. The possibility exists that these peptides could act in a paracrine way or have an exocrine function by being secreted into the ducts rather than serve as endocrine hormones released in to the circulation.

Taken together, in Paper I, we have identified ghrelin/obestatin-IR cells in the human GI tract, pancreas and mammary glands. Our results indicate that it is important to further characterize mammary epithelial cells concerning the presence of NE markers. Such studies could provide useful information in revealing the physiological function of ghrelin and obestatin in mammary glands.

Neuroendocrine Markers in the Human Mammary Glands (Paper II)

Considering that ghrelin and obestatin were present in the human mammary glands, which we described in Paper I, we sought to evaluate this event in more detail.

In Paper II we have characterized the distribution of several NE markers (including ghrelin and obestatin) in normal human mammary glands. The presence of NE cells in the breast has not been proven conclusively, and the existence of breast cancers with NE differentiation has been reported only rarely.

Tissue specimens from 28 cases were collected. Samples were represented by both men ($n = 8$) and women ($n = 20$, all non-lactating). By IHC, several markers were found to be expressed in the breast, including CgB, SgII, SgIII and VMAT2 (Figure 6). The presence of ghrelin and obestatin was confirmed, as described in Paper I, together with the previously reported peptides apelin and adrenomedullin, in specific luminal epithelial cells in the mammary gland (Figure 6). For an overview of the antibodies used in the study and those resulting in immunoreactivity in the breast, see Paper II, Tables 1 and 2.

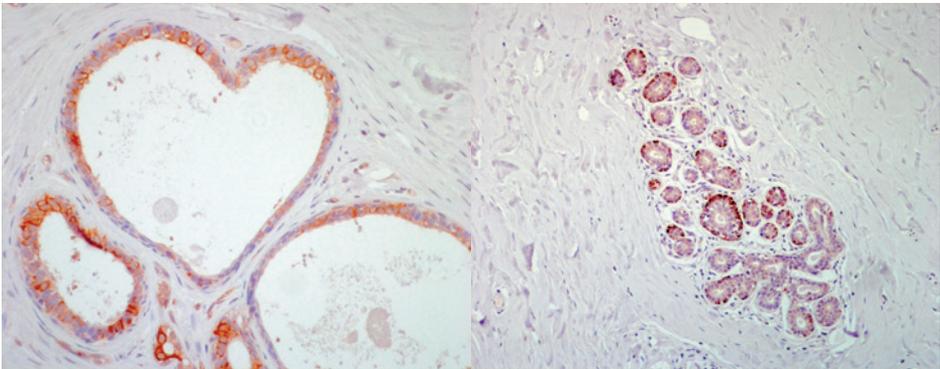


Figure 6. Human mammary gland, immunostained for obestatin (left) and SgIII (right).

Furthermore, using consecutive sections, the immunostaining patterns of obestatin, ghrelin, adrenomedullin, apelin and VMAT2 were demonstrated to be very similar, indicating that mammary epithelial cells are able to express several peptide hormones (Paper II, Figure 1).

In this study members of the granin family were also detected in the human breast. SgII and SgIII showed immunoreactivity in the outer layer of myoepithelial cells lining the luminal epithelium. Using double immunofluorescence and staining for a myoepithelial cell marker, smooth muscle actin (SMA), the co-localization of these markers and SMA in the same cells was confirmed (Paper II, Figure 2).

CgB had a broader staining pattern than that of the above-mentioned IR peptides, showing immunoreactivity in both the epithelial and myoepithelial cells (Paper II, Figure 3). SgII and CgB also stained endothelial cells surrounding vessels in the stroma. SgII has previously been demonstrated to exert effects on the endothelium; it can induce direct migration of endothelial cells, stimulate adhesion of human monocytes to endothelial cells and act as a direct angiogenic cytokine [153-155]. The strong immunostaining of SgII in the endothelial cells in the breast could indicate autocrine functions, regulation of vessel formation and/or inflammation.

Interestingly, adrenomedullin, ghrelin and CgB have been shown to have antimicrobial effects [30, 156, 157], and since they seem to be present in the same specific cells, it could be speculated that the cells expressing these molecules in the breast, may be involved in some form of microbial defense.

