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Gene expression studies in gastrointestinal pathophysiology and neoplasia

Doctoral thesis
for the degree of philosophiae doctor

Trondheim, February 2006

Norwegian University of Science and Technology
Faculty of Medicine
Department of Cancer Research and Molecular Medicine

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List of papers

I. Eva Hofsli, Liv Thommesen, Kristin G. Nørsett, Sture Falkmer, Unni Syversen, Arne K. Sandvik and Astrid Lægreid. Expression of chromogranin A and somatostatin receptors in pancreatic AR42J cells. *Molecular and Cellular Endocrinology*, 194: 165-173, 2002.

II. Kristin G. Nørsett, Astrid Lægreid, Herman Midelfart, Fekadu Yadetie, Sture Falkmer, Jon E. Grønbech, Helge L. Waldum, Jan Komorowski, Arne K. Sandvik. Gene expression based classification of gastric carcinoma. *Cancer Lett,* Jul 16;210(2):227-237, 2004.

III. Kristin G. Nørsett, Astrid Lægreid, Mette Langaas, Sara Wörlund, Reidar Fossmark, Helge L. Waldum, Arne K. Sandvik. Molecular characterisation of rat gastric mucosal response to potent acid inhibition. *Physiological Genomic,* Jun 16;22(1):24-32, 2005.

IV. Kristin G. Nørsett, Astrid Lægreid, Waclaw Kusnierczyk, Mette Langaas, Sonja Ylving, Reidar Fossmark, Simen Myhre, Sture Falkmer, Helge L. Waldum, Arne K. Sandvik. Changes in gene expression of gastric mucosa during therapeutic acid inhibition. Submitted.

Abbreviations

CCK-2	cholecystokinin-2
CgA	chromogranin A
CRE	cAMP responsive element
D cell	somatostatin producing cell
EC	enterochromaffin cell
ECL cell	enterochromaffin like cell
EGF	epidermal-like growth factor
G cell	gastrin cell
GI	gastrointestinal
GO	gene ontology
HDC	histidine decarboxylase
HB-EGF	heparin-binding epidermal-like growth factor
ICER	inducible cAMP early repressor
NE	neuroendocrine
NSE	neuron-specific enolase
PACAP	pituitary adenylate cyclase activating peptide
PPI	proton pump inhibitor
Reg	regenerating gene protein
RT-PCR	reverse-transcriptase polymerase chain reaction
SST	somatostatin
SSTR	somatostatin receptor
SYN	synaptophysin
TGF-α	transforming growth factor α
VIP	vasoactive intestinal peptide
VMAT-2	vesicular monoamine transporter type 2

Introduction

1 Morphology of the stomach and gastric mucosa

The stomach may be divided into three parts. The cardia is a narrow zone bordering the oesophagus characterised by mucus secreting cells and the absence of parietal and chief cells. The body of the stomach consists of the fundus and corpus with the acid producing or oxyntic mucosa. This part of the mucosa produces hydrocloric acid and numerous regulatory peptides. In the antrum (or pyloric part of the stomach) the mucosa mainly consist of mucus cells. The G cells of the antrum produce the acid secretagogue hormone gastrin.

The stomach, like other parts of the gastrointestinal (GI) tract, consists of four principal layers: mucosa, submucosa, muscularis and serosa. The mucosal cells are organised in glands. The tissue between the glands in the mucosa is called lamina propria and contains blood vessels and lymph vessels, nerves and connective tissue. The muscularis mucosae is located below the bottom of the gastric glands and forms the boundary to the submucosal layer. Outside the submucosa, the stomach wall consists of two muscle layers and an outer serosal layer.

The morphology of the gastric mucosa has been thorougly reviewed by Helander (Helander 1981). The glandular mucosa of the mammalian stomach invaginates the lamina propria, forming the gastric pit on the luminal surface and the gastric glands from the bottom of the pits. Each oxyntic gland is divided in to three regions: the isthmus, the neck and the base. Two main types of cells (exocrine and neuroendocrine (NE)) can be found in the gastric gland (Figure 1). The neck and isthmus region is also referred to as the proliferative or regenerative zone and harbours endodermal stem cells, which may be the origin of all cells in the mucosa (Modlin *et al* 2003a). The stem cells proliferate and produce linage precursors that differentiate into the

different mucosal cell types of the gastric mucosa (Karam 1995). As these stem cells divide, they migrate upwards or downwards as they differentiate into the different cell types.

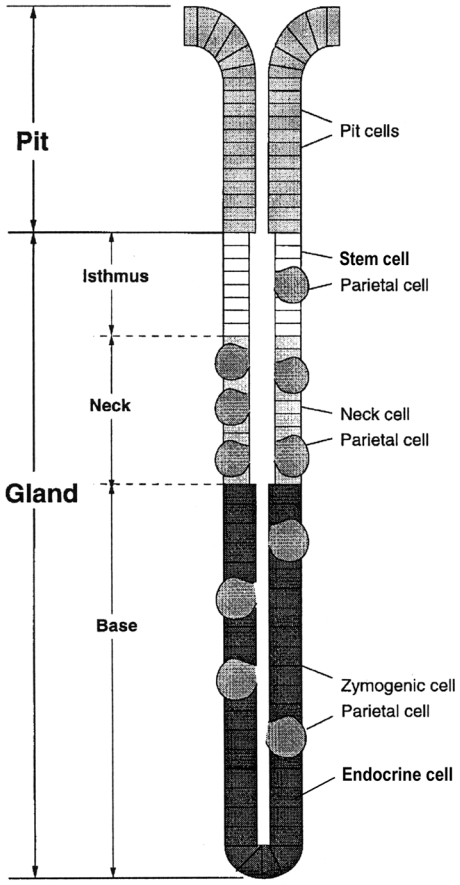

Figure 1: Schematic illustration of a glandular unit from the oxyntic mucosa (modified from (Karam *et al* 2001)).

1.1 Exocrine cells

Surface mucous cells cover the surface of the gastric mucosa and the gastric pits, and secrete mucus and bicarbonate. In the neck and isthmus region mucus neck cells and endodermal stem cells are found. Chief cells secrete pepsinogen, which is a precursor of the proteolytic enzyme pepsin. They are found in the basal part of the oxyntic glands (Helander 1981).

The dominant cell population in the oxyntic mucosa are the hydrochloric acid-secreting parietal cells. They may be found in all parts of the glands, but are most

numerous in the middle third. The apical cell membrane has tubular invaginations, thus increasing the surface. Acid is secreted by the gastric H^+/K^+-ATPase (proton pump) which is translocated to the apical secretory canaliculi upon stimulation, by fusion of the cytoplasmatic tubulovesicles holding the proton pump in the resting state. The proton pump (H^+/K^+-ATPase) can be used as a marker for parietal cells. The transport of protons from the interior of the cell to the glandular lumen is highly energy-consuming, and parietal cells contain an unusually high number of mitochondria.

1.2 NE cells

The NE cells constitute about 2% of the cells in the gastric mucosa (Bordi & D'Adda 1991). They are usually situated in the lower half of the glands and are classified into *open type cells* (cells extending to the gastric lumen) and *closed type cells* (cells not reaching the lumen) (Capella *et al* 1991).

About 65-75% of the NE cells in rat oxyntic mucosa are enterochromaffin like (ECL) cells (Håkanson *et al* 1976). The corresponding number in man is 30-35% (Simonsson *et al* 1988). Like other gastric NE cells, the ECL cells are predominantly located in the basal half of the glands, and ECL cells are capable of self-replication (Tielemans *et al* 1989; Ryberg *et al* 1990). The main function of the ECL cells is production of histamine which stimulates acid secretion. Biosyntesis of histamine is done in the cytosol by histidine decarboxylase (HDC) (Modlin & Tang 1996). Both histamine and HDC are used as ECL cell markers. However, histamine and HDC are also found in mast cells (Suzuki-Ishigaki *et al* 2000), thereby somewhat reducing the specificity for ECL cells in the oxyntic mucosa. ECL cells exhibit a characteristic ultrastructure, with a large eccentric nucleus surrounded by numerous electron-dense granules and electron-lucent vesicles (Tanabe *et al* 2003). Histamine is sequestered in secretory vesicles by the vesicular monoamine transporter type 2 (VMAT-2). In addition to histamine, the secretory granules of ECL cells also contain chromogranin A (CgA) (Capella *et al* 1991) and pancreastatin (product of CgA), which are both released into the blood stream (Syversen *et al* 1994; Håkanson *et al* 1995).

In the stomach the gastrin cells (G cells) are exclusively located in the antral mucosa and are found in the mid portion of the glands (Sundler & Håkanson 1991). G cells are also found in the duodenum (Rehfeld & van Solinge 1994), but only the antral G cells seem to participate in the regulation of gastric acid secretion. G cells are of the open type and the luminal surface is capable of responding to gastric luminal content. The G cells' main function is to synthesise gastrin, which makes the G cells essential in the regulation of both gastric acid secretion and growth of the oxyntic mucosa. Gastrin is released into blood from secretory granules along the basolateral membrane, which is close to mucosal blood vessels (Larsson 2000).

D cells are found both in the antrum, where the open type predominate, and corpus, mainly as closed cell type (Holst 1991). They secrete somatostatin (SST), a regulatory peptide hormone which inhibits numerous physiological processes. It inhibits gastrin release from the G cell, histamine release from the ECL cell and hydrochloric acid secretion from the parietal cell. In addition the somatostatin peptide plays an important role in the control of cell proliferation in normal and tumourous tissue (Patel 1997). The D cells are prototypical paracrine cells and have long cytoplasmatic processes that directly contact neighbouring cells (Larsson *et al* 1979).

Enterochromaffin (EC) cells are distributed all along the GI tract and play a pivotal role in several aspects of gut function including secretion, motility and sensation (Sjölund *et al* 1983; Gershon 1999). The EC cells show argyrophilia (Dawson 1948) and synthesise, store and release the biogenic amine serotonin, or 5-hydroxytryptamine (5-HT) (Rapport *et al* 1948). These cells are found both in the antrum and the corpus. They constitute about 25% of the gastric NE cells in man (Simonsson *et al* 1988), but are nearly absent in rat stomach (Capella *et al* 1991). The EC cells are belived to function as sensory transducers that activate mucosal processes of both intrinsic and extrinsic primary afferent neurones through their release of serotonin (Gershon 1999; Lundgren 2000). Secreted serotonin may also influence adjacent cells by paracrine actions and exert hormonal effects on distant cells via the blood circulation.

A-like cells are found predominantely in the oxyntic mucosa. At the ultrastructural level the secretory granules resemble those of the pancreatic glucagon (A) cell. Recently, the peptide ghrelin has been localised to the A-like cells in rats and humans

(Kojima *et al* 1999; Date *et al* 2000). The most important role of ghrelin appears to be stimulation of appetite and regulation of energy homeostasis. Ghrelin has also been suggested to play a role in regulation of gastric acid secretion (Peeters 2005). It appears to be an endocrine signal, possibly reaching the central nervous system via the bloodstream. However, it also uses neural pathways, in particular the vagus (Peeters 2005).

2 The NE system

The diffuse NE system can be defined as a set of cells which are diffusely dispersed in several organ systems including the GI and respiratory systems (Langley 1994). More than twenty different types of NE cells are found throughout the body, and common features of them are: the production of signal substanses such as regulatory peptides or bioactive amines, the presence of dense-core secretory granules from which the hormones are released by exocytosis in response to an external stimulus, and the absence of axons and synapses (Langley 1994).

NE cells have mainly two specific tasks; synthesis of peptide hormones and uptake of amino acids and transformation of these into biogenic amines by decarboxylation. Thus, the NE cells are also known by the acronym APUD (amine precursor uptake and decarboxylation) (Pearse 1969). The embryological origin of the NE cells is disputed. Because these cells show many similarities with neurons, it was claimed that they all originated from the neural crest (Pearse & Polak 1971). This was found not to be the case for all NE cells (Le Douarin 1988). The common theory today is that also these cells derive from a common endodermal stem cell (Fontaine & Le Douarin 1977; Rawdon & Andrew 1993; Andrew *et al* 1998; Montuenga *et al* 2003). However, this has not been fully determined (Andrew *et al* 1998; Waldum *et al* 1999).

The gastroentero-pancreatic system is the richest source of regulatory peptides outside the brain (Polak & Bloom 1986). Due to common cytochemical and functional characteristics, the pancreatic islet cells and other endocrine cells of the gut, including the EC cell, were brought together by Pearse (Pearse 1969). Today, the ECL cell in the oxyntic mucosa is probably the best characterised NE cell (Waldum *et al* 1996b).

In pancreas, the endocrine tissue (the pancreatic islets of Langerhans) is composed of four different cell types, α, β, δ and PP cells. These cells produce the hormones glucagon, insulin, somatostatin, and pancreatic polypeptide (PP), respectively. Under physiological conditions, the pancreatic islets control blood glucose homeostasis.

2.1 NE markers

CgA and its product pancreastatin are found in a variety of NE cells and are widely used as general markers of NE cells (Nobels *et al* 1998; Wick 2000). CgA is an acidic secretory glycoprotein that belongs to the granin family (Taupenot *et al* 2003). It is expressed in most types of NE cells and in most NE tumours (Nobels *et al* 1998; Wick 2000). CgA is typically bound to the membrane of the NE secretory granules. Its precise biological functions are not fully elucidated, but there is a number of hypotheses (Gorr *et al* 1987; Syversen *et al* 1994; Nobels *et al* 1998): CgA is thought to be a precursor to biologically active peptides with autocrine, paracrine and/or endocrine functions. One of these peptides is pancreastatin, which has a regulatory effect on secretion of both endocrine and non-endocrine cells (Nobels *et al* 1998). CgA itself appears to modulate the proteolytic processing of peptide hormones and neuropeptides. This glycoprotein takes part in the condensation of the content of secretory granula (Huttner *et al* 1991). It also may play a part in the osmotic regulation.

Other general markers are synaptophysin (SYN) and neuron-specific enolase (NSE). SYN has been identified as an integral membranous glycoprotein of pre-synaptic vesicles in neurons and in NE cells (Wick 2000). It is expressed by a variety of normal and neoplastic NE cells and is therefore used as a marker of NE differentiation in various tumours. It has also been found in non-NE tumours, and should therefore not be used alone to characterise NE differentiation, but in addition to e.g. CgA (Wick 2000). NSE is a soluble neuronal protein which is known as a cell specific isoenzyme of the glycolytic enzyme enolase (Marangos & Schmechel 1987). It is used as a marker of NE cells, although less specific than CgA (Lloyd 2003).

Histamine and HDC, SST, and serotonin can be used as specific markers for the NE ECL cells, D-cells and EC-cells, respectively. Moreover, VMAT-2 can be used as a marker of ECL-cells, although not being entirely ECL cell specific (Zhao *et al* 1997).

3 Control of gastric acid secretion

3.1 Gastrin and histamine

Gastrin was the second hormone postulated to exist (Edkins 1905), and gastrin-17 was finally characterised by Gregory and Tracy in 1964 (Gregory & Trazy 1964). Preprogastrin is rapidly altered during translation to generate progastrin, which is cleaved to generate the COOH-terminal Gly-extended gastrins (G34Gly, G17Gly). These are then converted to their corresponding COOH-terminally amidated peptides G34 and G17 which act at the cholecystokinin-2 (CCK-2, or gastrin-CCK_B) receptor.

Postprandial increase in acid secretion is mediated by the release of gastrin (Walsh 1994; Beltinger *et al* 1999). Gastrin releases histamine from the ECL cells and this histamine subsequently stimulates the parietal cells directly to produce acid (Figure 2) (Waldum *et al* 1991b; Shankley *et al* 1992).

In addition, gastrin increases histamine synthesis and storage in ECL cells by inducing the expression of HDC and VMAT-2. CgA mRNA abundance is also regulated by gastrin (Dimaline *et al* 1993). Gastrin receptor mRNA has been found expressed in parietal cells (Tømmerås *et al* 2002), but observations are diverging and there is no consensus on the question of a direct effect by gastrin on the parietal cell. In mice in which the gastrin gene has been deleted, the parietal cells are immature, and there is reduced acid secretion and insensitivity to acute secretagogue stimulation (Koh *et al* 1997; Friis-Hansen *et al* 1998). An infusion of gastrin reverses the insensitivity, suggesting that gastrin regulates the final steps in parietal cell maturation, which might be mediated by CCK-2 receptors expressed on these cells.

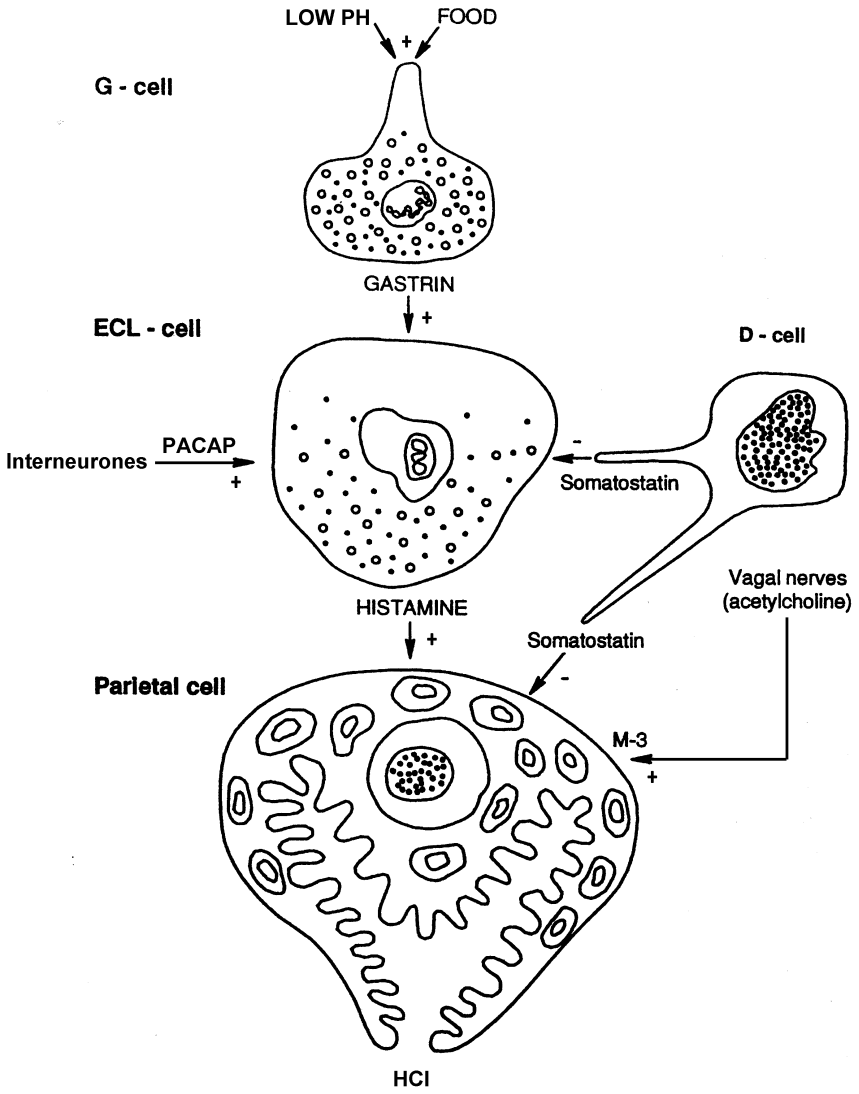

Figure 2: Schematic illustration of the main cells and mechanisms regulating gastric acid secretion (modified from (Waldum *et al* 1998b)).

3.2 Somatostatin

This peptide is widely expressed throughout the central and peripheral nervous systems and in peripheral tissues (Olias *et al* 2004). Somatostatin (SST) regulates neurotransmission in the brain, and secretory processes in the anterior pituary gland, the pancreas, and the GI tract. SST is released from D cells and is the main inhibitor of histamine release, shown by studies on the vascularly perfused stomach (Sandvik & Waldum 1988; Fykse *et al* 2005) and isolated ECL cells (Lindström *et al* 1997).

Five subtypes of somatostatin receptors (SSTR) are known at the present. These five subtypes of SSTR belong to the superfamily of G-protein coupled receptors that have seven transmembrane domains (Patel 1997). SSTRs are found in a range of tumour cell lines and are expressed in most human NE tumours. Different SST analogs (e.g. octreotide) are widely used both in diagnosis and therapy of these tumours. Functionally, somatostatin inhibits gastrin release by binding to the somatostatin receptor 2 (SSTR-2) on the ECL cells (Borin *et al* 1996).

3.3 Vagal activity

The nervous system is implicated in the regulation of gastric acid secretion (Walsh 1988). Activation of the vagal nerves and subsequent stimulation of postganglionic cholinergic fibers can stimulate gastric acid secretion by a direct effect of acetylcholin on muscarinic receptors type 3 on the parietal cells (Pfeiffer *et al* 1990; Sandvik *et al* 1998). Intrinsic neurons also contain different peptides, such as pituitary adenylate cyclase activating peptide (PACAP), gastrin releasing peptide (GRP), galanin and vasoactive intestinal peptide (VIP). They innervate the D, ECL and parietal cell of the oxyntic mucosa. PACAP induces histamine release from ECL cells via the PACAP-1 receptor (Pisegna *et al* 2000; Sandvik *et al* 2001). It has also become apparent that PACAP is even more potent than gastrin in the stimulation of ECL cell proliferation (Lauffer *et al* 1999). GRP seems to have a dual role in regulation of acid secretion. The vagal nerves stimulate gastrin release via GRP. The gastrin release is balanced by GRP stimulated release of somatostatin from D-cells. Inhibition of gastric acid secretion induced by the GRP-homologue bombesin is found to be SSTR-2 receptor-mediated (Piqueras *et al* 2003). A recent study found that bombesin-induced release of gastrin was potentiated by a somatostatin antagonist, suggesting that the G cells posess SSTR-2 receptors and further indicating a SSTR-2 receptor-mediated inhibition of gastrin release (Fykse *et al* 2005). Moreover, GRP may inhibit ECL cell histamine output indirectly by stimulating SST release from corpus D-cells. Galanin is a negative neural regulator of the ECL-cell (Zeng *et al* 1998).