In this study we could not detect CgA or synaptophysin expression in any of the tissue samples. These two polypeptides are among the most commonly used markers to identify NE differentiation. These markers typically identify a rare type of breast cancer, i.e. breast cancer with NE differentiation. However, since CgA and synaptophysin could not be identified in normal breast tissue, it is possible that other markers might prove to be a better choice in identifying NE breast cancers.

To summarize, in Paper II, we have demonstrated the presence of several NE markers in the normal human mammary gland. Further studies are needed to clarify the function of these peptides in the normal breast.

Ghrelin and Obestatin in Invasive Breast Cancer (Paper III)

In Paper II, we identified the presence of several NE markers in normal human mammary glands. The clinical implications of the expression of these peptides in breast cancer are unclear. Against this background we sought to clarify the distribution of ghrelin and obestatin expression in breast cancer. Using TMAs consisting of 144 invasive breast cancer samples, we evaluated their relation to established biomarkers and clinical outcome.

Both ghrelin and obestatin were detected with different intensities in the samples. Each of the TMA cores was scored separately as 0=negative, 1=weak, 2=intermediate and 3=strong expression of either of the two peptides (Paper III, Figure 1 and Table 2). A large proportion of breast cancers expressed ghrelin and obestatin.

Statistical correlation tests revealed significant negative correlations between both peptides and factors associated with bad prognosis in breast cancer (i.e. large tumor size, high Ki67-index and high tumor grade) indicating that in patients in which ghrelin and/or obestatin expression is present a better prognosis would be expected (Paper III, Table 3).

Cox regression proportional hazards models were used to estimate the impact of peptide expression on RFS and BCSS in both univariate and multivariate analysis, adjusted for tumor size, age, lymph node status and tumor grade. Both peptides were correlated to an improved RFS and BCSS in the univariate analysis (Paper III, Table 4).

The multivariate analysis further strengthened that ghrelin may be an independent prognostic factor associated with longer BCSS and time to relapse, whereas obestatin was no longer significant (Paper III, Table 5).

In addition, survival curves compared by Kaplan-Meier estimates and the log-rank test showed that patients expressing ghrelin had significantly longer RFS (Paper III, Figure 2A) and BCSS (Paper III, Figure 2B) than patients with no ghrelin expression.

The optimal cut-off value was determined by investigating HR for different cut-offs. The cut-off resulting in the best separation of the poor prognostic group and the good prognostic group was demonstrated to be IR (1+2+3) vs. non-IR (0). This information makes the evaluation easy for possible future use in clinical practice.

A reproducibility study of the evaluation of staining was performed on the scored data. For both the ghrelin and obestatin immunostainings the agreement of the evaluation of staining intensity between the investigators was very good (the kappa values were 0.94 and 1.00 for ghrelin and obestatin, respectively). The combination of easily scored material and good reproducibility would make this method easy to adapt to routine use.

In conclusion, ghrelin could be a new factor correlated to good outcome in patients with invasive breast cancer. Patients with tumors expressing ghre-

lin has approximately 3 times lower risk for recurrence or breast cancer death than those with no ghrelin expression. Ghrelin immunohistochemical analysis is easily assessable with high reproducibility. However, more studies are needed to establish the role of ghrelin as a biomarker in breast cancer.

Ghrelin and Obestatin in Neuroendocrine Tumors (Paper IV)

The aim of Paper IV was to characterize the expression of obestatin in relation to ghrelin in a comprehensive panel of NETs and other endocrine-related disorders. Totally, 131 cases were included in this study. All patients had a histopathologically confirmed diagnosis. The expression of ghrelin and obestatin was evaluated using IHC.

Both peptides were displayed in only a minority of the included tumors (Paper IV, Table 1). Tumors showing immunoreactivity for the peptides originated from the GI tract, endocrine pancreas and lung (Paper IV, Figure 1). However, we were unable to detect any IR cells in tumors derived from the small intestine.

In most tumor specimens expressing the peptides only occasional tumor cells were IR. There was a slight heterogeneous pattern between tumors expressing obestatin (14/131) and ghrelin (13/131). This difference is difficult to explain but might be due to the complex ghrelin gene locus, which recently has been revised. This complexity is thought to allow for different expression of the peptides within a certain cell.

IR cells were also found in two cases of rectal NETs. This finding is somewhat surprising since neither ghrelin nor obestatin have previously been found in normal rectal tissue. One possible explanation for to this observation could be that ghrelin and obestatin are expressed by multipotent neoplastic NE stem cells in these tumors.