4 Control of proliferation

Gastrin has a general trophic effect on the corpus mucosa (Ekman *et al* 1985; Havu 1986). In addition to the general trophic effect, gastrin has a specific and pronounced effect on proliferation of ECL-cells (Koh & Chen 2000). There is evidence that gastrin might act directly on ECL cells via the CCK-2 receptor to stimulate their proliferation (Mahr *et al* 1998). In rats and mice, it has been well demonstrated that ECL cells are able to undergo self-replication (Ryberg *et al* 1990). Gastrin also increases the proliferation rate of stem cells in the mucosal progenitor zone (Ryberg *et al* 1990), and a role for gastrin in differentiation of the gastric progenitor cells into the different mucosal cell lineages, particularly the parietal cell lineage, has been proposed (Friis-Hansen *et al* 1998). The general trophic effect of gastrin may be due to a direct action on the endodermal-derived stem cell, although the gastric stem cells have not yet been conclusively shown to express the CCK-2 receptor. This suggests that the general trophic effect may also be mediated indirectly by substances released from other target cells expressing the CCK-2 receptor, e.g., the ECL cell (Waldum *et al* 1991c; Miyazaki *et al* 1999; Dockray *et al* 2001).

Gastrin activates several paracrine cascades downstream of the CCK-2 receptor. The epidermal-like growth factor (EGF) family members and regenerating gene protein (Reg) have attracted particular attention because they may mediate some of the effects of gastrin on cell proliferation (Fukui *et al* 1998; Higham *et al* 1999; Miyazaki *et al* 1999; Varro *et al* 2002b). In both experimental animal models of hypergastrinemia (omeprazole-treated rats), and in patients with hypergastrinemia (pernicious anemia) there is increased expression of gastric Reg (Fukui *et al* 1998; Higham *et al* 1999). In both rat and man, Reg is expressed in ECL cells, but in human stomach it is also found in chief cells. Reg increases proliferation of gastric epithelial cells and mutations of Reg are also associated with ECL cell carcinoid tumours (Fukui *et al* 1998; Higham *et al* 1999). Moreover, in trangenic mice that overexpress Reg there is increased differentiation of parietal and chief cells (Miyaoka *et al* 2004). In a model system designed to allow studies of paracrine signalling pathways there is evidence that gastrin acts via shedding of heparine-binding epidermal-like growth factor (HB-

EGF) to stimulate proliferation (Miyazaki *et al* 1999; Varro *et al* 2002b). Gastrin may also stimulate HB-EGF gene expression (Sinclair *et al* 2004).

There are studies demonstrating that ECL cells both secrete and are stimulated by transforming growth factor α (TGF-α) (Lawton *et al* 1996; Modlin *et al* 1996). Basic fibroblast growth factor **(**BFGF) is thought to be secreted by the ECL cells and is found in the majority of human ECL cell hyperplastic lesions and carcinoid tumours (Bordi *et al* 1994). It is postulated to have autocrine properities as it increases the number of ECL cells in culture (Mahr *et al* 1998). Recent findings indicate that gastrin, acting via CCK-2 receptors, also regulates the expression of a number of genes in gastric mucosa that potentially influences the organisation of the mucosa, including a trefoil factor, matrix metalloproteinases and inhibitors of extracellular proteolysis (Wroblewski *et al* 2002; Varro *et al* 2002a; Khan *et al* 2003).

In addition to inhibition of histamine release, somatostatin also inhibits mucosal growth. The SST analogue octreotide inhibits ECL cell proliferation (Raynor *et al* 1993; Tsutsui *et al* 1995; Bakke *et al* 2000; Fykse *et al* 2004) and has been shown to reduce serum gastrin concentration in hypergastrinemic mastomys (Modlin *et al* 1992).

Recent work indicates that progastrin and biosyntetic intermediates with C-terminal Gly (G-Gly) stimulate the proliferation of a varity of tumour cells through mechanisms independent of the CCK-2 receptor (Hollande *et al* 1997; Singh *et al* 2003).

5 Tumours of the gastric mucosa

5.1 Adenocarcinomas

Gastric adenocarcinoma is the second most common cancer worldwide. Despite a decreasing incidence, gastric cancer accounts for 3-10% of all cancer related deaths (Crawford 1994). Ninety percent of all tumours of the stomach are malignant, and gastric adenocarcinoma comprises 95% of the total number of malignancies

(Schwartz 1996). Curative therapy involves surgical resection. The overall 5-year survival rate of patients with resectable gastric adenocarcinoma ranges from 10-30% (Harrison *et al* 1998; Msika *et al* 2000; Green *et al* 2002).

Currently diagnosis of gastric cancer requires histopathologic examination of tissue or cytologic assessment of gastric brushing/washing. Several classification systems have been proposed to aid the description of gastric cancer either through macroscopic features or on the basis of microscopic structure. The two most commonly used are the Laurén and World Health Organization (WHO) systems (Fenogilo-Preiser *et al* 2000).

The Laurén classification divides gastric cancer into two major histologic types: intestinal and diffuse (Laurén 1965). This system describes tumours on the basis of microscopic features. Diffuse-type cancers have noncohesive tumour cells diffusely infiltrating the stroma and often exhibit deep infiltration of the stomach wall with little or no gland formation (Fenogilo-Preiser *et al* 2000; Werner *et al* 2001). In comparison to intestinal-type gastric cancers, diffuse-type gastric cancers are less related to environmental influences, have increased in relative incidence, occur more often in young patients, more common in women, and are associated with worse prognosis (Fenogilo-Preiser *et al* 2000). Intestinal-type cancers show recognisable gland formation and are belived to arise secondary to chronic atrophic gastritis (Fenogilo-Preiser *et al* 2000; Werner *et al* 2001).

Helicobacter pylori infections and autoimmune gastritis are the most common predisposing conditions to gastric cancer. If gastritis persists, gastric atrophy occurs followed by intestinal metaplasia, which in turn may lead to dysplasia and eventually carcinoma.

Some studies have suggested that a proportion of gastric adenocarcinomas develop from the ECL cell, especially those classified as diffuse type according to Laurén (Waldum *et al* 1991a; Waldum *et al* 1998a). Gastric adenocarcinomas are associated with hypergastrinemia (Rakic *et al* 1991), and the association is particularly strong in patients with severe hypergastrinemia secondary to chronic gastritis, without or with pernicious anemia (Borch *et al* 1985; Sipponen *et al* 1985; Hsing *et al* 1993; Kokkola

et al 1998). Furthermore, some gastric adenocarcinomas in hypergastrinemic patients show signs of NE differentiation (Qvigstad *et al* 2002).

Several factors have been associated with increased local recurrence and decreased survival in gastric carcinoma. Putative tumour markers (p53, E-cadherin, CD-34, c-ErbB2, CA 72-4, CEA, c-met, K-sam) have recently gained popularity as potential prognostic indicators for predicting tumour behavior (Starzynska *et al* 1996; Allgayer *et al* 2000; Pinto-De-Sousa *et al* 2001; Gaspar *et al* 2001; Lee *et al* 2002). Of these, only E-cadherin has been linked definitively as a marker of heriditary diffuse gastric cancer (Machado *et al* 1999; Ascano *et al* 2001). These markers are likely to gain importance as the field of gene-expression analysis continues to expand (Starzynska *et al* 1996).

5.2 Carcinoids

Gastric carcinoid tumours arise from proliferating ECL-cells. Elevated levels of plasma gastrin initiate and maintain neoplastic change in these cells (Gough *et al* 1994). The basis of the hypergastrinemia is usually hypoacidity due to chronic gastritis, but it can also be caused by a gastrin-secreting neoplasm. The sequence hyperplasia, dysplasia and neoplasia characterises the progression (Solcia *et al* 1988). Carcinoid tumours are distinct from adenocarcinomas in their biological behavior and, in general, exhibit a relatively favourable outcome. Gastric carcinoids are reported to comprise 8.7% of all GI carcinoid tumours (Modlin *et al* 2003b).

Gastric ECL cell carcinoids have been classified into three types (Rindi *et al* 1993). Type I carcinoid tumours are associated with chronic atrophic gastritis, which is characterised by chronic inflammation of the oxyntic mucosa resulting in atrophy of the oxyntic glands and achlorhydria with hypergastrinemia. It often occurs in association with autoimmune diseases such as pernicious anemia. This is the most common type of gastric carcinoid. They are usually small, multicentric and often localised to the fundus. Type II carcinoids are associated with Zollinger-Ellison syndrome (ZES) and type I multiple endocrine neoplasia (MEN-1); type II carcinoid is less common than type I, and tumours are usually small and multiple. The prognosis is intermediate between the relatively aggressive type III carcinoids and the

more benign tumours of type 1 (Rindi *et al* 1993). Both type I and type II are related to gastrin, are considered semi-malignant, but also have metastatic potential (Borch *et al* 1985). Type III sporadic carcinoid tumours are usually single, isolated tumours arising in normal gastric mucosa (Lehtola *et al* 1985). They are not associated with hypergastrinemia, and are thus independent of the trophic stimulus of gastrin (Modlin & Tang 1996). Metastatic spread is common for this type of carcinoid.

Poorly differentiated, high grade NE carcinomas comprise a distinct, potentially unrelated subset of gastric carcinoids. Some have found that the majority of patients with such tumours also have chronic atrophic gastritis and hypergastrinemia (Rindi *et al* 1993), implying that these more aggressive carcinomas could have carcinogenetic pathways in common with ECL cell carcinoids. Several decades ago, it was suggested that there is a morphological continuum, ranging from adenocarcinomas to typical carcinoids (Bates & Belter 1967).

5.3 Consequences of hypergastrinemia

”The gastrin concept” (*hypoacidity→hypergastrinemia→ECL cell hyperplasia→carcinoid*), explaining the relationship between hypergastrinemia induced by acid hypo secretion and gastric carcinoids, has been known for several years (Håkanson & Sundler 1990). Whereas the role of gastrin in the ECL cell hyperplasia-dysplasia-neoplasia sequence is well documented in rodents (Ekman *et al* 1985; Poynter *et al* 1985; Havu 1986), it is more difficult to study in humans as the time perspective of carcinoids is prolonged, and also due to confounding factors. However, gastrin induces ECL cell hyperplasia and ECLomas also in humans (Solcia *et al* 1990; D'Adda *et al* 1996). It is known that patients with chronic atrophic gastritis have an increased risk of developing gastric carcinoids (Borch *et al* 1985; Kokkola *et al* 1998). Patients with hypergastrinemia due to sporadic gastrinomas (Zollinger-Ellison Syndrome (ZES)) without multiple endocrine neoplasia type 1 (MEN1) may also develop advanced ECL cell changes and dysplasia (Peghini *et al* 2002) as well as carcinoids (Cadiot *et al* 1995). Gastric adenocarcinomas have also been associated with hypergastrinemia (Rakic *et al* 1991).

6 Gene expression analysis

6.1 A new era in biomedical research

The sequencing of the complete human genome (International Human Genome Sequencing Consortium 2001; Venter *et al* 2001) has led us into a new era in biomedical research. The post-genomic challenge is to move from genomic sequence to a complete understanding of gene function and complex biological processes. Increasingly, research in the life sciences is moving from a gene-by-gene approach to a Systems Biology approach (Kitano 2002b), essentially driven by a push-pull mechanism: the capability to generate high-throughput data for data-driven hypothesis generation, and the growing need to understand the dynamic behaviour of biological systems. ”Systems Biology” approaches, a novel paradigm for modelling of systems function, will for a large part hinge on the availability of robust technology for data production.

Several methods have been developed to be able to meet the growing need for high-throughput analysis. The microarray technology is a rapidly advancing field gaining popularity in many biological diciplines. The technology allows simultaneous measurement of the expression of thousands of genes from a single sample, providing us with an enormous amount of information compared with more traditional studies of single genes. Microarray analysis is a particularly useful tool in cancer research based on the complexity of the disease, where many genes and mechanisms are involved (Kitano 2004). The cDNA microarray was pioneered by Patric Brown and David Botstein (Schena *et al* 1995), while Affymetrix developed the GeneChip arrays (oligo arrays) (Chee *et al* 1996).

Other techniques, like serial analysis of gene expression (SAGE) (Velculescu *et al* 1995), were also developed to study high throughput gene expression at RNA level. However, the microarray methods now seem to be dominating. Microarray-based functional genomics technology is a key component of a ”Systems Biology” approach for biological function discovery.

In addition to sequence information, knowledge of gene function and how gene expression modulates cellular phenotype and response to the environment is important for understanding tumour biology, the improvement of diagnosis and general management of cancer. Variation in gene expression reflects biological function of physiological and patophysiological samples, and the use of microarrays to study expression patterns in tumours offers a systematic and detailed way of exploring such complex biological systems. An advantage of microarray is that it is a translational tool that incorporates functional interactions in an attempt to understand biology, not simply to identify the component parts of a pathway (Lockhart & Winzeler 2000). The ability of cataloguing and classifying genes with characteristic patterns of expression in several model systems as well as human cells gives insight into which regulatory systems are operative and provides clues to the function of unknown genes (Hvidsten *et al* 2001; Lægreid *et al* 2003).

6.2 DNA microarray

PCR-amplified cDNA sequences or presynthesised oligonucleotide sequences can be printed on the array with the aid of a robotic arm, or alternatively oligonucleotide sequences may be synthesised directly on the array (e.g. Affymetrix, Rosetta Biosoftware/Agilent Technologies). A ”one sample, one array” approach is applied in single colour platforms such as Affymetrix and Applied Biosystems Expression Array System (ABI). In two-colour platforms, two different fluorophore labels are used. The DNA microarray methods are based on complementary base paring, and only spots with hybridised sample will have a quantitative signal. In the single colour systems test and control samples are hybridised separately to two different microarrays with subsequent comparison of signals. In a two-colour-based method there is a competitive binding of reference and sample, and the ratio between these two signals for each spot are calculated and used for further analysis.

The different steps in a two-colour experiment will be outlined in the following (Figure 3). Total RNA or mRNA is isolated from properly preserved tissue or cell samples (snap frozen or RNAlater®). If the sample is limited in size, RNA amplification may be necessary to obtain the amount of material needed for the

Errors in the IMAGE collections of clones used on cDNA printed microarrays have been reported, and additional errors may have occurred during production. For this reason, clones representing genes of particular interest, should be sequence verified. Duplicate spots and external spike controls printed on the arrays are important issues for technical validation. Parameters like input and output ratios, signal linearity, hybridisation specificity and consistency across an array can be evaluated by the utilisation of exogenous control genes. Moreover, replicate hybridisations can also be quite useful as a means of identifying problematic hybridisations in a study, where the correlation coefficient between replicates can provide a means of assaying relative quality of the arrays. Replicate experiments should also include multiple ”biological repeats”. Biological repeats show a higher degree of variability in comparison to mere technical replicates of one biological sample but are essential to assess biological variation, for statistical analysis and for the generalisation of conclusions. In two-colour platforms, exchange of the dyes between the experiment and the reference sample (a so-called ”dye-swap” experiment) provides additional control of labeling and other biases. Microarray observations which are important for the scientific interpretation will usually be verified by an alternative method such as Northern Blot, dot blot methods, real-time quantitative RT-PCR , Western blot or immunohistochemistry (Chuaqui *et al* 2002; Yadetie *et al* 2004).

Translation of individual mRNA species into their encoded proteins is regulated at multiple levels: transcriptional control, RNA processing control, RNA transport control, translational control, mRNA degradation control and protein activity control (Figure 4), producing discrepancies between mRNA and protein levels. As mRNA is eventually translated into protein, one might assume that there should be some sort of correlation between the level of mRNA and that of protein. Attempts to correlate protein abundance with mRNA expression levels have had variable success (Greenbaum *et al* 2003). Some studies found a positive correlation, ranging from $r = 0.48\text{-}0.76$ (Anderson & Seilhamer 1997; Futcher *et al* 1999; Ideker *et al* 2001). Other studies show no significant correlation (Greenbaum *et al* 2003). However, correlations have been found between the mRNA expression levels of different protein subunits within protein complexes (Jansen *et al* 2002). This implies that there should be, in general, a correlation between mRNA and protein abundance, as the subunits provide a special case as they have to be available in stoichiometric amounts

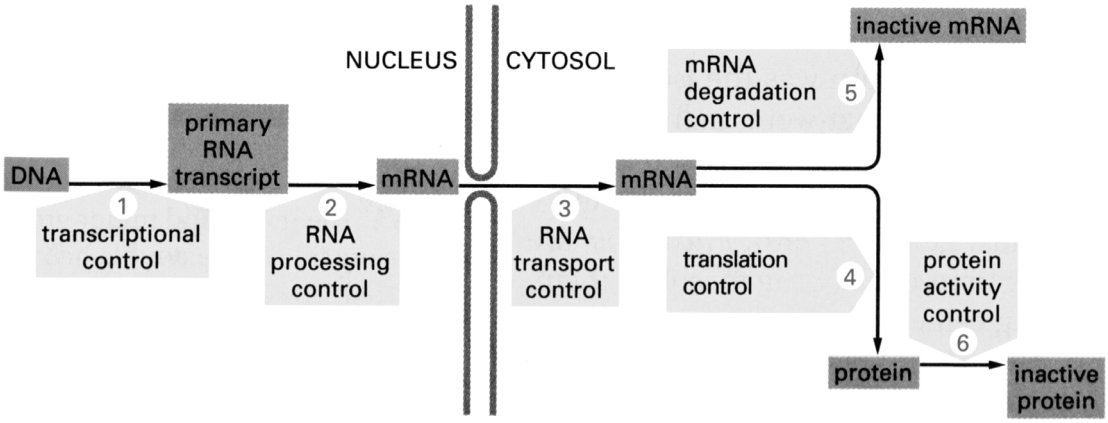

Figure 4: Six steps at which eucaryote gene expression can be controlled (Alberts *et al* 1994).

of proteins for the complexes to function. Thus, a major limitation to find correlation is belived to be due to experimental challenges, such as technological limitations in mRNA and protein expression experiments (Greenbaum *et al* 2003). In addition, the measurements of mRNA and protein abundance were in some experiments not carried out using identical samples, and typically only a small set of protein abundance data was used in the analysis. The comparisons will probably become more informative, as relatively large datasets of mRNA and proteomic measurements are obtained from the same cell samples (Nie *et al* 2006).

Observations underscore that analysis at the transcript level, albeit important, is insufficient by itself to describe completely the phenotype of cells under different conditions (MacKay *et al* 2004). However, the quantification of protein abundance and mRNA expression is not an exercise in redundancy; measurements taken from both of these molecular populations are necessary for a complete understanding of how the cell works (Hatzimanikatis *et al* 1999). High-throughput protein identification and quantification still lags behind the high-throughput experimental techniques used to determine mRNA expression levels as the proteomic arrays are still struggling with technical problems.

6.3 Knowledge discovery

Typical array experiments create hundreds of thousands of data points in highdimensional space defined by the number of samples analysed. This represents a

Unsupervised methods are suitable for class and pattern discovery, but they are dependent on several parameter settings such as distance matrices, clustering methods and types of inter cluster distances which may give rise to a wide range of qualitatively different clusters/patterns observed. Presently there is no universal standard for how the data analysis should be done, and often the researcher is left to explore the different methods and different databases before deciding on a preferred approach, whithout really being able to apply stringent, preconceived criteria for the choice.

Biological background information such as gene networks, gene pathways and gene ontology are partly available through public databases. An increasing number of companies and academic institutions are further developing the technique, making complete packages consisting of e.g. statistical tools, ontology and pathway analysis (microarray analysis reviewed in; (Wildsmith & Elcock 2001; Stears *et al* 2003)).

6.4 Molecular profiling

Breast cancer was among the first malignant diseases subjected to large-scale gene expression analysis. Breast cancer is a complex disease, with large variability between breast cancer patients regarding prognosis and treatment response. A search for predictive and prognostic markers has been going on for a long time and clinical markers in present use are variables like age, lymph node status, receptor status, proto-oncogenes, and gene mutations. However, these methods need further refinement and breast cancer was an obvious choice when the first large-scale gene expression analyses of solid malignant tumours were done. Several laboratories have reported that unsupervised clustering of microarray-generated gene expression data from breast cancer consistently separates estrogen receptor positive and negative tumours into two clusters (Perou *et al* 2000; Gruvberger *et al* 2001), strongly suggesting that genomewide expression analysis can indeed identify clinically important disease subclasses and discover new subclasses (Sørlie *et al* 2001). Moreover, the supervised methods applied by Hedenfalk *et al* could distinguish sporadic breast cancer from breast cancer, early onset (BRCA) mutant cases (Hedenfalk *et al* 2001). Most interesting, studies using supervised learning have demonstrated that microarray analysis can predict overall and relapse-free survival

based on "predictive gene sets" that are superior to currently available clinical and histologic prognostic models (van de Vijver *et al* 2002; van't Veer *et al* 2002; Lossos *et al* 2004).

The application of unsupervised methods to diseases such as nonsmall cell lung cancer, lymphoma, oesophageal carcinoma, and Barrett's oesophagus have similarly shown the use of microarray in documenting tumour subgroups that correlate with clinicopathological parameters and survival (Alizadeh *et al* 2000; Wigle *et al* 2002; Selaru *et al* 2002). On the other hand, supervised methods have e.g. been applied to predict recurrence rates for hepatocellular carcinoma (Iizuka *et al* 2003), and to classify and predict leukemia subtypes, drug resistance and treatment outcome (Golub *et al* 1999; Yeoh *et al* 2002; Holleman *et al* 2004).

Although most microarray analysis on disease states has concentrated on malignancies, the method is increasingly being utilised in studies also on other diseases. Subgroups of patients with glomerulonephritis (Peterson *et al* 2004) have been identified by means of unsupervised learning. In patients with heart failure, gene expression profiling combined with supervised learning could predict cardiomyopathy etiology (Kittleson *et al* 2004). Microarray profiling and significance analysis have been applied as tools to provide better understanding of nonalcoholic steatohepatitis (Sreekumar *et al* 2003) and autoimmune diseases (Qing & Putterman 2004; Jarvis *et al* 2004).

In addition to exploring disease, genome profiling has been applied to study normal physiological processes in humans and several model systems. Microarrays containing virtually all yeast genes have been used to study coregulated genes linked to a variety of conditions as differences in metabolic state (DeRisi *et al* 1997), growth conditions (Wodicka *et al* 1997), sporulation (Chu *et al* 1998) and cell cycle regulation (Spellman *et al* 1998), by use of clustering algorithms (unsupervised learning). *In vivo* effects of growth hormone have been studied in liver, heart and kidney of rats (Flores-Morales *et al* 2001; Tollet-Egnell *et al* 2001). In these studies, differentially expressed genes and involved biological processes were identified. There have been several reports on the use of microarrays to study expression patterns in cultivated human cells under different experimental conditions, for example heat shock responses in T-cells (significance analysis) (Schena *et al* 1996), and the

response to fibroblast serum stimulation (clustering of genes) (Iyer *et al* 1999). de Veer *et al* compiled data from many experiments on human and murine cell lines, using two different microarray formats to obtain a more comprehensive list and a better understanding of genes regulated by interferons (de Veer *et al* 2001).

Aims of the studies

- to characterise NE features of AR42J cells and elucidate whether this cell line can be used as a model system for NE tumours (I)
- to establish microarray methods for small amounts of biological material (II)
- to establish methods for data analysis and supervised learning/classification, and develop classifiers for gastric carcinoma with respect to several clinical parameters (II)
- to examine the effects of potent acid inhibition (proton pump inhibitor, PPI) on genome-wide gene expression in rats, on biological process and single gene levels (III)
- to investigate the effects of therapeutic doses of a potent acid inhibitor (proton pump inhibitor, PPI) on genome-wide gene expression in patients, on biological process and single gene levels (IV)

Summary of papers

Paper I:

Expression of chromogranin A and somatostatin receptors in pancreatic AR42J cells.

In this paper, some NE features of the rat pancreatic acinar cell line AR42J were studied. The results show that AR42J cells express CgA mRNA, and secrete its cleavage product pancreastatin. Gene expression of CgA was upregulated by gastrin and EGF. Some of the cells have NE secretion granules and are faintly immunoreactive to NE markers, indicating that the cells are of a rather poorly differentiated type. AR42J cells express SSTR subtype 1, 2, 3 and 5. Proliferation studies showed that the SST analog octreotide inhibits gastrin-induced proliferation, confirming the expression of functional SSTR in AR42J. The results suggest that AR42J cell line could be a useful experimental model to study the regulation of CgA and SSTRs.

Paper II:

Gene expression based classification of gastric carcinoma.

Gastric carcinoma is the second most frequent cause of cancer death world-wide. Using cDNA microarray analysis with a 2504 gene probe set, we have shown in this paper that microarray analysis of gastric carcinoma can produce data that are significantly related to important clinicopathological features. Using a supervised learning method, we were able to generate reliable classifiers for prediction of several clinically important parameters, including histopathological classification (Laurén), presence of lymph node metastasis and location of tumour. Our work also identified several genes that were not previously known to display characteristic expression patterns in gastric carcinoma.

Paper III:

Molecular characterisation of rat gastric mucosal response to potent acid inhibition.

Inhibitors of gastric acid secretion are among the most commonly used drugs in clinical practice. Proton pump inhibitors (PPIs) cause profound changes in the

intragastric environment with near-neutral pH, and increase serum concentration of the gastric secretagogue hormone gastrin. Long-term hypergastrinemia increases mucosal thickness and ECL cell density in gastric corpus mucosa, and results in development of gastric carcinoids in experimental animals. The aim of this study was to elucidate molecular responses in gastric mucosa of rats receiving the proton pump inhibitor omeprazole for 10 weeks by measuring genome-wide transcript level changes using cDNA microarrays with probes representing 11848 genes. Our results indicate a global change in the induction of proliferation, apoptosis, inflammatory, immune and stress response in the presence of proton pump inhibitors. Several of the identified genes were previously known to be affected by potent acid inhibition. However, many genes were identified that were not previously known to respond to inhibition of gastric acid secretion or that have unknown biological functions. Characterisation of the roles of these genes may give new insight into molecular responses to treatment with PPIs.

Paper IV:

Changes in gene expression of gastric mucosa during therapeutic acid inhibition.

Knowledge of the effect on mucosal gene expression of PPIs given to humans in ordinary, therapeutic doses is limited. Eight patients suffering from gastro-oesophageal reflux disease were included in this study. Endoscopic biopsies were taken from the corpus mucosa before and towards the end of a three-month treatment period with the PPI esomeprazole. Using cDNA microarrays with probes representing 5346 genes, a large set of candidate genes not previously associated with acid inhibition was identified, which underlining the complex molecular responses to this therapeutic intervention. Our results indicate a global change in the induction of the same biological processes as identified in paper III. Moreover, eight genes were found to be regulated in the same direction in rats and patients in response PPIs. The genes presented in this study are likely to be associated with acid inhibition. Further studies specifically targeting the genes implicated in this study will further our understanding of the molecular responses to potent acid inhibition, including the mucosal response to moderately increased gastrin levels encountered in clinical practice.

General discussion

7 Methodological considerations

DNA microarray analysis is the main method used in the present study. Thus, only microarray will be considered in this chapter. For all other methods used, standard procedures were performed.

The four studies presented in this thesis reflect the development in technology that has taken place over the last few years, from studies focusing on single gene analysis to whole genome studies. As the techniques are constantly developing, new variants of the methods were used for each paper. The milestone of sequencing the human genome was achieved during this period (International Human Genome Sequencing Consortium 2001; Venter *et al* 2001), and a whole new world of knowledge has become available through the enormous increase in available bioinformatics tools.

7.1 cDNA microarrays

Only cDNA microarrays were available when the present studies were performed. Thus, glass microarrays with cDNA clones (200-2000 nucleotides in length) printed by the Norwegian Microarray Consortium, were used in the studies presented in this thesis. The cDNA clones spotted on the arrays were from publicly available clones obtained from the IMAGE consortium (Lennon *et al* 1996) and some clones were generated in-house for our specific areas of interest. Errors in the IMAGE collections of clones have been reported, and additional errors may have occurred during production. For this reason, in paper III and IV, clones representing genes of particular interest were sequence verified.

One of the major concerns of cDNA microarrays is cross-hybridisation of the labeled RNA (or cDNA) to non-target homologous probe sequences on the array (Evertsz *et al* 2001; Afshari 2002). Due to poly(A)-tail of mRNA, PCR-products (cDNAs) contain poly (dA/dT) sequences. Cross-hybridisation and non-specific hybridisations that arise due to poly (dA/dT) sequences or repetitive elements were reduced or eliminated using blocking reagents like cot-1 and LNA dT blocker®. Generally, only a minimal background noise from the array can be accepted, and small variations in production of arrays and experimental conditions can create immense background staining. Moreover, the uneven length of the cDNA printed on the arrays makes it difficult to find an optimal hybridisation temperature. As cross-hybridisation is known to be one of the main sources of errors in cDNA microarrays, particularly interesting results from the microarray experiments in paper II and III were validated using RT-PCR or Northern blot analysis, respectively. The long length of the cDNA probe makes hybridisation more stable compared to oligo probes, allowing the use of more stringent washing conditions in order to reduce noise signal.

Oligonucleotides offer greater specificity than cDNAs or PCR products, having the capacity to distinguish single-nucleotide polymorphisms and discern splice variants. Compared with the cDNA probes, a smaller percentage of the oligonucleotide probes show a potential for cross-hybridisation (Flikka *et al* 2004). This is because the oligonucleotide probe sequences are much shorter (60-70 nucleotides), designed from specific regions of cDNA to minimise cross-hybridisations. However, conserved gene sequences coding for functionally related proteins could be sufficiently long to cross-hybridise in arrays using shorter oligos (Hughes *et al* 2001). For oligo nucleotide arrays, quality control for the immobilised sequence is done during syntesis, making it unnecessary to perform sequence verification. Also, the fact that the production of oligonucleotide microarrays does not require handling of a large clone collection reduces the need of a costly infrastructure.

7.2 3DNA labeling method

In the present study we used a microarray labeling and hybridisation protocol (Figure 6) that enables the use of very small amounts of RNA without amplification (Stears *et al* 2000). The 3DNA labeling system provides a more predictable and

consistent signal than direct or indirect dye incorporation for two reasons. First, since the fluorescent dye is part of the 3DNA dendrimer reagent, it does not have to be incorporated during the cDNA preparation. This avoids the ineffective cDNA synthesis observed when direct incorporation methods are used. Direct incorporation also generates a risk of unequal incorporation of Cy3 and Cy5 labeled nucleotides and dye-specific hybridisation artefacts. Second, because each 3DNA molecule contains an average of about 375 fluorescent dyes and each bound cDNA will be detected by a single 3DNA molecule, the signal generated from each message will be largely independent of base composition or length of the transcript. In contrast, the signal generated from each message labeled through direct dye incorporation will vary depending on the base composition and length of the message.

A narrow window of change of expression is observed in most of our microarray analyses. This appears to be a result of low dynamic range of fluorescent signals obtained using the dendrimer labeling and hybridisation protocol, compared with other labeling methods (unpublished results from our laboratory). This has been confirmed in studies comparing the dendrimer labeling method with other labeling methods, concluding that the dendrimer method has a lower dynamic range (Manduchi *et al* 2002; Richter *et al* 2002). Despite these limitations, the method can identify differentially regulated genes with reasonable accuracy, as we show using Northern blotting and spiked external controls. However, efforts were made to limit the compression effect in the present study, as Genisphere has provided improved protocols. A new reagent, the “high-end differential enhancer” addressed a portion of the compressed differentials. The “high-end differential enhancer” block the unlabled arms (meaning arms that did not receive eighter a fluorescent dye labeled oligo or a complementary sequence oligo to the primer). Another blocking reagent, the Locked Nucleic Acid blockers (LNA dT Blocker), was also added to the kit. PCR-products (cDNAs) contain poly (dA/dT) sequences. The LNA dT Blocker is a high-performance poly T based blocking reagent designed to completely block all the poly dA sequences present in the cDNA probes (Singh *et al* 1998). A regular oligo dT blocker, which we initially used, have an on-off interaction. The LNA dT blocker, once they hybridise on to a string of A’s it is locked, so it is able to completely block all poly dA spots. In addition, a “two-step” hybridisation protocol was introduced,

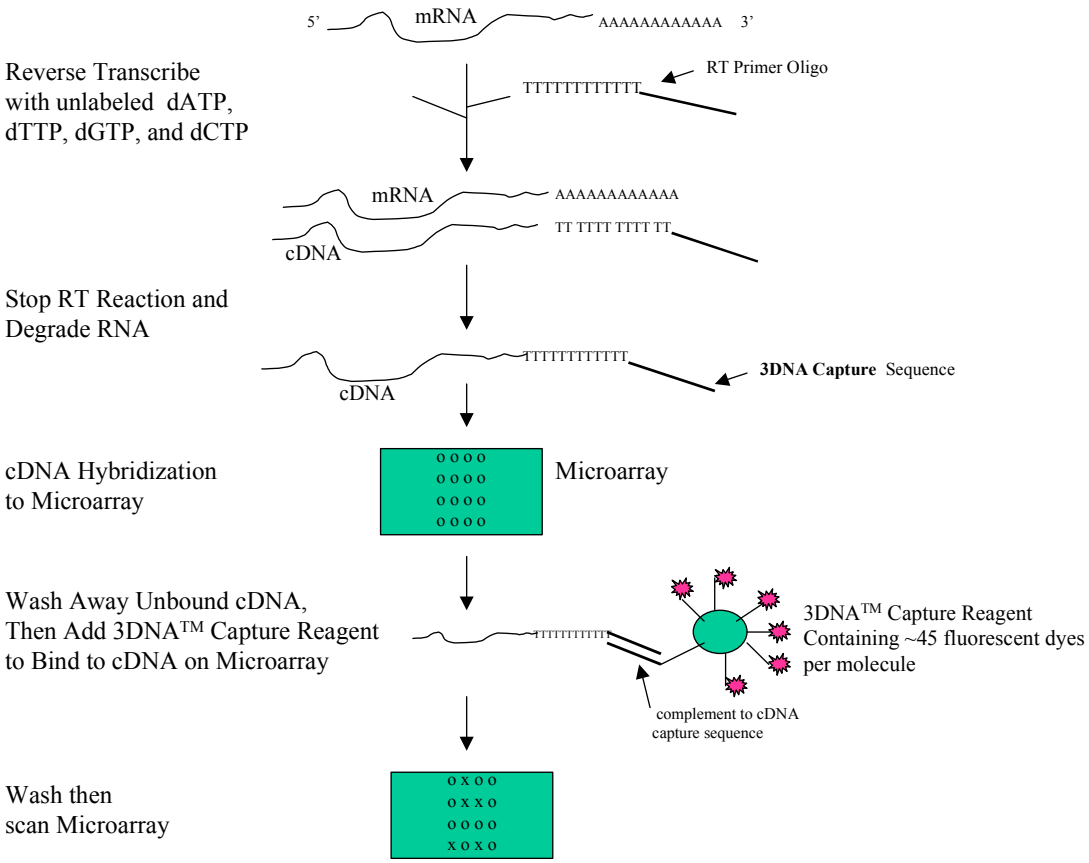

Figure 6: Outline of the different steps in a "two-step" microarray detection protocol with 3DNA™ reagent. Alternatively, a "one step protocol" can be applied, in which the cDNA is pre-hybridised to the fluorescent 3DNA to produce a cDNA/3DNA complex with subsequent hybridisation of the complex to the array (Genisphere®).

adding the bulky 3DNA fluorescent probe *after* cDNA hybridisation, and thus circumventing any sterical hindrance during hybridisation. In the previous "one-step protocol", the cDNA is pre-hybridised to the fluorescent 3DNA to produce a cDNA/3DNA complex with subsequent hybridisation of the complex to the array. The two-step hybridisation improves the signal intensities and the dynamic range, mainly for low level expressors. The one-step protocol was used in paper II. In paper IV, the one-step protocol and the "high-end differential enhancer" were used. In paper III, the two-step protocol, the "high-end differential enhancer" and LNA dT Blocker were applied.

7.3 Quality control

7.3.1 Spike-in controls

In general, microarrays pose tremendous challenges to the user, and the technology is still struggling to achieve good quality standards. The commercial SpotReport Array Validation System (Stratagene) (spike-in controls) was used in paper III and IV for assessment of experimental performance. The spike-in controls consist of exogenous RNA transcripts, which were added to the RNA samples prior to reverse transcription and labeling. The arrays were spotted with probes for these exogenous transcripts. Different amounts of each exogenous transcript was added to the test and reference samples to generate pre-determined signal ratios between fluorescent dyes upon scanning of the array. This information was used to evaluate reverse transcription and labeling procedures, as well as for assessing the dynamic range of the assay.

Labeled reverse complementary oligonucleotides offer another candidate for reference sample purposes. Oligonucleotides are stable, easy to quality control and quantify, can be designed to contain specific sequences and can produce consistent signals across arrays. Such controls can be used to monitor the hybridisation step in a very controlled manner.

7.3.2 Replicates

Due to cost and low amount of sample material (paper II and IV), technical replicates such as repeated hybridisations and dye-swaps were not performed. Compared with direct incorporation of labeled nucleotides, the 3DNA method enables a more even hybridisation of the two dyes. For this reasons, dye-swap replicates were regarded as less important, and hence not included. Labeling has caused debate the past couple of years, as one of the fluorescent colours (Cy5) used worldwide seemed to bleach very fast, and finally ozone was suggested to cause this problem (Fare *et al* 2003). Artefacts due to different sensitivity to photobleaching could have been monitored better if dye-swaps had been included. However, the normalisation process can balance many of the systematic variations. Each individual clone was replicated two or more times at different locations of the array. With this layout, random and

systematic measurement errors in the microarray process that might affect the data can be easily assessed, and more reliable information can be gained from a single experiment. As a consequence, the number of hybridisation experiments can be reduced. Several biological replicates were included in the present microarray papers.

In the present study, all hybridisations were performed manually. Today, to ensure a more even and reproduceable hybridisation and subsequent washing procedure, and reduce the "hands on" time, the use of a hybridisation station is preferable.

7.3.3 Universal reference samples

In paper II, universal human reference RNA obtained from Strategene was used to generate a reproducible hybridisation control over a series of arrays, thus providing a base level against which the relative abundance of transcripts from test samples can be measured. Pooled RNA from cell lines were used as a universal reference sample. Cell lines provide an abundant source of RNA, and a mixture of RNA from several cells is used for production of several commercially available universal reference RNAs. There is an ongoing effort to standardise the reference material (Novoradovskaya *et al* 2004; Cronin *et al* 2004). The ability to perform comparisons between experiments, platforms and laboratories can be greatly enhanced if all users incorporate such a standard into their process. Concerns over this approach, however, have been raised as the pooling of RNA from several cell lines may result in some low abundance transcripts being diluted below the limit of detection.

From a standardisation point of view, the use of this cellular RNA is sub-optimal for several reasons: 1) the relative expression of transcripts within and between cell lines is not necessarily stable over time and may result in batch-to-batch variation; 2) the application of a primary RNA standard against which rigorous quality control can be performed is challenging due to the unstable nature of the raw material; 3) the inadequate reliability when measuring absolute quantities of each individual transcript within the reference sample makes sample characterisation a challenge; 4) the statistics behind the indirect comparison method utilising a common reference are less favourable than that of direct methods, due to an elevated variation (four-fold).

However, although the material may be sub-optimal, it may still help in the pursuit of array standardisation as no better universally accepted standards are currently available. Several other materials can be used as a reference, such as a mixture of cDNA products spotted onto arrays, a mix of labeled oligos complementary to every microarray probe or genomic DNA (Novoradovskaya *et al* 2004).

8 Results and discussion

In the last few years several lines of evidence have emerged which suggest that the biology of gastrin is much more complicated than indicated by earlier works. In particular, studies of (1) the various peptides generated during gastrin biosyntesis, (2) genetically modified mice that overexpress the gastrin gene or in which the genes encoding gastrin or its receptor have been deleted, (3) the phenotype of patients with hypergastrinemia, and (4) new targets indicated by functional genomic methods (gene arrays and proteomics) all indicate that gastrin, and possibly its variants, are implicated in a wide variety of biological processes, including effects outside the stomach. We have only a limited knowledge of molecular mechanisms involved in the complex physiological and pathophysiological responses to gastrin. There is abundant evidence to suggest that gastrin may play an important role in tumour biology (Dockray *et al* 2005). Hypergastrinemia is associated with the occurrence of gastric ECL cell carcinoid tumours and with an increased risk of gastric carcinoma (Dockray *et al* 2005). The present work examines some aspects of the biology of the gastrin-responsive cell line AR42J, molecular responses to acid inhibition in gastric mucosa, and gene expression based classification of gastric carcinoma.

8.1 Molecular profiling of gastric carcinoma

To produce a metastasis, tumour cells must complete a multistep progression through a series of sequential and selective events. The metastatic process consists of tumour cell detachment, local invasion, motility, angiogenesis, vessel invasion, survival in the circulation, adhesion to endothelial cells, extravasation, and regrowth in different organs. In each step, causative molecules have been identified, including cell-

adhesion molecules, various growth factors, matrix degradation enzymes, and motility factors, and most of these can be regarded as prognostic factors since they are related to local and distant dissemination of tumour. A recent study aimed to identify metastasis related genes in gastric cancer comparing cell lines from low and highly metastatic gastric cancer, using an unsupervised clustering method (Fukui *et al* 2005). However, cluster analysis is a very subjective analysis strategy. In paper II, we instead used a supervised learning method to develop classifiers for several steps in the metastatic process; penetration of gastric wall, lymph node metastasis and remote metastasis. Not surprising, these classifiers include genes like cadherins, integrins and laminin binding genes. In addition, several novel metastasis-related genes were identified that might give indications on how to uncover the precise mechanisms of the development and progression of gastric carcinoma.

The majority of microarray studies examining gastric adenocarcinoma have been aimed at developing exploratory gene profiles of gastric tumour or gastric cancer cell lines to identify gastric cancer-related genes, delineate molecular phenotypes, demonstrate tumour subclasses, and identify functional gene clusters as potential markers of biologic behavior, using unsupervised learning methods (El-Rifai *et al* 2001; Hippo *et al* 2001; Lee *et al* 2002; Hippo *et al* 2002; Liu *et al* 2002; Ji *et al* 2002; Meireles *et al* 2003; Yu *et al* 2005). Results by Wu *et al* and Boussioutas *et al* indicate that Lauréns classification reflects significant molecular differences in gastric carcinomas (Boussioutas *et al* 2003; Wu *et al* 2006). However, these studies did not apply supervised methods to create classifiers. Like our results in paper II, modeling for prediction of diffuse type and intestinal type of gastric cancers using a supervised method, identified genes that may represent distinct molecular signatures of each histological type (Jinawath *et al* 2004). Recent studies have shown that microarray, using supervised methods in combination with statistical modeling, accurately predicted tumour behavior with respect to tumour progression, metastatic potential, tumour recurrence, and overall prognosis/survival (Inoue *et al* 2002; Hasegawa *et al* 2002; Meireles *et al* 2004; Chen *et al* 2005). However, to my knowledge, paper II is still the only study on gastric carcinoma in which molecular classification has been achieved for several clinicopathological parameters based on microarray gene expression profiles. Although in its infancy, gene expression analysis, combined with

predictive models, holds promise in extending our understanding and possibly improving classification and treatment of gastric carcinoma.

8.2 Heterogenous tissue

In paper II, III and IV, tissue biopsies were used. When assaying a tissue sample, the microarray result reflects the sum of gene expression in all cell types in the sample. There has been some concern around the question of the heterogenity of solid tissue. Some researchers have tried to approach the problem by methods like laser microdissection of tumour material before mRNA is extracted (Klur *et al* 2004; Mizuarai *et al* 2005). One should bear in mind, however, that the disease process often is the result of interaction between the pathological cells themselves and surrounding stroma, like for instance in the processes of invasion, metastasis and angiogenesis. Thus, analysis of the gene expression in the whole tissue may actually in some instances be more informative than examination of only the distinctly pathological cells (Nakagawa *et al* 2004). In the case of solid tumours as those which are used as a source of RNA in paper II, the tissue is composed not only of tumour cells but also comes together with normal cells, stromal cells, infiltrating inflammatory cells, endothelial cells, etc. This heterogenity does interfere with gene expression profile but one could argue that the biology of the tumour is a result of the interaction of these various cell types and hence, it could be relevant to have them all in the sample under examination. Moreover, one study indicates a low degree of expression profile variability within gastric tumour samples isolated from one gastric cancer patient. Those data suggest that tumour tissue heterogenity is not a dominant source of error for microarray analysis of large tumour samples, making total RNA extraction an appropriate strategy for performing gene ship expression profiling of gastric cancer (Trautmann *et al* 2005).

A possible complication by a whole tissue approach is if different cell types regulate the same genes in opposite directions. If a gene is up-regulated in one cell type and down-regulated in another, the overall sum of gene expression may cancel out giving the impression of not being important. Further studies are needed to clarify this aspect of gene expression measurement in complex systems.

Interestingly, as shown in paper III, gene-expression arrays can indeed provide a sensitive measure of gene changes within a minor subpopulation of cells, as the increased gene expression of the ECL cell-specific genes HDC and Reg was clearly observable, even though the ECL cells only comprise about 1% of the total cell mass in the rat gastric mucosa. Another study has found that microarrays can detect gene expression in merely 5% of the total cell population (Hamadeh *et al* 2002). These findings indicate that, if a cell culture or tissue is heterogenous, significant changes may be related to only a small fraction of the cells; and investigators may wish to confirm localisation with a microscopic technique, as we did for some gene products in paper III and IV.