Other endocrine-related disorders that were also included in the study were nesidioblastosis and thyroid c-cell hyperplasia. Noteworthy was that the nesidioblastosis case was IR for both peptides (Figure 7).

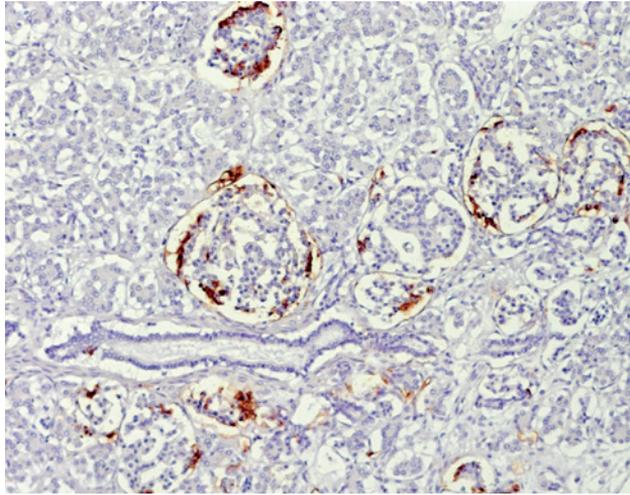


Figure 7. A case of nesidioblastosis, immunostained for obestatin. Islet cell hyperplasia is seen in close association with pancreatic ducts. Hyperplasia of obestatin immunoreactive cells is seen in some islets.

Double immunostainings were performed on a variety of tumors, including a duodenal, a rectal and a MEN-1-associated pancreatic NET (Paper IV, Figure 2). Co-localization of the peptides was demonstrated in all cases, which is in accordance to what is seen in normal tissues (Paper I).

In summary, ghrelin and obestatin do not seem to be abundantly expressed in NETs since they are only present in occasional tumors originating from the GI tract, endocrine pancreas and the lung. From this study, we cannot recommend that ghrelin and obestatin should be included in the general work-up of patients with NETs.

Major Findings

Paper I

- Ghrelin-IR cells and obestatin-IR cells are present in the human gastrointestinal tract, endocrine pancreas and mammary glands.
- Ghrelin co-localizes with obestatin and shows the same cytoplasmic distribution pattern.
- Most normal tissues do not express ghrelin or obestatin.

Paper II

- VMAT2, together with the previously reported peptides ghrelin, obestatin, apelin and adrenomedullin, are present in specific epithelial cells in the human mammary gland.
- Secretogranin II and secretogranin III are expressed in the myoepithelial cells.
- Secretogranin II is also detected in the endothelium.
- Chromogranin B has a broad immunoreactive pattern that includes the epithelial cells, the myoepithelial cells and the endothelial cells of the mammary gland.

Paper III

- Both ghrelin and obestatin are expressed in invasive breast cancer.
- Both peptides are correlated to previously recognized prognostic factors for breast cancer.
- In this study ghrelin was shown to be an independent prognostic marker for prolonged BCSS and RFS.
- Ghrelin could be a new prognostic factor for a good clinical outcome in invasive breast cancer.

Paper IV

- A minority of neuroendocrine tumors express ghrelin and obestatin.
- The peptides are expressed predominately in tumors originating from the foregut.
- From this study we suggest that ghrelin and obestatin should not be included in the general work-up of patients with NETs.

Future Perspectives

This thesis has opened up for questions about the significance of ghrelin and obestatin expression in breast cancers. We are therefore planning further studies to address the role of NE markers in breast cancer. We will study the expression of both ghrelin and obestatin in a larger panel of patients with breast cancer to confirm the prognostic value of these peptides. For this purpose, other breast cancer collections with more events, i.e. more patients that have had recurrence or died from breast cancer, will be used. This approach will hopefully yield higher statistical power in the statistical analyses. We hope this will establish whether the NE markers could be used for prognosis assessment of breast cancer.

We are also planning to evaluate other NE markers (Cgs and Sgs, as well as VMAT2, adrenomedullin and apelin) detected in normal breast tissue in terms of their expression in breast cancer. This evaluation will be done in order to establish whether their expression also might be of prognostic value in a similar way to that of ghrelin.