8.3 PPI-induced acid inhibition

The consequences of profound and sustained PPI-induced acid inhibition have been carefully evaluated in a number of recent reports; (Lamberts *et al* 1988; Solcia *et al* 1989; Lamberts *et al* 1993; Solcia *et al* 1993). Such studies have, as yet, revealed no irreversible pathological changes directly related to ECL cell neoplasia in patients with longterm hypergastrinemia. However, treatment with PPIs increases fasting serum gastrin about 2-3 fold after 1 month (Genta *et al* 2003) or 3 months (Eissele *et al* 1997), and very high gastrin levels are seen in 10% of the patients (Klinkenberg-Knol *et al* 1994; Eissele *et al* 1997). Furthermore, multiple measurements of serum gastrin in patients undergoing similar treatment as described in paper IV, showed that the 24-h gastrin exposure is far higher during PPI treatment than is reflected by a single fasting gastrin measurement (Waldum *et al* 1996a). Thus, the gastrin exposure of the gastric mucosa in paper IV is much more pronounced than suggested by the relatively modest increase in fasting serum gastrin. A clear quantification of what is an unsafe exposure to gastrin has not yet been established, but the maximal trophic effect of gastrin is reached at levels lower than previously realised (200-500 pM) (Sjöblom *et al* 1991; Brenna & Waldum 1992). Provided long-term hypergastrinemia, it seems that all conditions with hypergastrinemia are associated with ECL cell hyperplasia, dysplasia or neoplasia and some researchers have warned against long-term inhibition of gastric acid secretion since the mid 1980s.

In paper IV, changes of gene expression in human gastric oxyntic mucosa were studied in patients receiving a therapeutic dose of the PPI esomeprazole. These were studied after a treatment period of three months. Interestingly, the treatment induced significant changes in the gene expression of a high number of genes. A higher dose of PPI and a relatively longer dosage period (compared with lifetime) were used in the corresponding rat study (paper III). In addition, different microarrays, representing different genes, were used. Thus, it is not surprising that few common genes were found to be differentially expressed in both rats and patients. However, a number of genes were indeed regulated in the same direction in both studies. These genes are considered highly relevant to the molecular responses to potent acid inhibition. Also, the endocrine-specific protein-18 (RESP18), which is upregulated in response to PPI in rats, is an interesting candidate gene for further analysis. RESP18 has lately been shown to be upregulated in hypergastrinemic rodent mastomys (Kidd *et al* 2004). Further characterisation of the functional roles of these genes may give new insight into the biological responses to this very common therapeutic intervention.

Moreover, looking at the PPI responses at a biological process level, using Gene Ontology (GO), similar results were observed in the two studies. GO provides information by structuring biological knowledge with a controlled vocabulary consisting of GO terms, that enable us to understand the molecular picture better. Of the differentially expressed genes with known function, 40% and 34% are involved in the biological processes proliferation and/or apoptosis in PPI-dosed patients and rats, respectively. The corresponding numbers of genes involved in stress and inflammatory/immune responses are 21% and 22%, respectively. The fraction of genes involved in these processes was remarkably similar for rats and patients. Other studies also show that comparable biological themes emerge from data across disparate platforms and laboratories when GO nodes are used to analyse collections of genes representative of biological themes instead of direct gene-by-gene comparisons (Segal *et al* 2004; Bammler *et al* 2005). A recent study showed that the biological representations such as those expressed in GO classifications of the genes are more important to understand molecular mechanisms than the genes themselves (Bammler *et al* 2005). Even if on a gene-by-gene basis they found inconsistency, when they looked at the processes themselves, the conserved prosesses represented by regulated genes were there. Looking at the level of the biological process annotation rather than

the gene gives a more biologically meaningful and statistically robust approach to data analysis (Petersen *et al* 2005).

The final outcome of human ECL cell hyperplasia in prolonged low-acid states awaits full characterisation. Only long-term studies can elucidate the consequences on the ECL cell system of potent acid-suppressive pharmacotherapy and elevated gastrin levels. Although the putative malignant potential of gastric carcinoids may ultimately be of only modest concern in a background of hypergastrinemia, its relationship to gastric adenocarcinoma is still enigmatic and worthy of further consideration. Valuable insights into the regulation of gastric mucosal turnover will undoubtedly be gained from continued study of the biology of this gastrin-generated neoplasm of the gastric mucosa. The results in paper III and IV may contribute to new insight into the biological responses to treatment with PPIs, both on biological process and single gene levels.

8.4 AR42J as a cell model system

To understand the molecular mechanisms governing normal physiological processes and oncogenesis is an essential first step in the design of effective therapies. Research into the molecular mechanisms underlying the various aspects of physiology and patophysiology requires the use of *in vivo* and *in vitro* model systems. Animal models are often beneficial in this regard, but present significant drawbacks, including ethical concerns and the lack of appropriate models for the mechanism being studied. Moreover, to elucidate spesific mechanisms, simplified model systems are necessary. Thus, cell culture models have been developed that mimic various physiological conditions and cancer types.

Reports show that gastrin is a major trophic factor for several neoplastic cell types, including the pancreatic acinar cell derived cell line AR42J (Seva *et al* 1994), which we characterise with respect to NE features in paper I. The CCK-2 receptor belongs to the seven-transmembrane domain, G-protein coupled, receptor superfamily. This receptor has been shown to be expressed in several GI and pancreatic tumour cell lines, including AR42J (Watson *et al* 1998). It is normally coupled to $G\alpha_{q/11}$ and activation leads to an increase in intracellular Ca^{2+} and protein kinase C (PKC). The

mechanisms have been studied in parietal cells and ECL cells (Sachs *et al* 1997; Kinoshita *et al* 1998). In addition, there is a considerable volume of work on signalling via CCK-2 receptors in other CCK-2 receptor-expressing tissues, like pituitary, pancreatic islets and cancer cell lines. The data indicate activation of the mitogen activated protein kinase (MAPK), phospholipase C (PLC) and phosphoinositide 3-kinase (PI3-K)/Akt signalling pathways (rewieved in (Todisco 2000)). Thus, it is clear that gastrin activates growth signaling pathways.

Gastrin is known to play a pivotal role in the regulation of genes containing cAMP responsive elements (CRE), including the CgA and somatostatin genes (Montminy & Bilezikjian 1987; Wu *et al* 1995). Proteins binding to this sequence (CREB) are activated by increase in intracellular cAMP. Todisco et al. (Todisco *et al* 1997) reported that gastrin-mediated proliferation in AR42J probably involves both PKC-dependent and PKC-independent mechanisms. Studies on this cell line show that gastrin-mediated proliferation also involves PKA, identified CRE as a gastrin responsive promotor element and found that CRE is indispensible for gastrin-induced activation of the c-fos promotor in AR42J (Thommesen *et al* 2001). We have previously shown that gastrin induces expression of the CRE-responsive gene inducible cAMP early repressor (ICER), which is suggested to play a role in negative feedback regulation of genes activated via CRE (Thommesen *et al* 2000). Thus CRE promoter elements may play a central role in gastrin-mediated modulation of gene expression involved in physiological effects of gastrin.

Gastric cell lines have been widely used as experimental models to study the genetics, pharmacology and biochemistry of gastric cancers. A study comparing gene expression profiles of 12 gastric adenocarcinoma cell lines to gene expression patterns of 15 cell lines derived from other epithelial cancers, found a marked heterogenity among the expression patterns of the cell lines with origin from the GI tract (Ji *et al* 2002). The heterogenity may reflect the underlying molecular characteristics or specific differentiation programs. In another study, gene expression analysis of six different gastric cancer cell lines with different metastatic potentials in terms of grade and target was examined to clarify the mechanism of gastric peritoneal dessemination (Sakakura *et al* 2002).

Moreover, molecular mechanisms underlying proliferative responses to gastrin, HGF, EGF and PACAP have been studied by transcript profiling in the carcinoid tumour cell line BON (Hofsli *et al* 2005). By identifying a number of growth factor-responsive genes in human NE GI tumour cells, useful hypotheses for further studies aimed to search for new therapeutic targets as well as tumour markers in NE GI tumours were provided.

AR42J is considered to be an interesting model for the study of a possible involvement of ICER in gastrin-mediated cellular responses and signaling mechanisms of CCK-2 receptor (Thommesen *et al* 2000). In paper I, we propose that AR42J, showing gastrin-induced proliferation and expressing CgA and several SSTRs, also might be a valuable experimental model to study molecular mechanisms involved in the biology of NE tumour cells, including the regulation of the NE cell marker CgA and SSTRs.

Concluding remarks and future perspectives

The four studies presented aimed to shed light on molecular aspects of gastric carcinoma, growth related gene expression of gastric mucosa and the NE cell line AR42J. The main conclusions are:

- AR42J displays several NE features and can be used as a model to study the regulation of CgA and SSTRs in response to gastrin.
- A model for classification of gastric carcinoma with respect to several important clinicopathological parameters was developed.
- The biological processes proliferation, apoptosis, stress, inflammatory and immune responses are shown to be affected by potent acid inhibition of gastric mucosa in both rats and humans. A large number of genes responding to inhibition of gastric acid secretion were identified.

These studies are an example of how future research in molecular biology may be performed, switching back and forth between detailed studies of genes/proteins and high-throughput analysis. Development of new technology and characterisation of the human genome sequence has made this approach possible, and one of the greatest challenges now is within bioinformatics and statistics. Also important is to continue building bridges between the different branches of science, to be able to develop the emerging field of Systems Biology (Kitano 2002b).

Several aspects of the findings presented will undergo follow up in future studies. The classification model for gastric carcinoma will be extended using a higher number of samples. Furthermore, the classifiers developed will be tested using an independent "test set" of samples. Moreover, differentially expressed genes in gastric tumours compared with gastric mucosa in healthy individuals will be explored in a new study. Some of the most interesting genes regulated by PPIs will be studied further to elucidate their role in acid secretion and proliferation of gastric mucosa.

It is conceivable that endoscopically obtained tissue samples may be used to generate preoperative predictive gene clusters. Full scale DNA microarray technology will probably be too advanced and laborious to be of any routine use in the clinic. However, a qualified assortment of markers elucidated from full-scale DNA microarray analysis will have great potential in routine determination of diagnosis and prognosis in the clinic. Characterisation of genes that are differentially expressed in gastric carcinoma is essential for accurate diagnosis and tumour characterisation and for informed surgical and adjuvant therapy decision-making, development of novel therapeutics, and delineation of tumour behavior for more accurate prognostication.

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Papers I-IV

Paper I

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Expression of chromogranin A and somatostatin receptors in pancreatic AR42J cells

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Abstract

The exocrine pancreatic cell line AR42J is also known to display some neuroendocrine (NE) features. We have extended this fact by showing that AR42J cells express mRNA of chromogranin A (CgA), display immunoreactivity (IR) to CgA, and secrete its cleavage product pancreastatin. A sparse occurrence of typical NE secretion granules, together with only a faint IR to conventional NE markers, indicates that the NE cells are of a poorly differentiated type. CgA promoter reporter plasmid experiments showed that gastrin, epidermal growth factor, and phorbol 12-myristate 13-acetate, induce upregulation of CgA after 24 h. By RT-PCR, it was found that AR42J expresses all of the five subtypes of the somatostatin (SST) receptor (SSTR) family, except SSTR4. The existence of functional SSTRs was confirmed by showing that the SST analog octreotide could inhibit gastrin-induced proliferation. Thus, the AR42J cell line may function as a valuable experimental model to study the regulation of CgA and SSTRs in poorly differentiated NE tumor cells.

Keywords: Pancreastatin; Neuroendocrine features; Somatostatin receptors; AR42J; Reporter-gene

1. Introduction

The rat AR42J cell line is a widely used experimental model system for studies of pancreatic acinar cells (Rosewicz et al., 1992; Christophe, 1994). The cell line derives from a chemically induced pancreatic adenocarcinoma (Longnecker et al., 1979). In addition to its well-known exocrine properties, it also displays some neuroendocrine (NE) features such as the expression of the typical NE vesicle protein synaptophysin (Syn), the synaptic vesicle protein type 2 (SV2), voltage-activated ionic channels (Kusano and Gainer, 1991), as well as the neurotransmitters GABA, glutamate and glycine (Rosewicz et al., 1992; Christophe, 1994). AR42J cells also express some transcription factors typically found in NE cells (Palgi et al., 2000). Furthermore, upon treatment with betacellulin, a member of the epidermal growth factor family (EGF), or with hepatocyte growth factor, in combination with activin, AR42J cells differentiate into insulin-producing cells (Mashima et al., 1996a,b). This has also been shown after treatment with glucagon-like peptide 1 and exendin-4 (Zhou et al., 1999). These observations suggest that this cell line can serve as an experimental model to study the formation and differentiation of pancreatic endocrine cells.

Chromogranin A (CgA) belongs to the granin family of acidic secretory glycoproteins that are expressed in most types of normal NE cells and in the parenchyma of most NE tumors (c.f. Nobels et al., 1998; Wick, 2000). CgA is typically bound to the membrane of the NE secretion granules, but its precise biological functions are not fully elucidated. CgA is processed in a tissue specific manner into biologically active peptides with various functions. One of these peptides is pancreastatin, which has a regulatory effect on secretion from both endocrine and non-endocrine cells (Reinecke et al.,

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1991; Nobels et al. 1998). CgA itself appears to modulate the proteolytic processing of peptide hormones and neuropeptides, and to be involved in both the packing of peptides and in directing them to the regulated pathway of secretion. In a clinical setting, CgA seems to be one of the most important markers of NE tumors (Syversen et al., 1993; Nobels et al., 1998; Öberg, 2000). CgA is used both as a serum and tissue marker, and a future application may be its use in visualization of NE tumors in patients.

Somatostatin (SST) is a widely distributed peptide hormone which plays a pivotal regulatory role in multiple target organs (c.f. Patel, 1999). It inhibits secretion from a wide variety of both endocrine and exocrine cells, it functions as a neurotransmitter in the central nervous system (CNS), and it plays an important role in regulation of cell proliferation and differentiation. An antiproliferative effect of SST has been demonstrated both in normal cells, in malignant cell lines, and in tumors (Hofland and Lamberts, 1997; Patel, 1999). SST exerts its effects through binding to specific surface membrane receptors. Five different SST receptor (SSTR) subtypes have been characterized (SSTR1-5) (Schonbrunn et al., 1995; Hofland and Lamberts, 1997; Patel, 1999).

The majority of NE tumors express a high density of SSTRs and different SST analogs (e.g. octreotide) are widely used both in diagnosis and therapy of these tumors (Hofland and Lamberts, 1997; Wulbrand et al., 1998; Pollak and Schally, 1998; Öberg, 2000). Usually more than one subtype is expressed, and the general pattern of expression suggests a high frequency of SSTR2 mRNA. Interestingly, a specific loss of SSTR2 subtype gene expression has been observed in human pancreatic and advanced colorectal adenocarcinomas, and this has been suggested to represent a growth advantage in these tumors (Buscail et al., 1996). In fact, it has recently been shown that SSTR2 gene transfer mediates antitumor effect both in animals and in-vitro (Rochaix et al., 1999). Thus, SSTR2 gene transfer may represent a new therapy for cancer.

In spite of the extensive clinical use of SST analogs in the management of NE tumors, the exact functional significance of the presence of SSTRs, and how these are being regulated, have still not been fully established (Patel, 1999). The same is true concerning knowledge about the function and regulation of the NE cell marker CgA (Nobels et al., 1998). To this end, well-characterized in-vivo and in-vitro models are needed. The aim of this study was to investigate whether the AR42J cell line expresses CgA, and to characterize this cell line with respect to the expression of the five known SSTRs. Our findings lead us to propose that the AR42J cell line might be a valuable experimental model to study molecular mechanisms involved in the biology of NE tumor cells.

2. Materials and methods

2.1. Cells and reagents

AR42J (rat pancreatic acinar cell derived, ATCC, Rockville, MD, USA), Rat-2 (rat fibroblast, ATCC), NRK-52E (rat epithelial, ATCC) and PC-12 (rat pheochromocytoma, ATCC) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose (Gibco BRL, Life Technologies, Paisley, Scotland), 1 mM Na-pyruvate (Gibco), 0.1 mg/ml L-glutamine (Gibco), 10 U/ml penicillin/streptomycin (Gibco), 1 μg/ml fungizone (Gibco) supplemented with 15% (AR42J), 10% (PC-12) or 5% (Rat-2, NRK-52E) fetal calf serum (FCS) (Biological Industries, Beit Haemek, Israel). RIN-5F (rat insulinoma, ATCC) and RIN-14B (rat somatostatinoma, ATCC) cells were grown in RPMI 1640 (Gibco) with 2 g/l glucose, 0.1 mg/ml L-glutamine, 0.04 mg/ml garamycin (Schering-Plough Labo, Heist-op-den-Berg, Belgium), supplemented with 10% FCS.

Recombinant epidermal growth factor (EGF) (stored lyophilized) was purchased from Biomedical Technologies (Stoughton, MA, USA). Gastrin-17 (stored lyophilized) was obtained from Sigma (St. Louis, MO, USA). Phorbol 12-myristate 13-acetate (PMA) (Sigma) was dissolved in DMSO (1 mg/ml) and stored at −20°. Octreotide (Sandostatin) was purchased from Novartis (Oslo, Norway).

2.2. Northern blot analysis

Total RNA from AR42J, PC-12, RIN-5F, RIN-14B, Rat-2 and NRK-52E cells was isolated by phenol extraction as previously described (Liabakk et al., 1993). Twenty μg of each total RNA was electrophoresed on a formaldehyde agarose gel and blotted onto nylon membranes (Roche Molecular Biochemicals, Mannheim, Germany). Plasmids containing cDNA fragments of CgA (Angelsen et al., 1997) or 18S (Bakke et al., 2000) were linearized, and antisense RNA probes labeled with ^{32}P were generated by in-vitro transcription according to standard protocols using SP6 or T7 RNA polymerases. Probes were purified on NucTrap columns (Amersham Pharmacia Biotech, Little Chalfont, UK). Membranes were prehybridized for 4 h at 65 °C in 5 × sodium chloride–sodium-phosphate–EDTA buffer (SSPE; 0.75 M NaCl, 0.05 M sodium phosphate, and 5 mM EDTA, pH 7.4), containing 50% formamide, 5 × Denhardt's solution (0.1% bovine serum albumine (BSA), 0.1% polyvinylpyrrolidine, and 0.1% Ficoll 400; w/v), 0.5% sodium dodecyl sulfate (SDS) and 200 μg/ml sonicated salmon sperm DNA (Sigma), then hybridized in the same solution containing RNA probe (2×10^6 counts per min per ml) for a further 18 h at 65 °C.

After hybridization, membranes were washed twice at room temperature (RT) for 20 min with 2 × SSPE containing 0.1% SDS and once at 65 °C for 20 min with 0.1 × SSPE containing 0.1% SDS. Washed membranes were exposed to a storage phosphor screen for 15 min (18S) or 18 h (CgA), and the screen was scanned on a Phosphorimager 425 (Molecular Dynamics, Sevenoaks, UK). Membranes were hybridized, first with the CgA riboprobe, then by 18S. Probes were removed between hybridizations by boiling in 0.1% SDS.

2.3. RT-PCR

AR42J was seeded out in growth medium at 0.9×10^6 cells per well in six-well plates and cultivated for 3 days (subconfluent). Then, the cells were washed twice with phosphate-buffered saline (PBS), and 500 μl lysis/binding buffer (100 mM Tris, pH 8.0, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM dithiothreitol) was added. DNA was sheared by forcing the lysate five times through a 21 gauge needle by a 1–2 ml syringe. PolyA+ RNA was isolated from lysate (2.5×10^5 cells) with 125 μl oligo dT Dynabeads (Dynal, Oslo, Norway) according to the protocol of the manufacturer, and eluted from the beads in 20 μl Tris–HCl (10 mM, pH 7.5). Total RNA from rat cerebral cortex was isolated and prepared as previously described (Sandvik et al., 1995).

RT-PCR CgA was performed with 0.5 μl eluate with 1.25 U rTth DNA polymerase (Roche) according to the procedure recommended by the manufacturer. cDNA synthesis was performed at 61 °C for 40 min, followed by PCR with 300 μM dNTP (Roche), 500 nM primers and 3.0 mM Mn $(OAc)_2$. PCR amplification was run for 28 cycles at 94 °C for 15 s, 55 °C for 15 s, and at 72 °C for 30 s, followed by a final extension step for 3 min at 72 °C. The following PCR primers were used (**S**; sense, **AS**; antisense): **CgA-S**: 5′-TCC ATG AAG CTC TCC TTC-3′ and **CgA-AS**: 5′-AGA AAG CTG CCT GTG TTC-3′. The number of PCR-cycles was selected on the basis of experiments with 28, 30, 32, 34 and 36 cycles, which showed that 28 cycles yield quantitative results within the linear range.

RT-PCR for SSTRs were performed by a two step procedure. For reverse transcription, 5 μl of polyA+ RNA eluate or total RNA in a final volume of 30 μl, containing 150 U MuLV reverse transcriptase (Roche), 3 μl of 10 × PCR buffer (500 mM KCl, 100 mM Tris–HCl, pH 8.3), 60 U RNAsin (Promega), 250 μM dNTPs (Boehringer Mannheim), 5 μM oligo-d(T) (Amersham Pharmacia Biotech) and 5 mM $MgCl_2$ (Roche), was incubated at 42 °C for 60 min, and then the enzyme was denatured at 95 °C for 2 min. To ensure that the specific RT-PCR products were exclusively dependent on mRNA transcripts present, controls were performed without reverse transcriptase.

For PCR amplification, 1 μl of cDNA was incubated in a final volume of 20 μl with 1 U of AmpliTaqGold (Roche), 2 mM $MgCl_2$, 500 nM of each sense- and antisense primer, 300 μM dNTP, and 2 μl GeneAmp 10 × PCR Buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$) (Roche). PCR amplifications were run for 28 (SSTR1), 35 (SSTR2), 34 (SSTR3), 40 (SSTR4) and 42 (SSTR5) cycles, respectively, at 94 °C for 15 s, 66 °C for 15 s, and at 72 °C for 30 s, followed by a final extension step for 7 min at 72 °C. The following primers were used: **SSTR1-S**: 5′ ATG GTG GCC CTC AAG GCC GG 3′, **SSR1-AS**: 5′GGC AGT GGC GTA GTA GTC AA 3′, **SSTR2-S**: 5′ TCA TCA AGG TGA AGT CCT CTG G 3′, **SSTR2-AS**: 5′ AGA TAC TGG TTT GGA GGT CTC CA 3′, **SSTR3-S**: 5′ TGC CAG TGG GTA CAG GCA CC 3′, **SSTR3-AS**: 5′ CTG GAG GGC CAG ACC CTG GC 3′, **SSTR4-S**: 5′ TGC GGG CTG GCT GGC AAC AA 3′, **SSTR4-AS**: 5′ GTA GTC CAG GGG CTC TTC CT 3′, **SSTR5-S**: 5′ CCT TTC CTG GCC ACG CAG AAC GC 3′, **SSTR5-AS**: 5′ GGC CAG GTT GAC GAT GTT GAC 3′.

To check whether comparable amounts of polyA+ RNA from each sample were used, RT-PCR reactions for the house-keeping gene GAPDH were performed using the following primers: **GAPDH-S**: 5′-CCCAT-CACCATCTTCCAG-3′ and **GAPDH-AS**: 5′-ACAGTCTTCTGAGTGGCA-3′. PCR was run for 28 cycles at 94 °C for 15 s, 50 °C for 15 s and at 72 °C for 30 s, followed by a final extension step for 3 min at 72 °C.

The identity of the SSTR PCR products was checked by informative restriction analysis using one or two different restriction enzymes (Table 1). In each case, 8 μl of the PCR product was treated at 37 °C for 2 h with the appropriate enzymes (9-16 U) and restriction enzyme buffers in a total volume of 20 μl.

2.4. Reporter plasmid experiments

The plasmid pXp100Luc containing 100 bp of the proximal CgA promoter (Wu et al., 1995) was a generous gift from Dr D. O'Connor (University of California, San Diego, CA, USA). Cells (2×10^4) per well were seeded out in 96-well plates and transfected after 24 h with 0.12 μg luciferase reporter plasmid DNA per well, using 0.35 μl Fugene transfection reagent (Roche). After culture for 2 days in the presence of plasmid and transfection agent, cells were treated with agonists for 24 h followed by PBS wash (twice) and lysis in 15 μl Promega lysis buffer. Luciferase activity was measured by Turner Luminometer model TD-20/20 (Turner Designs, Sunnyvale, CA, USA) using the Luciferase reporter Assay System (Promega Corp., Madison, WI, USA) as recommended by the manufacturer.

Table 1
Restriction enzymes used, and expected post-cleavage product length (bp), for verification of amplified SSTR (SSTR1-5) products

PCR-product	Expected length (bp)	Restriction enzymes	Expected post-cleavage length (bp)
SSTR1	318	BsaH1	149 + 169
		PvuII	269 + 49
SSTR2	414	BamH1	22 + 392
		PvuII	220 + 194
SSTR3	328	BamH1	269 + 59
		PvuII	209 + 119
SSTR4	311	PvuII	184 + 127
SSTR5	549	BsaH1	174 + 375
		Sph1	426 + 123

2.5. Detection of pancreastatin

AR42J cells were grown in 75 cm^2 culture flasks for 4 days to reach confluence. The cells were then cultivated for 24 h in serum free media (3 ml), before medium was collected, centrifuged, and kept frozen at −80 °C until assay. Cell lysates were prepared by lysis in distilled water after one wash in PBS. Determination of rat pancreastatin was performed by using a commercial RIA kit (Peninsula Laboratories, Inc., San Carlos, CA, USA) as described by the manufacturer. Briefly, primary antibody (rabbit anti-peptide serum) was added to standards and unknown samples, followed by incubation overnight (4 °C). On the next day, ^{125}I-peptide was added and incubated for 24 h (4 °C). Then, addition of goat anti-rabbit IgG, incubation 90 min at RT and admixture of RIA buffer followed. The samples were centrifuged for 20 min, supernatant aspirated, and assay tubes counted. The detection limit of the assay was 5 pg/ml, and the intra- and inter-assay variations were 4.9 and 3.8%, respectively, (Syversen et al., 1993).

2.6. Light-microscopical, immunohistochemical, and ultrastructural examinations

Trypsinized AR42J cells were centrifuged at 4.000 × *g*. For the light-microscopical (LM) and immunohistochemical (IHC) investigations, the pellet was conventionally fixed in 10% neutral formalin, dehydrated, and embedded in paraffin. Sections, about 4-5 micron thick, on poly-L-lysine-coated slides, were used for the IHC examinations. They were performed both by means of the conventional avidin-biotin peroxidase procedure, using the Vectastain ABC kit (Vector Lab., Burlingame, CA, USA), and by applying the Tyramid Signal Amplification (TSA) technique, using the 'TSA indirect kit' (NEN LifeSci. Products, Boston, MA, USA), as recently described (Qvigstad et al., 1999). The CgA antiserum was provided by Incstar (Stillwater, MN, USA), known to be immunoreactive (IR) in rat NE cells. It was applied at a dilution of 1/500. For the Syn IHC examination, the monoclonal mouse anti-Syn antiserum, provided by Dako (Glostrup, Denmark) was employed with the dilution 1/20. For the neuron-specific enolase (NSE) IHC studies, the 'anti rat NSE' antiserum, provided by Polysciences (Warrington, PA, USA) was used, dilution 1/500.

For the electron microscopical (EM) investigations, the pellet was fixed in 2% neutral glutaraldehyde, post-fixed in 2% osmium tetroxide, contrasted with 1% lead citrate and 4% uranyl acetate, and conventionally embedded in Epon. Semi-thin sections were cut and stained with toluidine blue for orientation and trimming of the blocks. Finally, from the areas selected, conventional ultrathin sections were cut and analyzed by means of our transmission EMs (JEOL 100CX and Phillips SEI Tecnai 12).

2.7. Proliferation assay

Proliferation rate was determined by measuring DNA synthesis using the Cell proliferation ELISA BrdU (5-bromo-2′-deoxyuridine) kit (Roche). AR42J cells (2×10^3) were seeded out in 96-well microtiter plates in 150 μl serum-containing medium. After 24 h the cells were washed with 200 μl serum-free medium before treatment with gastrin and/or octreotide in a final volume of 100 μl. After 24 h, BrdU-labeling solution (10 μl per well) was added, and the cells were cultured for an additional 18 h before incorporation of BrdU was measured as described by the manufacturer. Briefly, after removing the labeling medium, the cells were fixed and DNA denatured in one step by adding 200 μl FixDenat-solution per well for 30 min at RT After removing FixDenat-solution, 100 μl anti-BrdU-POD working solution was added to each well, and incubated at RT for 90 min. The cells were then rinsed three times with 200 μl washing solution before 100 μl substrate solution was added to each well. After 3 min the light emission of the samples (RLU = relative luminiscence units) was measured in a microplate luminometer (Fluoroskan Ascent FL, Labsystems, Helsinki, Finland).

2.8. Statistics

Statistical analysis were performed using the Student's *t*-test.

3. Results

3.1. AR42J cells express CgA mRNA

To determine whether AR42J cells express the NE cell marker CgA, we used Northern blot analysis. As shown in Fig. 1, a distinct hybridization band of a size corresponding to the reported length of rat CgA mRNA (2.1 kb) was detected in AR42J. This was confirmed by RT-PCR (data not shown). The rat NE cell lines PC-12 (pheochromocytoma) and RIN-5F (insulinoma) were used as positive controls, as these cell lines have been shown to express CgA mRNA (Rausch et al., 1988; Swarovsky et al., 1994). The level of CgA in AR42J was comparable to the level in PC-12. We found CgA gene expression also in the somatostatinoma cell line RIN-14B (Fig. 1). This has not been reported earlier. The rat fibroblast Rat-2 and the rat epithelial NRK-52E cell lines were used as negative controls (Fig. 1).

3.2. Gastrin and EGF upregulate CgA gene expression

In order to study the regulation of CgA gene expression in AR42J, we performed CgA promoter reporter plasmid experiments. Gastrin, EGF and PMA induced a moderate (136, 77 and 114%, respectively) transcriptional upregulation of CgA after 24 h ($P < 0.001$) (Fig. 2). Gastrin and EGF are both known to activate protein kinase C (PKC) in AR42J cells (Stepan et al., 1999). Since PMA is known to exert its effect via both classical and novel diacylglycerol (DAG)-responsive PKCs (Newton and Johnson, 1998), the PMA-induced increase (114%) in CgA promotor activation suggests that PKC is involved in mediating CgA promoter transactivation in AR42J cells.

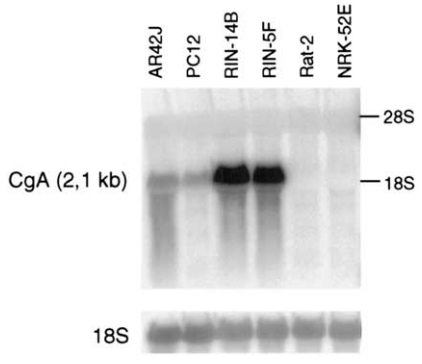

Fig. 1. Northern blot analysis of CgA. 20 μg of total RNA from AR42J, and from the NE cell lines RIN-5F (insulinoma), PC-12 (pheochromocytoma) and RIN-14B (somatostatinoma) were electrophoresed in 1% agarose-formaldehyde gels, electroblotted onto nylon membranes, and hybridized with RNA probes for CgA and 18S. The indicated size (kb) of CgA was obtained by comparison with the sizes of 18S and 28S rRNA. As negative controls were used the Rat-2 (fibroblast) and NRK-52E (epithelial) cell lines. The results shown are representative for three independent experiments.

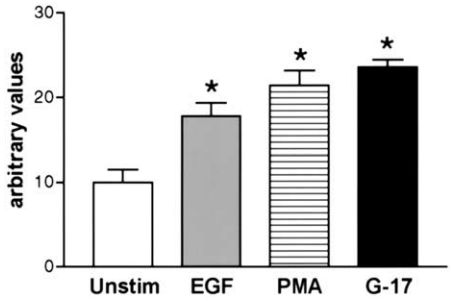

Fig. 2. Activation of the CgA reporter plasmid pXp100 AR42J cells were transfected with CgA promoter reporter plasmid pXp100 (Wu et al., 1995) and treated with either 50 ng/ml EGF, 50 nM gastrin (G-17) or 100 ng/ml PMA for 24 h with quadriplicate parallels per condition. Results are shown as mean value ± S.E.M. of one representative experiment, and are expressed as fold induction compared with untreated cells (* indicates significant difference with untreated cells; $P < 0.001$). Similar results were obtained in two other experiments.

3.3. AR42J cells secrete pancreastatin

In order to confirm translation of CgA mRNA into protein, we looked for the cleavage product pancreastatin in the cell medium. After incubation of confluent AR42J cells (grown in 75 cm^2 culture flasks in a volume of 3 ml) in serum free media for 24 h, the concentrations of pancreastatin were found to be in the range of 69-162 pg/ml, with a mean level of 96.5 ± 30.5 pg/ml ($n = 6$). These ranges lie within the steep part of the standard curve. We also detected pancreastatin in AR42J cell lysates (data not shown).

3.4. Some AR42J cells are equipped with NE secretion granules and are faintly IR to NE markers

In order to examine whether our AR42J cells fulfil the classical histopathological criteria of being a NE parenchymal cell, we examined the LM, IHC, and EM features of the cells. We focused our attention on four major NE criteria, namely the LM/IHC detection of CgA, Syn and NSE IR, as well as the actual EM demonstration of typical NE secretion granules in the cytoplasm of clear cells of NE appearance.

Only a faint IR could be discerned in a small minority of the AR42J cells when antisera against CgA were used. After the application of the TSA technique, a more convincing IR was seen. However, the CgA/TSA IR was still confined only to a small minority of the cells. This was also the case when antisera against Syn and NSE

were used. The Syn IR was more distinct than those of CgA and NSE.

EM revealed that only a minority population of AR42J displayed a fine structure, compatible with the idea that they might be of NE nature (Fig. 3). Like the clear cells of the 'Helle-Zellen-System' originally discovered by Friedrich Feyrter in the 1930's in the gastro-entero-pancreatic (GEP) organs (Falkmer, 1993; Falkmer and Wilander, 1995), their cytoplasm was found to be less electron dense than that of their non-NE counterparts. They were observed to be well equipped with cytoplasmic organelles, mainly mitochondria, lysosomal bodies, and an endoplasmic reticulum (ER) of both rough and smooth type. In addition, a few typical NE secretion granules appeared (Fig. 3; inset). They were displaying a fine structure that was rather commonplace, an electron dense, homogeneous core of non-crystalline shape, surrounded by a moderately broad halo and a thin granule membrane. Practically no cells were found containing numerous NE secretion granules. The overall structure of the AR42J cells was that of cells from a poorly differentiatied neoplastic parenchyma with numerous mitotic figures. The absence of any densely granulated NE cells indicated that the cells with NE features were rather immature.

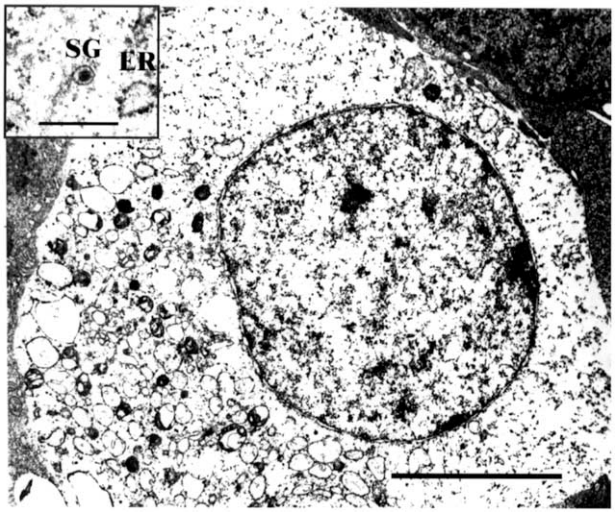

Fig. 3. Fine structure of an AR42J cell and one of its secretion granules (inset). Low-power EM, showing the ultrastructural features of an AR42J cell equipped with secretion granules of NE type ('SG'; inset; upper left corner). The cytoplasm of the cells of this minority population cell type was found to be of lower electron density than that of the adjacent AR42J cells in the pellet (upper right corner), forming the majority cell population of the pellet. In addition to mitochondria, a well developed endoplasmic reticulum ('ER'; inset, upper left corner), both of smooth and rough type, and several kinds of lysosomal bodies occurred. The secretion granules of NE type formed only a small minority of the cytoplasmic organelles. The bars give the actual lengths of 5 μm (main electron micrograph) and 0.5 μm (inset), respectively. Thus, the diameter of the secretion granule in the inset amounts to approximately 136 nm.

3.5. AR42J cells express SSTR subtype 1, 2, 3 and 5

There are conflicting results as to the question of which SSTR subtypes AR42J cells express (Vidal et al., 1994; Froidevaux et al., 1999). By RT-PCR analysis, we could show the presence of mRNA of the four SSTR subtypes 1, 2, 3 and 5 in AR42J cells (Fig. 4). This is the first report of SSTR5 expression in AR42J. SSTR4 mRNA was not detected. Each analysis was repeated at least five times. Contamination with genomic DNA was ruled out by performing PCR on samples where reverse transcriptase had been omitted in the cDNA synthesis step. The PCR reaction for each of the five SSTR subtypes was verified by using rat cerebral cortex as positive control, as this tissue has been shown to express transcripts of all the five SSTRs (Bruno et al., 1993; Thoss et al., 1995). The specificity of each PCR product was verified by informative restriction analysis. Fig. 5 shows that all specific SSTR PCR products exhibited the expected restriction fragment sizes (see Table 1).

3.6. Proliferation studies

Expression of SSTR mRNA in cancer cell lines is not always coupled with the expression of functional cell surface receptors, as assessed by classic competitive binding or proliferation studies (Fisher et al., 1998). In order to confirm the existence of functional SSTRs in AR42J cells, we examined the effect of the SST analog octreotide on gastrin-induced proliferation (Scemama et al., 1987; Seva et al., 1990; Watson et al., 1992). Octreotide binds with a high affinity to SSTR2, with a moderate affinity to SSTR3 and 5, and with a very low affinity to SSTR1 and 4. Octreotide, at a concentration of 0.1 nM, strongly inhibited gastrin-induced proliferation (Fig. 6). The effect was highly significant ($P = 0.0011$) at 0.4 nM gastrin. Our finding confirms the expression of functional SSTRs in AR42J.

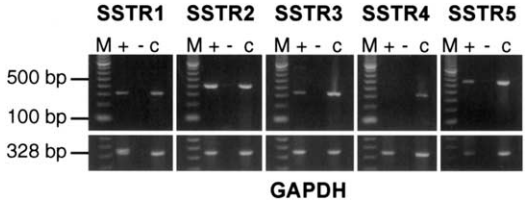

Fig. 4. RT-PCR analysis of the five SSTR subtypes (SSTR1-5) in AR42J cells. RT-PCR of polyA+RNA from AR42J was performed with (+) or without (−) reverse transcriptase (RT), to rule out contamination with genomic DNA. RT-PCR of total RNA from rat cerebral cortex (c) was used as positive control. PCR products were visualized in ethidium bromide stained 1.2% agarose gels. The marker (M) is a 100 bp DNA ladder molecular weight standard (Gibco), and the estimated PCR products are the following: SSTR1: 318 bp, SSTR2: 414 bp, SSTR3: 328 bp, SSTR4: 311 bp, SSTR5: 549 bp. To ensure that comparable amounts of RNA were used in RT^+ and RT^- PCR, GAPDH RT-PCR was performed. The results shown are representative of at least five experiments.

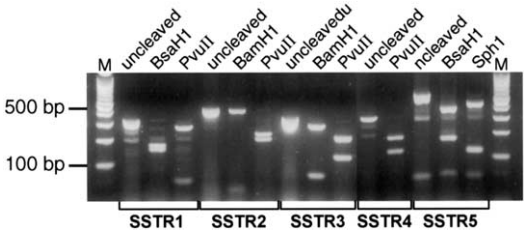

Fig. 5. Informative restriction enzyme analysis of amplified SSTR (SSTR1-5) products. Reactions were performed as described in Section 2. M: 100 bp ladder. Shown are uncleaved PCR product of each receptor, and the restriction enzyme pattern obtained with the different enzymes used. See Table 1 for expected post-cleavage product length (bp).

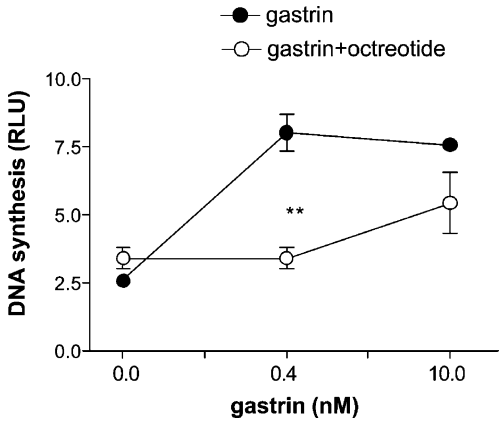

Fig. 6. Effect of octreotide on gastrin-induced proliferation in AR42J cells. Proliferation was measured by using the BrdU proliferation kit, as described in Materials and methods. AR42J cells were stimulated with gastrin (0.4 and 10 nM G-17) in the absence (●-●) or presence of octreotide (0.1 nM) (○-○) with quadriplicate parallels per condition. Results are shown as the mean value ± S.E.M. of one representative experiment, and are expressed as relative light units (RLU). Similar results were obtained in two other experiments. $*P = 0.0011$.

4. Discussion

Here we demonstrate, for the first time, that pancreatic acinar AR42J cells express the rather specific NE marker CgA, and that this expression is transcriptionally regulated by the growth factors gastrin and EGF. The sparse occurrence of typical NE secretion granules, supposed to contain CgA, combined with the faint CgA, Syn, and NSE IR, indicate that the cells are of a rather poorly differentiated type. Furthermore, we show that AR42J cells express mRNA of all SSTR subtypes, except SSTR4. Our findings indicate that the AR42J cell line could serve as a valuable experimental model to study the regulation of CgA and SSTRs in poorly differentiated NE tumor cells.

The fact that Rosewicz et al. (1992) were unable to detect CgA in AR42J cells by immunoblotting may be due to the low sensitivity of the immunoblotting method. Our analyses show that the CgA mRNA levels in AR42J cells are comparable to the levels in the NE pheochromocytoma cell line PC-12 (Fig. 1). We also detected CgA mRNA in the rat somatostatinoma cell line RIN-14B. To the best of our knowledge, this is the first report of CgA expression in RIN-14B. However, the finding was not surprising, since others (Funakoshi et al., 1990) have reported that CgA is expressed in the human somatostatinoma cell line QGP-1.

We found a moderate transcriptional activation of CgA in AR42J upon treatment with gastrin and EGF (Fig. 2). Gastrin-induced regulation of CgA promotor activity has previously been shown in gastric carcinoma cells (Höcker et al., 1998). However, Weiss et al. (2001) could not detect any upregulation of neither CgA mRNA (Northern blot), nor CgA protein (RIA), upon treatment with EGF in neuroblastoma cell lines. This may be due to a lower sensitivity of the Northern blot method as compared with reporter plasmid analysis used in the present study. However, it may also indicate that CgA expression is not inducible by EGF in all NE cell types. In conclusion, the demonstration that CgA gene expression is regulated by gastrin and EGF, and that CgA mRNA is translated into protein in AR42J, strongly suggest a physiologically important role of CgA in this pancreatic cell line.

Even though AR42J cells are known to express both functional SSTRs (Viguerie et al., 1988) and their mRNAs (Taylor et al., 1994; Vidal et al., 1994; Froidevaux et al., 1999), there are conflicting results as to which subtypes these cells express. Vidal et al. (1994) found high levels of SSTR2 PCR products, and only low levels of SSTR1 and 3. Froidevaux et al. (1999), on the other hand, were unable to detect SSTR3 or SSTR5 mRNA, and concluded that AR42J cells can be considered to be cells expressing exclusively SSTR2. Their study was, however, limited to examining the expression of just the three octreotide-binding receptor subtypes, SSTR2, SSTR3, and SSTR5. Our finding that the AR42J cells express four of the five SSTRs has, as far as we know, not been reported previously. At least any SSTR5 expression has not previously been detected in AR42J cells. We were unable to detect SSTR4 mRNA, which has not been reported investigated by others.

Wulbrand et al. (1998) found a widely varying expression pattern of the five SSTR subtypes in different types of NE gastro-entero-pancreatic (GEP) tumors. However, almost 20% of the GEP tumors were found to express four or five SSTR subtypes. Thus, it is obviously of great importance in investigations of the exact functional significance of SSTR expression to have access to experimental models in which the NE tumor cells express several SSTR subtypes. The AR42J cell line fulfils these criteria, and thus has an advantage over SSTR transfected cell lines which in most cases express only one receptor subtype.