Mainly conventional IHC will be used; however, double immunofluorescence stainings could be of particular importance to verify the simultaneous occurrence of different peptides. In some cases *in situ* hybridization might be used to confirm the presence of mRNA coding for the peptides.

Further statistical analyses will be performed to identify correlations to different breast cancer-specific factors. Clinical data of the patients included in the material are available and will be used in the analyses. Furthermore, survival analyses will be performed.

This project will lead to new knowledge about the expression of ghrelin and obestatin, as well as other NE markers in breast cancer. It is important to verify the role of ghrelin in breast cancer and with a larger material, we hope to verify the prognostic relevance of ghrelin, in addition to finding more markers that may be important in breast cancer.

Summary of the Thesis in Swedish

Populärvetenskaplig sammanfattning på svenska

Ghrelin är ett hormon som främst produceras av celler i magsäcken och som stimulerar aptiten. Ghrelin stimulerar också utsöndringen av tillväxthormon från hypofysen [87]. Det har visat sig att genen som bär på information för tillverkning av ghrelin, även kodar för en annan peptid, obestatin. Obestatin upptäcktes år 2005 och från början trodde man att obestatin hade motsatta fysiologiska effekter som ghrelin [111]. Senare rapporter har dock inte kunnat verifiera detta så den exakta biologiska funktionen av obestatin är fortfarande okänd [112, 113].

Immunhistokemi är en teknik där man använder antikroppar som är riktade mot specifika delar av ett protein (hormon) vilka får reagera med en vävnad där man tror att detta hormon produceras. Om hormonet finns i vävnaden kan man med olika hjälpmedel påvisa att antikroppen fastnat där. Denna metod gör det möjligt att visa att olika proteiner förekommer i vävnader.

Detaljer om ingående delarbeten

Vi har studerat uttrycket av hormonerna ghrelin och obestatin i både normala vävnader och tumörer. Studierna är främst utförda med immunhistokemi med specifika antikroppar riktade mot de olika peptiderna. Även andra neuroendokrina markörer har ingått i vissa arbeten för ytterligare karakterisering.

Delarbete 1

I delarbete 1 har vi använt oss av ett antal olika normala vävnader för att studera uttrycket av ghrelin och obestatin i kroppen. Vi har kunnat visa att ghrelin och obestatin finns främst i mag-tarmkanalen, bukspottkörteln och i bröstkörteln.

Med dubbel immunofärgningsteknik kunde vi visa att ghrelin var samlokaliserad med obestatin. Alla celler som var positiva för ghrelin var positiva för obestatin och vice versa.

Tippelfärgningar med ghrelin, obestatin och chromogranin A visade att de flesta ghrelin/obestatinpositiva cellerna var negativa för chromogranin A. Det verkar som att avsaknad av chromogranin A inte utesluter förekomst av ghrelin/obestatinceller.

Celler med produktion av ghrelin och obestatin fanns också i normal bröstvävnad. Immunoreaktivitet kunde ses i specifika epiteliala celler, medan myoepitelcellerna var negativa. Funktionen av ghrelin och obestatin i bröstet är fortfarande okänd, men det stora antalet av positiva celler antyder att dessa celler kan påverka nivåerna av ghrelin och obestatin i blodet. Man har också sedan tidigare visat att både ghrelin och obestatin finns i bröstmjölk, men deras funktion där är oklar.

Sammanfattningsvis har vi identifierat ghrelin och obestatin i magtarmkanalen, bukspottkörteln och bröstet. Resultaten från denna studie tyder på att det är viktigt att fortsätta karakterisera dessa celler vad gäller förekomsten av neuroendokrina markörer.

Delarbete 2

I det första delarbetet fann vi att ghrelin och obestatin fanns i normalt bröst. I delarbete 2 har vi studerat uttrycket av hormonerna i ett större material av normal bröstvävnad. Vi undersökte också förekomsten av andra neuroendokrina markörer.

Vi har i denna studie visat att neuroendokrina markörer förekommer i normal bröstvävnad. Vi kunde bekräfta uttrycket av ghrelin och obestatin, samt de tidigare rapporterade peptiderna apelin och adrenomedullin. Förutom dessa kunde vi visa att flera andra peptider finns uttryckta i bröst, bl.a. VMAT2, chromogranin B samt secretogranin II och III.