SSTR scintigraphy is a well-established diagnostic tool for staging of NE tumors, and may indicate sensitivity to treatment with SST analogs (Chiti et al., 2000). More recently, radiotherapy of SSTR positive tumors with radiolabeled SST analogs has been carried out with survival benefit (McCarthy et al., 2000). In addition, because of their nearly universal inhibitory actions on the release of peptide hormones and growth factors, SST analogs are regarded as the main choice for symptomatic treatment of hormone-related syndromes often related to NE tumors (Hofland and Lamberts, 1997; Öberg, 2000). In light of this extensive clinical use of SST analogs, it will be of great interest to elucidate molecular mechanisms involved in regulation of the expression of the different SSTR subtypes, since this may provide strategies to upregulate SSTRs in vivo. Our results lead us to suggest that the AR42J cell line could be a valuable experimental model for this purpose.

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Paper II

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Gene expression based classification of gastric carcinoma

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Abstract

The aim of the present work is to identify molecular markers that allow classification of gastric carcinoma with respect to important clinicopathological parameters. Gastric adenocarcinomas were subjected to cDNA microarray analysis with a 2.504 gene probe set. Using the Rosetta rough-set based learning system, good classifiers were generated for gene-expression based prediction of intestinal or diffuse growth pattern according to Laurén's classification and presence of lymph node metastases. To our knowledge, this is the first study on gastric carcinoma in which molecular classification has been achieved for more than one clinicopathological parameter based on microarray gene expression profiles.

Keywords: cDNA microarray; Gastric cancer; Learning; Prognosis

1. Introduction

Although the incidence of gastric adenocarcinoma is declining, this neoplastic disease is still the second most frequent cause of cancer death world-wide. Gastric carcinomas are often not detected until at an advanced stage; consequently, the 5-year survival rates are low and most often in the order of 10–20%. Variables such as size, microscopic differentiation and growth pattern, depth of infiltration as well as metastases in regional lymph nodes or in remote organs and tissues, all play important roles in treatment and prognosis. Carcinomas of the stomach have been the subject of numerous kinds of clinicopathological classifications, often based on gross features and/or microscopic growth pattern and differentiation. In Scandinavian countries, the prevalent classification is that of Laurén from 1965, subdividing the gastric adenocarcinomas into two major types, the intestinal and the diffuse [1].

Knowledge about the molecular features of gastric carcinoma has increased rapidly. Genetic changes include amplification of the *c-erbB2* gene, mutations of *ras*, *APC* and *p53* genes [2] and truncation of

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E-cadherin [3]. Loss of heterozygosity in advanced gastric carcinomas frequently implicates loci on chromosomes 1, 5, 7, 12, 13 and 17 [2]. The tumor cells also often show overexpression of the *Ras* oncogenes and cyclins [4,5]. Multiple autocrine loops may be involved, cytokines may be overexpressed, and gastric carcinomas may express regulatory peptides, like epidermal growth factor (EGF) [6,7], transforming growth factor alpha (TGF-α) [6,7], platelet-derived growth factor (PDGF) [6] and insulin-like growth factor II (ILGF-II) [6]. Hepatocyte growth factor (HGF) and its receptor *c-met* are frequently overexpressed [8,9]. The classification according to Laurén also corresponds to some degree with genetic abnormalities [6,10,11]. The objective of the work presented here was to examine the gene expression patterns of primary tumors in patients with gastric carcinoma by DNA microarray in order to search for correlations between gene expression and selected clinical and tumor parameters. We sought patterns that characterize both aspects of biological interest, like levels of serum gastrin and localization of tumor in the stomach, and gene expression-based classifiers for parameters important for treatment and prognosis. To this end we analysed gene expression data with a machine learning formalism based on rough sets [12], and the Rosetta toolkit [13]. The quality of the classification was assessed with a cross-validation scheme and tested on random data.

2. Materials and methods

2.1. Patients

The study was approved by the local Institutional Review Board, and the patients gave written informed consent. Tumor samples were taken from patients with gastric carcinoma who underwent gastric resection. The tumors were selected according to Laurén's classification, including approximately equal numbers of tumors with intestinal and diffuse growth pattern and avoiding tumors with an indeterminate histopathological pattern. Preoperative blood samples were taken for gastrin measurement. The extent of the disease was assessed preoperatively by chest X-ray, abdominal ultrasound and CT scan, and the abdominal cavity was explored during the surgery. The resectates were inspected and the tumors described by localization (cardiac, corpus, antral), penetration of the gastric wall and lymph node metastases. Histopathological assessment included tumor classification according to Laurén, depth of invasion and examination of lymph nodes in the resectate. Radioimmunoassay for gastrin was done as previously described [14].

2.2. Tumor material

Tumor samples were collected in the operating room as soon as possible after resection. Tumor tissue was identified macroscopically, dissected from the resectate and preserved on formaline, or snap frozen and stored on liquid nitrogen. The formaline-fixed material was processed using routine histopathological procedures and stained with hematoxylin–eosin before examination by an experienced pathologist (SF). Frozen tissue was homogenized in a guanidinium-isothiocyanate buffer with a rotating-knife homogenizer, total RNA was extracted by ultracentrifugation on a cesium chloride cushion, precipitated, purified using TRIzol (phenol-guanidinium-thiocyanate) (GIBCO BRL Life Technologies, New York, NY), and examined for degradation by agarose electrophoresis with evaluation of the 18S and 28S ribosomal RNA bands. There was no degradation in any of the samples used for microarray analysis.

2.3. Microarray procedures

Arrays were prepared using cDNA probes representing 2.504 sequence verified human genes (Research Genetics, Huntsville, AL) (supplementary material at http://www.lcb.uu.se/~herman/gastric/gastric.html), including 1.500 genes defined in the NCI Oncochip selection (http://resresources.nci.nih.gov/). Additional information of cDNA clone preparation is described in Ref. [15]. The probes were printed in duplicate onto amino-silane coated glass slides (Corning CMT-GAPS; Corning, Corning, NY) using a printing robot constructed in collaboration with NEMKO (Trondheim, Norway) after a prototype developed at the National Human Genome Research Institute (NHGRI), Bethesda, MD (http://www.nhgri.gov/DIR/LCG/15K(HTML/).

Universal Human Reference RNA from Stratagene (La Jolla, CA) consisting of total RNA from 10 different

cell lines selected to optimize gene coverage on human microarrays, and tumor sample total RNA (1 μg each), were reverse transcribed and labeled with Cy3- and Cy5-attached dendrimer, respectively, using the Genisphere 3DNA dendrimer kit (Genisphere, Montvale, NJ) as described in the manufacturer's protocol and previously by us [15]. Arrays were scanned separately at 532 and 633 nm using a confocal laser scanner constructed in collaboration with NEMKO (Trondheim, Norway) according to a prototype developed at NHGRI (http://www.nhgri.nih.gov/DIR/LCG/15K/HTML/).

2.4. Data analysis

The microarrays were analyzed using Scanalytics' MicroArray Suite with default settings. Several normalization techniques, including global and print-tip normalization [16], were tested on each array. We found that global normalization most often gave the highest correlation between the duplicate spots. Hence, each array was globally normalized and further analysis done on $\log_2$ transformed, background corrected ratios. Unreliable spots were removed from the arrays after scatter plot analysis.

The microarrays were analyzed with regard to the following parameters: histopathological classification (Laurén, diffuse or intestinal), site of primary tumor (cardia, corpus or antrum), penetration of the stomach wall or not, lymph node metastasis or not, remote metastasis or not, and high or normal serum gastrin. For each parameter, the tumor data contained two or three classes, a 'class' is a value of a parameter that may be assigned to a tumor sample (example: yes or no for remote metastasis). Genes that were differentially expressed between the classes of each parameter, were identified using a bootstrap *t*-test [17]. The measurements from each gene probe were tested separately by collecting the corresponding $\log_2$-ratios from the microarrays, and the $\log_2$-ratios from the probe duplicates were averaged. A gene was not tested, if the ratios for both duplicate spots were missing on more than 50% of the microarrays.

2.5. Generation of gene expression based classifiers

In order to generate classifiers for the six parameters we used Rosetta [13], a rough set theory [12] based supervised learning system. A classifier is trained on a set of tumors with known classes (e.g. the presence or absence of lymph node metastasis). The trained classifier may then assign a class to a new tumor (e.g. indicate from the gene expression pattern of the new tumor if there is lymph node metastasis or not). A training set was built using the $\log_2$-ratios of the differentially expressed genes with the highest t-statistic from the bootstrap analysis. Genes significant at the $P \leq 0.01$ level were primarily chosen, and if these were very few, genes at the $P \leq 0.05$ or $P \leq 0.10$ level were also used. Classifier performance was optimized by adjusting the maximum number of genes allowed in each classifier within a range of 10–40 genes. The $\log_2$-ratios of each gene were then discretized using frequency binning or Fayyad and Irani's discretization algorithm [18], converting quantitative (numerical) data into qualitative (categorical) data (eg. low, medium, high). Frequency binning divides the range of the $\log_2$-ratios into k intervals (or bins) so that the frequency of ratios is the same in each interval. In our case, we used k from 2 to 4 intervals. Rosetta provides several learning algorithms for producing rules. These algorithms and discretization methods were tested for each clinical parameter in order to determine the best classifier in each case.

The classifiers were evaluated using leave-one-out cross-validation, a method that has also been used by others [19,20] for tumor classification. A new classifier was learned for each sample by excluding the sample from the training set and training the classifier on the remaining samples. This classifier was then used for classifying the left-out sample. This process was repeated for all samples, and the quality of the classifiers (sensitivity, specificity, area-under-curve-(AUC)) was estimated on the basis of the predictions made for each sample. Note that the gene bootstrap selection step was included in the cross-validation procedure so that for each iteration of this procedure a new set of genes was selected and a classifier was trained using these genes. This is important since if the genes had been selected prior to cross-validation procedure, the estimated performance could have been optimistically biased. Details of the data analysis are given in the supplementary material (http://www.lcb.uu.se/~herman/gastric/gastric.html) and in Midelfart et al. [21].

2.6. RT-PCR analysis

Confirmatory reverse-transcription polymerase chain reaction (RT-PCR) analysis was done on four different genes. The primer sequences used can be found in supplementary material (http://www.lcb.uu.se/~herman/gastric/gastric.html). RT-PCR analysis was performed with 250 ng tumor total RNA and 1.25 U rTth DNA polymerase (Perkin Elmer, Boston, MA), with cDNA synthesis at 61 °C for 40 min, followed by PCR with 29 cycles at 94 °C for 15 s, 50 °C for 15 s, and at 72 °C for 30 s, and a final extension step for 3 min at 72 °C. The number of PCR-cycles was selected on the basis of preliminary experiments which showed that 29 cycles yielded quantitative results within the linear range. PCR products were visualized by electrophoresis on a 2% ethidium bromide agarose gel.

3. Results

3.1. Patient/tumor characteristics

Tumor samples were taken from 17 patients, 6 female (aged 45–80, median 70 years) and 11 male (aged 49–93, median 73 years), all Caucasian. Nine tumors were classified as intestinal and 8 as diffuse according to Laurén; 4 tumors were localized to the cardiac, 7 to the corpus and 6 to the antrum region. Thirteen patients had tumors penetrating the gastric wall and 10 had lymph node metastases. Incomplete clinical data made the presence of remote metastasis evaluable for only 13 patients, of these 3 had discernible remote metastases. Serum gastrin measurements were available for 14 patients, of these 5 had serum gastrin above the upper normal value of 40 pM. In these patients median serum gastrin was 104 (range 43–350) pM. Both sexes were similarly distributed between the classes in each parameter.

3.2. Microarray analysis-development and quality assessment of the classifiers

The raw data from the microarray experiments can be found as supplementary material (http://www.lcb.uu.se/~herman/gastric/gastric.html). The genes identified by bootstrap analysis were used to develop classifiers for the six selected parameters (Table 1). Several classifiers had a very good accuracy and a high AUC value, indicating that the classes of these

Table 1
Classifiers for clinical parameters

Parameter	Predicted[a]	Accuracy	Sensitivity	Specificity	AUC[b]	Total no. of genes in CV[c]	Max. genes in classifier	Prevalences in the classes
Histopathological classification (Laurén)	16/17	0.94	1.00	0.88	0.93	17	10	9/8[d]
Lymph node metastasis	14/17	0.82	0.70	1.00	0.90	73	20	10/7[e]
Penetration of gastric wall	16/17	0.94	1.00	0.75	0.85	75	20	13/4[e]
Remote metastasis	13/13	1.00	1.00	1.00	1.00	161	40	3/10[e]
Localization of tumor	17/17	1.00	1.00	1.00	1.00	72	20	4/13[f]
Serum gastrin	11/14	0.79	0.89	0.60	0.66	14	10	5/9[g]

Classifiers obtained by 'Rosetta'. The quality (accuracy, sensitivity, specificity, area-under-curve-AUC) of the classifiers is shown. Each algorithm was evaluated with cross-validation, and it is the performance of the best algorithm which is presented. The number of genes that occurs in at least one of the classifiers generated during cross validation is given. No rules or classifiers were combined, but the number of times each gene was used during cross-validation was examined. This is reported in Table 3.

[a] No. predicted vs no. samples.
[b] Area-under-curve.
[c] Cross-validation.
[d] Intestinal/diffuse.
[e] Yes/no.
[f] Cardiac/noncardiac.
[g] High/normal.

parameters could be predicted with a high level of confidence using the microarray data. The best results were usually obtained when not more than 10 or 20 genes with the highest bootstrap *t*-statistic were used in a single classifier.

There is a considerable risk of overfitting the classifier when there are only 3–5 samples in one class, as is the case for penetration of the gastric wall, remote metastasis, serum gastrin and localization. Therefore, the significance of each classifier was assessed with a permutation test, which estimated the probability that the results had arisen by pure chance. For each clinical parameter, we created 2000 random data sets by shuffling the class labels of the parameter. The full cross-validation procedure (including gene selection with bootstrapping and learning with rough set algorithms) was then repeated on each random data set so that the AUC could be computed. A *P*-value was estimated by counting the number of random data sets that had an AUC greater than, or equal to, the AUC obtained on the original data (Table 2). This analysis showed that the classifiers for Lauren's histopathological classification, and lymph node metastasis were convincingly significant. The classifier for localization of tumor also showed a *P*-value below 0.05 and should be considered significant. Penetration of the gastric wall had a *P*-value slightly greater than 0.05 and was a borderline case. This classifier should thus be treated with more caution. The classifiers for remote metastasis and serum gastrin had *P*-values well above 0.1 and are probably not usable.

Table 2
The probability of obtaining similar classification performance on random data

Parameter	*P*-value
Histopathological classification (Laurén)	0.007
Lymph node metastasis	0.007
Localization of tumor	0.031
Penetration of gastric wall	0.059
Remote metastasis	0.195
Serum gastrin	0.391

The *P*-values are the estimated probability that the learning algorithm (which was selected individually for each parameter) will obtain an AUC value greater or equal to the AUC that it obtained on the experimental data.

3.3. The genes in the classifiers

From a molecular biological point of view, it is highly interesting to examine the genes used by each of the classifiers. The genes used in a given classifier can distinguish between a tumor sample of one class and a tumor sample of another class within a clinical parameter (e.g. distinguish between presence and absence of lymph node metastasis). Thus, these genes are likely to encode proteins that play a role in the underlying molecular biology of the parameter in question. Table 3 shows a list of genes used by each of the classifiers generated by cross-validation. It is important to note that leave-one-out cross-validation creates one classifier for each sample with this parameter (that is 17 classifiers for all parameters except for gastrin level and remote metastasis where data were available for only 14 and 13 patients, respectively). Thus, the number of classifiers in which a given gene is used, indicates the general importance of this gene in predicting the class of a given patient sample for the parameter in question. Genes that occur in a high proportion of the classifiers for a given parameter are generally useful for separating the classes within that parameter. These genes are thus characteristic for that parameter and may be of particular biological interest. For example, *ISG15* appeared in each of the 17 classifiers that were created during cross-validation of the best learning algorithm for lymph node metastasis. *FAT*, on the other hand, occurred in only two of the classifiers.

The classifier genes code for proteins with many different functions; such as intracellular signal transduction, protein synthesis, cell division and differentiation, extracellular matrix components, cell adhesion molecules and several more. We also find several genes with unknown biological function. The classifier genes are of clinical and biological interest, since their expression is related to gastric carcinoma tumor biology. In the following, classifier genes for the different parameters are discussed in some detail.

3.4. Histopathology (Laurén)-intestinal or diffuse

Only three genes were used in more than two classifiers for these two histopathological classes. One is *BRCA2*, which was expressed at a higher

Table 3
Genes of classifiers for clinically relevant parameters

Symbol	Name	GeneBank AccNo	No classifiers	Highest level in	
	Intestinal (I) or diffuse (D)-Laurén			**I**	**D**
BRCA2	Breast cancer 2, early onset	H48122	17	×	
SCAND1	SCAN domain-containing 1	W69127	17	×	
RIN	Ric (*Drosophila*)-like, expressed in neurons	N53351	15	×	
	Lymph node metastasis (yes or no)			**Y**	**N**
LOC51058	Hypothetical protein	AA053665	17	×	
ISG15	Interferon-stimulated protein, 15 kDa	AA406020	17	×	
	Homo sapiens cDNA FLJ14959 fis, clone PLACE4000156	AA159900	16	×	
	Homo sapiens, clone IMAGE:3948563	AA043772	16	×	
DKFZP434J1813	DKFZp434J1813 protein	AA504844	16	×	
CACNB1	Calcium channel, voltage-dependent, beta 1 subunit	W72250	15	×	
	Homo sapiens, clone MGC:2492, mRNA, complete cds	AA620408	15	×	
NAP4	Nck, Ash and phospholipase C binding protein	AA625859	15	×	
PPP1CC	Protein phosphatase 1, catalytic subunit, gamma isoform	AI015359	14	×	
	ESTs, Mod similar to JC5238 galactosylceramide-like prot	AA071075	13	×	
HAT1	Histone acetyltransferase 1	AA625662	13	×	
MGC8471	Hypothetical protein MGC8471	AA447502	13	×	
SEC4L	GTP-binding prot homo to Sacc cerevisiae SEC4	T60109	12	×	
DUSP3	dual specificity phosphatase 3	AA190339	11	×	
NOLA2	Nucleolar protein family A, member 2	AA485675	11		×
RAB11A	RAB11A, member RAS oncogene family	AA025058	10	×	
SNRPE	Small nuclear ribonucleoprotein polypeptide E	AA678021	10	×	
TRIP10	Thyroid hormone receptor interactor 10	R49671	9	×	
	ESTs, Moderately similar to S47073 finger protein HZF2	AA281890	8	×	
	Homo sapiens, clone MGC:18257	AA495746	5	×	
DARS	Aspartyl-tRNA synthetase	AA481562	5	×	
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	W49619	5	×	
CA150	Transcription factor CA150	AA045180	4	×	
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	AA458838	4	×	
NDUFAB1	NADH dehydrogenase 1, alpha/beta subcomplex	AA447569	4	×	
CAMLG	Calcium modulating ligand	AA521411	2	×	
PP	Pyrophosphatase (inorganic)	AA608572	2	×	
IGSF3	Immunoglobulin superfamily, member 3	AI002566	2		×
MID1	Midline 1 (Opitz/BBB syndrome)	AA598640	2	×	
FAT	FAT tumor suppressor (*Drosophila*) homolog	AA159194	2	×	
	Cardiac (C) or non-cardiac (NC) location			**C**	**NC**
CDH2	Cadherin 2, type 1, N-cadherin (neuronal)	W49619	17	×	
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	AA458838	17	×	
MRPL4	Mitochondrial ribosomal protein L4	AA490981	17	×	
DUSP4	Dual specificity phosphatase 4	AA444049	17	×	
CYP3A4	Cytochrome P450, subfamily IIIA, polypeptide 4	R91078	16	×	
DUSP3	Dual specificity phosphatase 3	AA190339	15	×	
SOS1	Son of sevenless (*Drosophila*) homolog 1	N51823	15	×	
LOC51058	Hypothetical protein	AA053665	14	×	
RBSK	Ribokinase	T69020	14	×	
	ESTs, moderately similar to S47073 finger protein HZF2	AA281890	14	×	
MTF1	Metal-regulatory transcription factor 1	AA448256	14		×

(Continued on next page)

Table 3 (*continued*)

Symbol	Name	GeneBank AccNo	No classifiers	Highest level in	
CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	AA630082	14	×	
PMS1	Postmeiotic segregation increased 1	AA504838	13	×	
NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1	AA406535	12	×	
UBE2E1	Ubiquitin-conjugating enzyme E2E 1	AA044025	12	×	
KIAA1595	KIAA1595 protein	AA496999	11	×	
REG1A	Regenerating islet-derived 1 alpha	AA625655	9		×
CSE1L	Chromosome segregation 1-like	N69204	9	×	
NOTCH3	Notch (*Drosophila*) homolog 3	AA284113	9		×
MGC8471	Hypothetical protein MGC8471	AA447502	7	×	
ABR	Active BCR-related gene	W24076	6	×	
RELA	v-Rel avian reticuloendotheliosis viral oncogene homo A	AA443546	5	×	
LAMB1	Laminin, beta 1	AA019209	4	×	
	Similar to TEA domain family member 2	AA669124	4	×	
PPAT	Phosphoribosyl pyrophosphate amidotransferase	AA873575	4	×	
RAB18	RAB18, member RAS oncogene family	AA156821	3	×	
EIF2S2	Eukaryotic translation initiation factor 2, subunit 2	AA027240	3	×	
	Tumor penetrating gastric wall (yes or no)			**Y**	**N**
ADK	Adenosine kinase	R12473	16		×
RXRG	Retinoid X receptor, gamma	W96099	13	×	
PRKCQ	Protein kinase C, theta	H60824	12	×	
ITGA3'	Integrin, alpha 3	AA424695	9	×	
SCEL	Sciellin	AA455012	8	×	
	ESTs	R44752	8		×
LGALS3	Lectin, galactoside-binding, soluble, 3 (galectin 3)	AA630328	7	×	
TRD@	T cell receptor delta locus	AA670107	6	×	
PEG3	Paternally expressed 3	AA459941	4		×
ZNF238	Zinc finger protein 238	R79722	3		×
RUNX3	Runt-related transcription factor 3	N67778	3	×	
PPARD	Peroxisome proliferative activated receptor, delta	N33331	3		×
HNF3G2	Hepatocyte nuclear factor 3, gamma	R99562	3	×	
OMD	osteomodulin	N32201	3	×	
RI58	Retinoic acid- and interferon-inducible protein (58 kD)	W24246	3		×
	ESTs, weakly similar to gonadotropin ind trans rep-1	R09497	2	×	
TRIP7	Thyroid hormone receptor interactor 7	AA431611	2	×	
DCTN1	Dynactin 1 (p150, Glued (*Drosophila*) homolog)	AA488221	2	×	
FLJ10808	Hypothetical protein FLJ10808	AA443582	2	×	
EDG4	Endothelial diff, lysophos acid G-prot-coup rec, 4	AA419092	2	×	
RAB1	RAB1, member RAS oncogene family	N69689	2	×	
ZNF228	Zinc finger protein 228	N62629	2	×	
GRIA1	Glutamate receptor, ionotropic, AMPA 1	H23378	2	×	

Genes that occur in two or more of the classifiers for one of these parameters: histopathological classification (Laurén), lymph node metastasis, localization of tumor and penetration of the gastric wall. The number of classifiers in which a given gene is used is given. For example, ISG15 appeared in one rule in each of the 17 classifiers that were created during cross-validation of the algorithm that had the best performance for lymph node metastasis and this frequency estimates the stability of a gene in the classifier. Total number of classifiers was 17 for each parameter. Two classes are given for each parameter. The class with the highest mean level of expression compared to a common reference material is indicated for each gene. UniGene Build 136 was used.

level in tumors with intestinal differentiation. The product of this gene probably takes part in DNA repair. Mutations in the *BRCA2* gene have been associated with increased susceptibility to several malignant tumors, among these also gastric carcinoma [22]. There is no previous information, however, on any association of specific gastric carcinoma subtypes with BRCA2 inactivation.

3.5. Lymph node

Most of the lymph node metastasis classifier genes that are included in more than two classifiers are expressed at a higher level in tumors with lymph node metastasis. The lymph node metastasis classifier gene N-cadherin (*CDH2*) has previously been found in gastric adenocarcinoma [23] and upregulation correlates with invasiveness in carcinomas of the breast and prostate [24,25]. The thyroid hormone receptor interactor 10 (*TRIP10*) regulates microtubular structure and may induce cellular motility and spreading by binding to CDC42 [26].

3.6. Localization—gastric cardia vs. other locations

Most of the genes used in these classifiers are expressed at a higher level in tumors from the cardia. Among these are N-cadherin (*CDH2*) which is expressed in a subgroup of gastric carcinomas [23], cyclin-dependent kinase inhibitor 1B (*CDKN1B*) which has been found underexpressed in advanced gastric carcinoma compared to early carcinoma [27], and the cytochrome p450 subfamily IIIA polypeptide 4 (*CYP3A4*) which is overexpressed in intestinal metaplasia and in some well differentiated gastric carcinomas [28]. The nuclear factor-kB (*RELA*) has been shown to be overexpressed in gastric adenocarcinoma of the proximal stomach [29]. Moreover, a low expression level of the regenerating islet-derived 1-α peptide (*REG1A*) is used in two of the classifiers that identify cardiac localization. This peptide is mainly found in the oxyntic mucosal ECL cells which are scarce in the cardia [30].

3.7. Penetration of the gastric wall

Several of the classifier genes that are expressed at a higher level in tumors penetrating the gastric wall, are associated with cellular adhesion and migration. Galectin 3 (*LGALS3*) binds to laminin and correlates to metastasis and local invasion in colorectal cancer [31] and in carcinoma of the breast [32], and integrin α3 (*ITGA3*) is essential for cellular adhesion and migration. Also, tumors penetrating the gastric wall exhibit higher expression levels of the glutamate AMPA 1 receptor (*GRIA1*), whose antagonists are reported to inhibit proliferation, motility and invasive growth of colorectal carcinoma-derived cell lines [33].

3.8. Verification of results

The four genes *DSC2*, *BM1*, *PPP1CC* and *IGF1*, were analysed by RT-PCR in five tumor samples each. For 80% (8 of 10) of the gene-tumor sample-measurements with a microarray ratio less than 0.6, RT-PCR also indicated underexpression relative to the reference RNA. None of the tested genes were significantly overexpressed (microarray analysis) in tumor samples compared to the reference RNA. Thus, the results of the RT-PCR analysis were consistent with the expression profiles obtained through cDNA array hybridization.

4. Discussion

In the present study we have shown that microarray analysis of gastric carcinoma, a complex solid tumor with multiple cellular elements, can produce data that are significantly related to important clinicopathological features. Moreover, we were able to generate reliable classifiers for several clinically important parameters. Our work also identified several genes that were not previously known to display characteristic expression patterns in gastric carcinoma.

The rough set based system Rosetta was used to build classifiers in this study. Other classification methods such as nearest neighbour, discriminant analysis, neural networks, support vectors machines, decision trees could also have been used. Our choice was made for two reasons. First, the rough set approach creates decision rules, making interpretation of the classifier easier. Second, we obtained good classifiers with the rough set approach. In a recent study, Dudiot et al. [34] compared several different classification methods on three different data sets. They found that diagonal linear discriminant analysis and nearest-neighbour gave the best performance. Therefore, we also trained classifiers using one of these methods, namely diagonal linear discriminant analysis, and compared its performance to that of the rough set classifiers. The rough set classifiers outperformed discrimant analysis and obtained the best AUC for all of the clinical parameters (described in

detail in Midelfart et al. [21]). Hence, the rough set approach seemed to be well-suited for this analysis.

Some of the genes which we found to be differentially expressed between gastric carcinoma tumor samples have recently been found by others to be highly expressed in gastric carcinomas [35,36], supporting the validity of our data. This applies to *IMPDH2, ITGA3, TRIP10, CSPG2, CDH17* and *COL6A3*. Moreover, *IGFBP2*, for which we found low levels of expression in tumors penetrating the gastric wall, has previously been reported to be downregulated in gastric carcinoma [36]. The gene expression measurements in the present study are derived from co-hybridization of tumor RNA with a common reference RNA sample. The results thus do not indicate whether a gene is over- or underexpressed in gastric carcinoma *per se*. However, expression levels can be compared between individual tumor samples, and related to parameters associated with these samples. Since gastric carcinomas are tumors with cellular heterogenity, it is not possible to know whether a specific gene is expressed in the malignant cells or in the stroma. However, the data reveal gene expression features that are important in the interaction between tumor and stromal cells. Knowledge on such interactions is necessary to understand tumor biology, like the regulation of metalloproteinases or their inhibitors in gastric carcinoma tumor cells and stroma [37,38].

Some of the classifier genes code for proteins that are important in the context of defined clinical parameters, others have no known relation to malignant disease. Moreover, many classifier genes code for proteins with poorly characterized or completely unknown biological functions. Thus, our study identifies new genes that may be of potential clinical and biological interest in gastric carcinoma.

Although the findings in a single study like this must be handled with great care, our methods constitute one possible approach towards developing a clinically useful molecular classification of tumors. From primary gastric tumors, we have developed classifiers that characterize gastric carcinomas with respect to clinico-pathologically important parameters such as lymph node metastasis. Moreover, with the molecular methods used here ordinary endoscopic biopsies give ample total RNA for analysis of factors that are important in the surgical decision-making process and in the choice of adjuvant cancer therapy. However, all the patients were of Norwegian/Caucasian origin, and at the present stage one should be careful concerning the general applicability of the classifiers. The results will have to be verified in a larger series of tumors, probably also in different patient populations.

Interestingly, we were able to produce a classifier that distinguishes the diffuse and intestinal forms of gastric carcinoma as described by Laurén [1]. Like Boussioutas et al. [39], our results indicate that Laurén's classification reflects significant molecular differences in gastric carcinomas. However, Boussioutas et al. made no attempt to use supervised methods to predict the class of samples for these parameters. Hippo et al. [35], could not find that their two-way clustering gene expression analysis provided classifiers for histopathological features in their gastric carcinoma material. If the results of Boussioutas et al. and ourselves can be confirmed, a gene expression based classification of gastric carcinoma will probably be more precise than the presently used histopathological examination. Moreover, the classifier genes may provide further insight in the molecular processes that differentiate these two histopathological forms of gastric carcinoma.

The classifier that distinguishes cardiac from non-cardiac gastric carcinomas is of particular biological interest, since cardiac adenocarcinoma shows a clinicopathological and epidemiological pattern different from the more distal gastric carcinomas [40]. In contrast to adenocarcinomas of the corpus or antrum, the incidence of cardiac adenocarcinomas increases [41], and the cardiac tumors are unrelated to infection with *Helicobacter pylori*. It is interesting to note that when we first tried to predict all locations of the tumors (cardiac, corpus, antral), we obtained poor results and no good classifiers were found. We could, however, train a classifier that separated cardiac tumors from corpus and antral tumors combined. This indicates that cardiac tumors have a gene expression pattern that is clearly different from tumors with corpus and antral localization. Some of the localization classifier genes are clearly related to gastric carcinoma, like N-cadherin, but the only gene known to be region-specific is regenerating islet-derived 1α peptide, which is only expressed in the ECL cells of the oxyntic mucosa, and barely

expressed in the cardiac region [42]. The localization classifier genes are obvious targets for further studies.

A number of studies have shown that microarray analysis may be used to establish gene expression profiles identifying tumor subgroups that correlate with clinicopathological parameters. This has been done for breast cancer [43], lymphoma [44], and esophageal carcinoma [45]. Moreover, microarray gene expression data have been used to classify and predict leukemia subtypes [19], breast cancer estrogen receptor status [46–48] and the prognosis for patients with renal clear-cell carcinoma [49]. In the present study, we have demonstrated classification and prediction of several different parameters based on one set of microarray gene expression profiles. To our knowledge, this is the most complex set of classifiers/predictors reported for different clinically and biologically relevant features of gastric adenocarcinoma. This work should be considered a first attempt to produce clinically and biologically useful molecular classifications for this type of cancer.

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Paper III

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Paper IV

Changes in gene expression of gastric mucosa during therapeutic acid inhibition

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Running title: Esomeprazole-induced gene expression changes

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Abstract

Long-term therapy with potent acid inhibitors is the most common treatment for gastro-esophageal reflux disease. Administration of proton pump inhibitors (PPI's) causes profound and continuous hypochlorhydria by inhibition of the proton pump (H^+/K^+-ATPase) in gastric parietal cells. Long-term hypergastrinaemia increases mucosal thickness and ECL cell density in oxyntic mucosa, and results in development of gastric carcinoids in experimental animals. Knowledge of the effect on mucosal gene expression of PPI's given to humans in ordinary, therapeutic doses is limited. Eight patients suffering from gastro-esophageal reflux disease were included in this study. Endoscopic biopsies were taken from the corpus mucosa before and towards the end of a three-month treatment with the PPI esomeprazole. Microarray analysis identified 149 differentially expressed genes. Of these 56 were induced, and 93 were repressed. A high proportion of genes with changed gene expression levels in the presence of proton pump inhibitors are involved in proliferation, apoptosis and stress response. This work identified many genes that were not previously known to be affected by inhibition of gastric acid secretion. Further characterization of the functional roles of genes whose expression is modulated by potent acid inhibition may give new insight into the biological responses to this very common therapeutic intervention, including the mucosal response to the moderately increased gastrin levels encountered in clinical practice.

Key words: cDNA microarray, gastrin, proliferation, esomeprazole, proton pump inhibitors

Introduction

The introduction of potent drugs (H_2-blockers and proton pump inhibitors) in the 1970s and 1980s for the control of gastric acid secretion completely changed medical therapy of peptic ulcer and reflux esophagitis. Maintenance therapy with proton pump inhibitors is widely used for patients with gastroesophageal reflux disease. In this patient group, long-term therapy is often used, as an alternative to surgery, to reduce the risk of complications from long-standing inflammatory and erosive processes like strictures, ulcers and bleeding. During the last decade, H_2-blockers have been largely replaced by proton pump inhibitors which, by inhibiting the enzyme responsible for the final step in acid secretion (18), can virtually extinguish acid secretion.

The profound hypoacidity induced by proton pump inhibitors results in significant changes in the intragastric environment. Colonization of the stomach by bacteria which catalyze nitrosation may occur (70), with formation of potentially carcinogenic N-nitroso compounds (47). Also, the barrier function of acid against microorganisms and possibly prions is reduced (43, 53). Secondary to this, the immune and inflammatory responses in the mucosa are affected. With an increased pH, it is reasonable to believe that the cellular stress response of the gastric mucosa is changed. Administration of proton pump inhibitors reduces the feedback inhibition of acid on the synthesis and release of the acid secretagogue hormone gastrin, leading to increased serum gastrin in these patients (77). Gastrin stimulates acid secretion via the release of histamine from the enterochromaffin-like (ECL) cell (62). In addition to a specific and pronounced effect on function and proliferation of ECL cells, gastrin stimulates growth in the oxyntic mucosa (17, 24). It is not clear whether this general

trophic effect of gastrin is due to a direct action on the endodermal-derived stem cell or whether it is mediated indirectly by histamine, regenerating gene protein (*Reg*) or other substances released from the ECL-cell. In rats, long-term hypergastrinemia induced by very high doses of PPI's not only increases mucosal thickness and the ECL cell density, but also results in the development of gastric carcinoids (17, 24). ECL cell hyperplasia, which is regularly found during the first year of omeprazole treatment in the rat, has also been reported in patients on proton pump inhibitor treatment (16), and carcinoid tumors have been seen in patients with hypergastrinemia induced by gastrin-producing tumors or atrophic gastritis (20). It is, however, debated whether carcinoid tumors may occur in humans in response to therapeutic acid inhibition.

There is only a very limited knowledge of the gene expression changes involved in the complex physiological and patophysiological responses to acid inhibition. The ECL-cells' response to PPI-induced hypergastrinemia is reflected by increased abundance of histidine decarboxylase (*Hdc*) and Chromogranin A (*CgA*) mRNA (13, 14). Moreover, both chronic and short-term hypergastrinemia stimulate gene expression and protein level of regenerating gene protein (*Reg*) in the fundic mucosa (45). However, ECL cells only represent a small fraction of the different exocrine and neuroendocrine cells present in gastric glands. Among the exocrine cells we find surface mucous cells, isthmus and neck cells, acid secreting parietal cells and pepsinogen secreting chief cells. The neuroendocrine cells, which constitute about 2% of the cells in the gastric mucosa, comprise ECL cells, which produce and release histamine, D cells (somatostatin), G cells (gastrin) and other neuroendocrine cells.

Thus, only a few genes responsive to acid inhibition have been identified and characterized, and a more global gene discovery approach will most likely identify novel genes responsive to this pharmacological intervention. In a previous study, we identified genes differentially expressed in response to the PPI omeprazole in gastric mucosa of rats (51). In the present study, we have used DNA-microarray technology to investigate changes in the transcriptional profile of the oxyntic mucosa during a three-month treatment course with the PPI esomeprazole given to patients with reflux esophagitis in an ordinary therapeutic dose. The objective of this work was to see whether this very common clinical intervention induces significant changes in the mucosal gene expression pattern, especially with regard to cellular proliferation in the gastric mucosa. Using cDNA microarrays of 5346 genes, we show that esomeprazole modulates many genes not previously known to respond to acid inhibition, including genes involved in proliferation, apoptosis and stress response.

Materials and methods

Patients. The study was approved by the local Institutional Review Board. The patients had typical symptoms of gastroesophageal reflux disease, and were enrolled from those admitted to the Gastrointestinal Endoscopy Laboratory at St. Olav's University Hospital for diagnostic upper endoscopy. Written, informed consent was obtained. The patients all had endoscopic signs of reflux esophagitis (degree 2 or 3 according to the Savary Miller classification), and were treated with 40 mg esomeprazole (Nexium®, Astra AB, Gothenburg, Sweden) once daily for 90 days. Before the study period 5 biopsy specimens were taken from the oxyntic mucosa at the major curvature, using a FB-13K E biopsy foreceps (Olympus, Japan). One of the specimens was preserved on formaline. The rest of the specimens were preserved in RNAlater buffer (Ambion, Austin, TX). During the last week of the study period 4 biopsy specimens were taken from the same site. These specimens were all preserved on RNAlater buffer. Fasting blood samples were taken before and during the last week of the study period for gastrin measurement.

Serum measurements. Radioimmunoassay for gastrin and chromogranin A (*CgA*) was done as previously described (35, 71). Mean values ± SEM were calculated and the significance of the differences was evaluated by Wilcoxon matched pairs test. $P<0.05$ was considered statistically significant. An ELISA assay (QUANTA Lite™ *H. pylori* IgG; INOVA Diagnostics, Inc., San Diego, CA) was performed in order to detect antibodies against *Helicobacter pylori*.

Northern blot analysis. Northern blot analyses were performed for *CgA*, *Gapdh* and *18S* ribosomal RNA as previously described (5, 22, 72). A fragment of human histidine decarboxylase (*Hdc*) cDNA was generated by RT-PCR of total RNA from the promyelocytic leukaemia cell line HL-60 using primers corresponding to the 1188-1205 and 1782-1799 stretches of the published (NM_002112) cDNA sequence of human histidine decarboxylase, with subsequent cloning of the fragment into the pCRII vector (Invitrogen, San Diego, CA, USA). The authenticity of the probe was verified by sequencing using standard methods. The significance of the differences was evaluated by Wilcoxon matched pairs test. $P<0.05$ was considered statistically significant.

RNA isolation. The specimens were homogenized in lysis buffer RLT provided in the RNeasy kit of Qiagen (Basel, Switzerland) and total RNA extracted using RNeasy according to the manufacturer's protocol. Total RNA was further purified using the TRIzol method (phenol-guanidinium-thiocyanate) (GIBCO BRL Life Technologies, New York, NY), and examined for degradation by agarose electrophoresis with evaluation of the 18S and 28S ribosomal RNA bands. There was no degradation in any of the samples used for microarray analysis.

Microarray procedures. Microarrays were obtained from The Norwegian Microarray Consortium (www.microarray.no, Norway). Briefly, 6262 plasmids with sequence verified human probes were obtained from Research Genetics (Huntsville, AL), amplified by PCR with M13 plasmid primers, purified by ethanol precipitation and analysed by agarose electrophoresis and redissolved in 50% DMSO at 0.05-0.50 mg/ml. Probes were printed in duplicate onto amino-silane coated glass slides

(Corning CMT-GAPS; Corning, Corning, NY) using a MicroGrid II printing robot (BioRobotics; Cambridge, UK). The complete list of gene probes is deposited in the Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE3240. Ten different cDNAs from *Arabidopsis thaliana* (Stratagene Spotreporter; La Jolla, CA) were included in all arrays. Each *Arabidopsis* spike was printed 12 or 13 times on each array. *Arabidopsis* mRNA (Stratagene SpotReporter) was added to the samples prior to labeling and hybridization and was used as controls of the performance of the analysis with respect to labeling and hybridization efficiency.

Total RNA from patients before and during esomeprazole treatment (2 μg each), were reverse transcribed and labelled with Cy3- and Cy5-attached dendrimer, respectively, using the Genisphere 3DNA submicro dendrimer kit (Genisphere, Montvale, NJ) as described in the manufacturer's protocol. The slides were pre-hybridized in 1% BSA, 3.5x SSC, 1% SDS at 65^{o}C for 20 min, then washed by dipping 5 times in deionized sterile filtered H_2O, 2 times in isopropanol and air dried. Hybridization was done in a humidified hybridization chamber (Corning) at 60 ^{o}C for 18 h using 0.25 M $NaPO_4$, 1 mM EDTA, 4.5% SDS, 1x SSC, 2x Denhardt's solution and 0.2 μg human COT1 DNA (GIBCO BRL Life Technologies), in a total hybridization volume of 35 μl. Post-hybridization washes were done for 15 min at 55^{o}C with 2x SSC and 0.2% SDS, for 15 min at room temperature with 2x SSC and finally for 15 min at room temperature with 0.2x SSC. The arrays were scanned with a ScanArray Express HT scanner (Packard BioScience, Billerica, MA).

Data analysis – filtering and normalization. The microarrays were analyzed using MicroArray Suite software version 2.1 (Scanalytics, Inc., Fairfax VA) with default settings. All subsequent statistical analyses were performed using the statistical package R (59). A signal to noise ratio based criterion was applied to remove the spots with spot intensity vs. background intensity ratio less than one in either channel. A spot area based criterion was applied to remove the spots with the area less than 10 pixels in either channel. Print tip-dependent lowess normalization was applied to the data in order to compensate for systematic errors (84). The normalized ratio of the duplicates was averaged. Further analysis was done on the $\log_2$ transformed ratios. A gene was considered undetected and not tested, if the averaged ratio was missing on more than 50% of the microarrays.

Statistical Analysis. Statistically significant effects (up- or downregulation) of esomeprazole treatment were found using a two-sided one-sample Student's t-test separately for each gene. Genes with *P* values less than 0.01 were considered statistically significantly regulated by esomeprazole treatment. To account for multiple testing we calculated adjusted *P* values controlling the False Discovery Rate (FDR) using the Benjamini-Hochberg (6) procedure.

Database submission of Microarray Data. The microarray data were prepared according to Minimum Information About a Microarray Experiment (MIAME) recommendations (8), have been deposited in NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE3240.

Histology and immunohistochemistry. For general histology, the formaline-fixed material was processed using routine histopathological procedures and stained with hematoxylin-eosin before examination by an experienced pathologist. For immunohistochemistry, tissue samples from the oxyntic mucosa were taken from corpus/fundus region and immersed in 4% phosphate-buffered formaldehyde and dehydrated in 80% ethanol before paraffin embedding. From paraffin blocks, 4 μm sections were cut and deparaffinised with xylene, rehydrated and treated with 3% hydrogen peroxide for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was achieved by heating the slides immersed in Tris-EDTA buffer pH 9.1 (*Hdc* and urokinase (*Plau*)) or in citrate buffer pH 6.0 (H^+/K^+*ATPase* and *NF-κB* (p50)) in a commercial microwave oven at 160 W for 15 minutes. Antibodies against rat *Hdc* (1:5000, Eurodiagnostica, Malmö, Sweden), *NF-κB* (p50) (1:200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), H^+/K^+*ATPase* (1:1000, Affinity Bioreagents, Golden, CO) and urokinase (*Plau*) (1:10, Cell Sciences Inc., Canton, MA) were diluted in Tris-buffered saline pH 7.4 with 0.025% Tween 20 (DakoCytomation, Glostrup, Denmark) and 1% bovine serum albumin (Sigma, St. Louis, MS), and the sections were incubated with antibodies for 60 minutes. The immunoreactions were visualised using an EnVision-HRP kit (K5007, DAKO, Glostrup, Denmark) and AEC peroxidase kit (SK4200, Vector, Burlingame, CA) or Vector SG (SK4700, Vector, Burlingame, CA).