Ghrelin, obestatin, adrenomedullin, apelin och VMAT2 var uttryckta i de mjölkproducerande epitelcellerna. Liknande färgningsmönster för dessa peptider kunde påvisas, vilket tyder på en celltyp som kan producera flera olika peptidhormon. Secretogranin II och III påvisades i de kontraktila myoepitelcellerna. Chromogranin B hade ett brett färgningsmönster och var uttryckt i både epitel- och myoepitelceller.

Adrenomedullin, ghrelin och chromogranin B har visat sig ha antibakteriella effekter, och eftersom att dessa verkar vara uttryckta i samma celler, kan man spekulera i att cellerna som uttrycker dessa peptider i bröstet kan vara involverade i ett infektionsförsvar.

Chromogranin A och synaptofysin kunde inte påvisas i normalt bröst. En ovanlig typ av bröstcancer, s.k. bröstcancer med neuroendokrin differentiering, identifieras vanligtvis med dessa markörer. Eftersom markörerna dock inte kunde identifieras i normal bröstvävnad kanske andra markörer skulle kunna vara ett bättre alternativ för att påvisa dessa ovanliga tumörer.

Sammanfattningsvis har vi visat på uttryck av flera neuroendokrina markörer i normal bröstvävnad. Fler studier behövs för att klargöra funktionen av dessa hormoner i både det normala och tumöromvandlade bröstet.

Delarbete 3

I delarbete 3 har vi studerat uttrycket av ghrelin och obestatin med vävnadsarrayer på patienter med invasiv bröstcancer. Vi har i denna studie kunnat visa att ghrelin kan vara en ny prognostisk faktor för bröstcancer.

Både ghrelin och obestatin visade sig vara uttryckta i varierade grad i tumörerna. Statistiska analyser visade på signifikanta korrelationer mellan peptiderna och prognostiska parametrar, vilket tyder på att de patienter som uttrycker ghrelin och obestatin har en fördelaktig prognos. Univariata och multivariata analyser genomfördes för att se hur peptiduttryck påverkade risken för återfall respektive död i bröstcancer. De univariata analyserna visade att patienter som inte uttrycker ghrelin och obestatin har ungefär 3 gånger så stor risk för återfall/död i bröstcancer. Efter korrigering för skillnader i tumörgrad, ålder, lymfkörtelstatus och tumörstorlek i den multivariata analysen kvarstod statistisk signifikans för ghrelin men inte för obestatin. Dessa data talar för att patienter som uttrycker ghrelin har en mindre risk för att återfalla eller dö i bröstcancer.

Sammanfattningsvis kan ett uttryck av ghrelin korreleras till god prognos och skulle därmed kunna användas för att identifiera patienter som i framtiden inte behöver lika intensiv postoperativ behandling som alla får idag. Dessa resultat behöver dock verifieras i större patientmaterial.

Delarbete 4

I delarbete 4 har vi studerat uttrycket av ghrelin och obestatin i en stor panel av neuroendokrina tumörer och andra endokrina tillstånd.

Ghrelin och obestatin visade sig förekomma i ett fåtal tumörer, och främst i tumörer i den övre delen av magtarm-kanalen och bukspottkörteln.

Av 131 fall som inkluderades i studien, var 13 positiva för ghrelin och 14 positiva för obestatin. I de fall som uttryckte hormonerna var antalet positiva tumörceller få. Skillnaden i antalet fall som uttrycker ghrelin respektive obestatin kan kanske bero på ghrelingen som nyligen har visat sig vara mycket mer komplex än vad man tidigare trott.

Med dubbel immunofärgningsteknik på ett antal tumörer kunde vi visa att ghrelin var samlokaliserad med obestatin. Detta överensstämmer också med vad vi ser i normal vävnad (Delarbete 1).

Det är tydligt att peptiderna mestadels förekommer i samma organ som uttrycker peptiderna under normala förhållanden.

Sammanfattningsvis verkar ghrelin och obestatin inte vara ofta förekommande i neuroendokrina tumörer, då de endast förekommer i tumörer som utgår från mag-tarmkanalen och bukspottkörteln.

Sammanfattning

I avhandlingen ingår studier som leder till en ökad förståelse av förekomsten av ghrelin och obestatin, både i normal vävnad och i tumörvävnad. Resultaten bidrar inte enbart till en större inblick i förekomsten, utan är även intressanta eftersom de tyder på att ghrelin kan fungera som prognostisk faktor vid bröstcancer.

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