Results

Patient characteristics. Eight patients were included in the study, 3 female (aged 44-70, median 54 years) and 5 male (33-72 years, median 42 years). All had endoscopic signs of reflux esophagitis, degree 2 or 3 according to the Savary Miller classification prior to esomeprazole treatment. At the 3-month control endoscopy the esophageal lesions were completely healed in all patients, indicating a clinically effective acid inhibition. Measurements of serum gastrin and *CgA* were available for seven of eight patients. Average serum gastrin before treatment was 4±1 pM (n=7) (mean±SEM), and after 90 days treatment with esomeprazole 20±3 pM (n=7) (mean±SEM) (P=0.0156). Average serum *CgA* before treatment was 18 ±4 ng/ml (n=7) (mean±SEM), and after 90 days with esomeprazole 33±5 ng/ml (n=7) (mean±SEM) (P=0.0156). Northern blot analyses of *Hdc* and *CgA* were available for all patients. Both *Hdc* and *CgA* mRNA expression increased (P=0.039) during treatment. Thus, esomeprazole induced a significant increase in serum gastrin, *Hdc* and *CgA*. This is in accordance with previous results (78). Histopathological examination showed gastritis in one of the patients. The same patient was infected by *H. pylori* and had the highest levels of gastrin and *CgA*. None of the other patients were infected by *H. pylori* and the gastric mucosa showed normal histology.

Genes differentially regulated in response to esomeprazole treatment. Genes differentially expressed in response to esomeprazole were identified by microarray analysis by hybridization of RNA from a patient before esomeprazole treatment against RNA from the same patient during esomeprazole treatment. We found that 149 genes exhibited changed expression levels (P<0.01) in patients treated with the

proton pump inhibitor for 3 months. Of these, 56 (38%) were induced and 93 (62%) were repressed. The False Discovery Rate (FDR) was estimated to be 10%. i.e. we expect on the average that 10% of the genes called significantly differentially regulated by esomeprazole treatment are false positives. The list of all 149 genes is shown in Supplemental Table S1 (available at the *Physiological Genomics* web site).[1]

To facilitate interpretation of the results, genes with changed expression in response to esomeprazole were grouped according to cellular processes in which they are likely to be involved, based on information from the literature and from the Norwegian Microarray Consortium Annotation Database (www.microarray.no) (Table 1). All genes regarded to be involved in the processes proliferation, apoptosis, stress, inflammatory and immune response are listed in Table 1. We chose to focus on these processes because they were considered to be the most interesting ones in the molecular response to acid inhibition. Of the 149 differentially expressed genes, 72 (48%) could be classified into these broad functional groups. Mediators of proliferation and apoptosis comprised the largest functional gene cluster (60 genes, 40%), whereas the numbers of genes involved in immune/inflammatory responses (33 genes, 22%) and stress response (32 genes, 21%) were somewhat lower. Several genes were involved in more than one of these biological processes (Table 1). Relatively, most annotations were found for the upregulated genes. This phenomenon has also been observed by others (30).

Validation of results by Exogenous RNA Spikes. Validation of our microarray protocol was done using the SpotReport Array Validation System (Stratagene). Analysis of exogenous (*A. thaliana*) mRNA spiked into labeling reactions at predetermined ratios

showed that the observed ratios highly correlated with the expected ratios (the median across arrays for the pairwise correlation of all observed RNA control spots ratios with expected ratios was 0.90) The observed RNA control spots were also highly correlated across experiments (median correlation of all observed RNA control spots for pairs of arrays of 0.96). The expected ratios for the RNA controls were 6, 5, 4, 3, 2, 1, 0.5, 0.33, 0.25, and 0.125. The external controls showed that a twofold change in mRNA levels could be reliably detected and correlated well with expected fold changes. However, the observed fold changes for the external controls were underestimated at both ends of the expected range (data not shown). This is similar to our observations in previous studies (51, 83).

Further verification of results using Northern Blot, semi-quantitative RT-PCR and real-time quantitative RT-PCR has previously shown that the false positives in our studies are within the range commonly found in microarray experiments (10, 26, 51, 52, 65, 83).

Sequencing. Errors in the IMAGE collections have been reported, and additional errors may have occurred during production. For this reason, 39 (26%) of the clones representing genes regarded as the most interesting, were sequence verified using standard methods. Good sequencing results were obtained for 27 of the clones and 21 (78%) of these showed the expected sequence. For 6 of the 27 clones the sequence was wrong. The incorrect clones were shown to represent a different gene than expected. The new gene information is reported in Table 1 and Supplemental Table S1 (Supplemental Table S1 is available at the *Physiological Genomics* web site[1]). Three of the incorrect clones (IMAGE nr 898109, 770858 and 360885) were shown to represent genes (*Ttc1*, *Gnai1*, and *Comm6*) already present in the list of differentially

expressed genes. Hence, these clones were removed from the table. Notably, for all three genes, both clones were found to be regulated in the same direction.

Rat Orthologs. In a previous study, we identified differentially expressed genes in gastric mucosa of omeprazole-dosed rats (51). In the present study, we tried to find rat orthologs for the 39 sequenced human genes. Rat orthologs were found for 37 of these 39 genes. 17 of the genes were found to have rat orthologs present on the rat arrays used. Notably, eight of these genes were found to be regulated in the same direction in rat and man in response to omeprazole/esomeprazole, although the omeprazole induction of rat genes was not found to be statistically significant, and hence not reported in the previous article. All eight genes are listed in Table 2.

Immunohistochemistry. The *Hdc* immunoreactive cells were found in the basal half of the glands (Fig. 1A). Some of the cells were seen with cytoplasmatic extensions (Fig. 1A, inset). The distribution and shape of these cells are characteristic of neuroendocrine cells of the oxyntic mucosa. Urokinase (*Plau*) immunoreactivity was seen in cells shaped and distributed like parietal cells (Fig. 1B). *NF-κB* (p50) immunoreactivity was only seen in the nucleus of H^+/K^+*ATPase* immunoreactive cells (Fig. 1C). Co-localisation to H^+/K^+*ATPase* immunoreactive cells indicates that *NF-κB* is located to parietal cells.

Discussion

In the present study, molecular responses in gastric mucosa of patients treated with the proton pump inhibitor esomeprazole were studied by measuring genome-wide transcript level changes using cDNA microarrays with probes representing 5346 genes. The acid inhibitory treatment with esomeprazole was clinically effective, as evidenced by the complete healing of esophageal lesions in all patients. Fasting serum gastrin and the serum concentration of *CgA* changed as expected, with significant increases within the range of normal values. In a previous study from our laboratory (78), treatment for a comparable period of time with an equivalent drug (omeprazole) showed a similar fasting serum gastrin and *CgA* response. In that study, histamine and *CgA* increased in tissue biopsies confirming that ECL cell stimulation takes place during therapeutic acid inhibition. Furthermore, multiple measurements of serum gastrin in each patient with area-under-curve calculations showed that the 24-h gastrin exposure is far higher during PPI treatment than is reflected by a single fasting gastrin measurement. Thus, the gastrin exposure of the gastric mucosa in this study is much more pronounced than suggested by the relatively modest increase in fasting serum gastrin.

The genes encoding *CgA* and *Hdc*, both known to be induced by gastrin, (13, 14), showed significantly elevated transcript levels during treatment with esomeprazole (data not shown). Because gene probes for these two genes were not present on the microarrays (*Hdc*), or were filtered away during the quality control (*CgA*), these measurements were performed by Northern blot. These results show that a gene expression response to gastrin was obtained in the study patients, and confirm earlier observations on gene expression changes of gastric mucosa in response to acid

inhibition. Results from a previous study from our laboratory indicate that microarray analysis can indeed be used to detect gene expression changes in a minor cell population of the complex gastric tissue such as ECL cells (51).

Of the 149 genes identified as differentially expressed, 39 were tested for rat orthologs. 17 of the tested 39 differentially expressed human genes were found to have rat orthologs present on the rat arrays used in our previous study on omeprazole-dosed rats. Notably, eight of these genes were found to be regulated in the same direction in rat and man in response to omeprazole/esomeprazole. All eight genes are listed in Table 2. Among these genes, it is interesting to find the upregulated genes plasminogen activator, urokinase (*Plau*) and BCL2-related *Mcl1*. *Plau* expression is known to correlate with tumor angiogenesis and poor outcome in gastric cancer (31). *Mcl1* is known to be upregulated in response to *IL-6* in the human gastric cancer cell line AGS (41). The gene Kruppel-like factor 4 (gut) (*Klf4*) was downregulated in response to acid inhibition. Highly interesting, *Klf4* is known to repress expression of *Hdc* (1). Decreased expression of this gene has been shown to be critical in development and progression of gastric carcinoma (80). All eight genes are annotated to one or more of the biological processes discussed below.

The 149 genes found to be differentially regulated in esomeprazole-treated patients are most likely directly or indirectly regulated by acid inhibition and/or hypergastrinemia. The functional diversity of the candidate genes suggests that many of them are likely to be differentially regulated as indirect consequences of the many processes affected by PPI's in gastric mucosa. One particularly interesting downregulated gene is radixin (*Rdx*), which has been suggested to be involved in

secretion of gastric acid (74). The present study is the first to report that expression of radixin is reduced in response to treatment with acid inhibitors. Another interesting gene, the upregulated cytochrome c oxidase subunit Va (*Cox5a*) has been suggested as a diagnostic marker for gastric carcinoma (50).

Since gastrin is known to stimulate proliferation of both the ECL-cells and the gastric mucosa in general, genes involved in this process were regarded as the most interesting. The immune/inflammatory cellular processes are also of great interest, since for instance *Helicobacter pylori* infection of the human stomach is very common, and related to various diseases such as gastritis, peptic ulcer, mucosa-associated lymphoid tissue (MALT) lymphomas and gastric cancer. The immune/inflammatory processes are most likely affected by the dramatic changes in the intragastric environment induced by potent acid inhibition. Moreover, with an increased pH, there is also reason to believe that the cellular stress response of the gastric mucosa is changed. In the following, these selected processes and the genes involved will be discussed.

Proliferation and apoptosis. Not surprisingly, mediators of proliferation and apoptosis comprised the largest functional gene cluster (Table 1). This finding is similar to results from our previous microarray study on omeprazole-dosed rats (51), although we report mostly different genes due to the low number of differentially expressed genes common to both arrays. Hypergastrinemia induced by potent and longterm inhibition of gastric acid secretion increases proliferation rate and mucosal tickness (17, 24). Apoptosis normally plays a role complementing proliferation and is considered to be essential for the maintenance of gastro-intestinal homeostasis and

health (58). Disturbance in the balance between these two processes may predispose to cell loss with mucosal damage or cell accumulation and cancer development (33, 85). In the normal gastric mucosa, approximately 1-3% of gastric epithelial cells in the antrum or corpus/fundus are apoptotic (61). The present study indicates an increased proliferation and decreased apoptosis in response to inhibition of gastric acid secretion and identifies genes possibly involved in these processes.

An enrichment of genes accosiated with proliferation and apoptosis was found for upregulated genes. Overproduction of tumor supressor *p53*, which is upregulated in the present study, has previously been shown in the ECL-cells during hypergastrinemia in the African rodent mastomys (42). Moreover, the plasminogen activator, urokinase (*Plau*), is known to stimulate proliferation and has previously been implicated in gastric carcinoma (15, 31). Most of the upregulated genes in this category are involved in positive regulation of cell proliferation (e.g. prostate tumor over expressed gene 1 (*Ptov1*) (63), tripeptidyl peptidase II (*Tpp2*) (69), minichromosome maintenance deficient 3 (*Mcm3*) (21), and GRB2-associated binding protein 1 (*Gab1*) (9)). A remarkably high number (13 genes, 23%) of upregulated genes have been reported to have anti-apoptotic effects. In this group we find tripeptidyl peptidase II (*Tpp2*) (69), presenilin 1 (*Psen1*) (3), brain and reproductive organ-expressed (*Bre)* (39), plasminogen activator, urokinase (*Plau*) (76), and BCL2-related gene *Mcl1* (41). Furthermore, Act1 (*C6orf4*) activates the anti-apoptotic transcription factor *NF-κB*, which is constitutively activated in human gastric carcinoma tissue (64, 82).

Among the downregulated genes, we found hydroxysteroid (17-beta) dehydrogenase 13 (*Sdcr9*), the E2F transcription factor 4 (*E2f4*), transcription factor *Klf4*, tumor

supressor *Rbl2*, and the ras homolog gene family member B (*Rhob*) which are all known to be negative regulators of cell proliferation (11, 38, 40, 54, 57). *Rhob* may also play a role in induction of apoptosis (57). Several others of the downregulated genes are also known to induce apoptosis. Among these are *Hmga2* (56), calreticulin (*Calr*) (44), the tumor supressor gene cylindromatosis (*Cyld*) (79), protein kinase C zeta (*Prkcz*) (19), and protein kinase c-like 2 (*Pkn2*) (32).

The fact that a remarkably high number of the differentially expressed genes are involved in proliferation and/or apoptosis supports our hypotesis that there is a significant change in cell turnover during chronic administration of PPI. The expression patterns of positive and negative regulators of proliferation and apoptosis indicate a complex involvement of the identified genes in increased proliferation and decreased apoptosis, inducing the mucosal hyperplasia. However, the complexity of the gastric mucosa makes precise interpretation of these results difficult. Numerous studies report that gastrin stimulates proliferation of both the ECL-cells and the gastric mucosa in general, the effect on ECL cells being more pronounced than the general trophic effect (4, 17, 24). The growth-related genes found here represent good candidates for further confirmation and follow-up studies that may shed light on the precise cellular molecular responses to potent acid inhibition.

Stress responses. Of 149 differentially expressed genes with known function, 32 encode proteins involved in stress responses. This observation is in agreement with the findings in our previous study on omeprazole-dosed rats (51). Several markers of stress response were significantly reduced during acid inhibition. Prolactin (*Prl*) (86), brain-derived neurotrophic factor (*Bdnf*) (2), the general transcription factor II, I

(*Gtf2i*) (55), glutathione-S-transferase-like protein (*Gsto1*) (37) and heat shock protein *Hsph1* (23) are all downregulated in response to acid inhibition. Downregulation of stress responsive genes may be due to the decreased level of gastric acid in the stomach. Gastric acid is the main aggressive factor in the intragastric environment and a prerequisite for disorders like peptic ulcer disease, stress ulcer and ulcerative gastritis. On the other hand, the success of pharmacological treatment to prevent or heal ulcerative lesions may not depend only on the blockade of acid secretion, but also on the enhancement of mucosal protective factors. Antioxidant depletion is belived to be a mechanism involved in the pathophysiology of several upper gastrointestinal disorders, and H^+/K^+-ATPase inhibitors can alter free radical production by neutrophils. Esomeprazole has been shown to be protective against oxidative stress due to its antioxidant properties (36). It is therefore interesting to note that the upregulated genes paraoxonase 1 (*Pon1*), fatty acid binding protein 5 (*Fabp5*), myeloid cell leukemia 1 (*Mcl1*) and selenophosphate synthetase 1 (*Seph5*) all are belived to be involved in the protection agains oxidative stress (7, 29, 46, 66). Moreover, inhibition of the downregulated gene encoding calreticulin (*Calr*) has been shown to enhance cytoprotection against oxidative stress (28). These proteins may be involved in the molecular mechanisms underlying acid-independent gastroprotective effects of proton pump inhibitors.

Inflammatory and immune responses. Altered gene expression was observed for surprisingly many genes known to be involved in immune and inflammatory responses. Many of these were upregulated. Patients with reduced gastric acidity are more susceptible to bacterial infections with enteropathogenic bacteria. The high number of genes involved in these processes may be due to gene expression in

immune cells infiltrating into the gastric mucosa. Examples of such genes are formyl peptide receptor 1 (*Fpr1*) (12), Interleukin 17 receptor B (*Il17rb*) (67) and interferon-stimulated transcription factor 3 (*Isgf3g*) (48). Reducing the amount of acid with potent proton pump inhibitors may also reduce inflammatory responses in the gastric mucosa since gastric acid is the main aggressive factor in the intragastric environment. Downregulation of chemokine (C-C motif) ligand 2 (*Ccl2*), interleukin 4 receptor (*Il4r*), *Cd58* and matrix metalloproteinase 7 (*Mmp7*) which all are involved in immune and/or inflammatory responses, may be interpreted to indicate that these proteins may be involved in protecting the gastric mucosa against acid-induced damage (34, 60, 75, 81). The downregulated gene product of prostaglandin E receptor 3 (*Ptger3*) and ATP-binding cassette, sub-family B (MDR/TAP), member 1 (*Abcb1*) are shown to play a central role in adaptive cytoprotection and host- bacterial interactions, respectively, in the gastrointestinal tract (25, 73). Gastric parietal cells are known to express *Ptger3* mRNA (73).

The gastric mucosa is very complex, with intermingling cells of different types with various roles in the acid secretory process. Parietal cells comprise about 32% of the corpus mucosa, whereas chief cells make up about 26%. The remainder of the gastric mucosal cells comprise about 17% surface mucous cells, 6% mucosal neck cells, and 20% lamina propria cells (27). Endocrine cells constitute about 2% of the cells in the gastric corpus region and mainly belong to one of the following types: ECL cells (35%), EC cells (25%) and somatostatin (D) cells (26%). The remaining 14% consists of A-like cells, D_1 cells and P cells (68). The present experiment gives a picture of the gene expression changes in the total cell population of the gastric mucosa, and must be followed by further analysis using other methods, like single-cell studies,

immunohistochemistry or in situ hybridization, to identify the cell types in which genes of interest are expressed. Thus, some of the differentially expressed genes, chosen to cover several biological functions, were studied further by immunohistochemistry. Plasminogen activator urokinase (*Plau*) encodes a serine protease involved in degradation of the extracellular matrix and possibly tumor cell migration and proliferation (15). This protein converts plasminogen to plasmin and is most likely localized to parietal cells. Localization of *NF-κB* to the nucleus of H^+/K^+*ATPase* immunoreactive cells indicates that *NF-κB* is located to parietal cells. This anti-apoptotic transcription factor has been shown to be activated by the upregulated transcription factor Act1 (*C6orf4*) (82). *NF-κB* is known to be expressed in parietal cells (49). Since the ECL cell is an undisputed target of gastrin, staining for ECL cell *Hdc* was done and as shown in the figure neither *Plau* nor *NFκB* is expressed in this cell type.

Inhibitors of gastric acid secretion are among the most commonly used drugs in clinical practice. In the present study, a comprehensive set of candidate genes not previously associated with acid inhibition was revealed, which underlines the complex nature of this therapeutic intervention. Our results indicate a global change in the induction of proliferation, apoptosis, inflammatory, immune and stress response in the presence of proton pump inhibitors. The genes presented in the present study are likely to be associated with acid inhibition. Further studies specifically targeting the genes implicated in this study will further our understanding of the molecular responses to potent acid inhibition, including the mucosal response to moderately increased gastrin levels encountered in clinical practice.

Text footnotes

[1] The Supplemental Table S1 is available online at

http://physiolgenomics.physiology.org

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Tables

Table 1 Differentially expressed genes in esomeprazole-treated patients involved in proliferation and apoptosis, stress response, and immune and inflammatory responses.

CloneNo	*Unigene 186 Cluster*	*Name*	*Symbol*	*Proliferation / apoptosis*	*Stress response*	*Inflammatory / immune response*	*Mean ratio*	*SD*
Upregulated								
727390	Hs.3260	Presenilin 1 (Alzheimer disease 3)	*Psen1*	x	x	x	1,5	0,4
714106	Hs.77274	Plasminogen activator, urokinase	*Plau*	x	x	x	1,7	0,6
81331	Hs.408061	Fatty acid binding protein 5 (psoriasis-associated)	*Fabp5*	x	x	x	1,9	0,6
843287	Hs.532826	Myeloid cell leukemia sequence 1 (BCL2-related)	*Mcl1*	x	x	x	1,6	0,4
207370	Hs.80720	GRB2-associated binding protein 1	*Gab1*	x	x	x	1,2	0,1
136821	Hs.1706	Interferon-stimulated transcription factor 3, gamma 48kDa	*Isgf3g*	x	x	x	1,5	0,3
144924	Hs.486228	TRAF3 interacting protein 2	*C6orf4*	x	x	x	1,6	0,4

739993	Hs.258314	Brain and reproductive organ-expressed (TNFRSF1A modulator)	*Bre*	x	x	x	1,3	0,2
24415	Hs.408312	Tumor protein p53 (Li-Fraumeni syndrome)	*Tp53*	x	x	x	1,6	0,3
840702	Hs.124027	Selenophosphate synthetase 1	*Sephs1*	x	x	x	1,6	0,4
898281	Hs.195464	Filamin A, alpha (actin-binding protein-280)	*Flna*	x	x		1,7	0,5
124795	Hs.461453	WW domain containing oxidoreductase	*Wwox*	x	x		1,9	0,5
782587	Hs.75275	Ubiquitination factor E4A (UFD2 homolog, yeast)	*Ubea4*	x	x		1,7	0,6
753620	Hs.274313	Insulin-like growth factor binding protein 6	*Igfbp6*	x		x	1,5	0,3
24085	Hs.432424	Tripeptidyl peptidase II	*Tpp2*	x		x	1,9	0,6
144802	Hs.49787	Latent transforming growth factor beta binding protein 1	*Ltbp1*	x		x	1,9	0,5
810372	Hs.32942	Phosphoinositide-3-kinase, catalytic, gamma polypeptide	*Pik3cg*	x		x	2,2	1,0
788566	Hs.80296	Purkinje cell protein 4	*Pcp4*	x			1,6	0,5
34773	Hs.506852	Protein tyrosine phosphatase, non-receptor type 11	*Ptpn11*	x			1,5	0,4
809557	Hs.179565	Minichromosome maintenance deficient (S. cerevisiae) 3	*Mcm3*	x			1,6	0,2
840658	Hs.515540	Prostate tumor over expressed gene 1	*Ptov1*	x			1,2	0,2
681948	Hs.196981	Nasopharyngeal carcinoma, down-regulated 1	*Fhit*	x			1,5	0,4
796542	Hs.43697	Ets variant gene 5 (ets-related molecule)	*Etv5*	x			1,7	0,5
40017§	Hs.437060	Cytochrome c, somatic	*Cycs*	x			2,1	0,6
235924	Hs.233552	Cell division cycle 2-like 5 (cholinesterase-related cell division	*Cdc2l5*	x			1,3	0,2

		controller)						
75254	Hs.530904	Cysteine and glycine-rich protein 2	*Csrp2*	x			1,2	0,2
296094	Hs.50308	Huntingtin interacting protein 2	*Hip2*	x			1,3	0,1
293328	Hs.432438	Echinoderm microtubule associated protein like 4	*Eml4*	x			1,2	0,1
838676	Hs.496593	Calpain 6	*Capn6*	x			1,8	0,6
128143	Hs.370995	Paraoxonase 1	*Pon1*		x	x	1,1	0,1
773236	Hs.753	Formyl peptide receptor 1	*Fpr1*		x	x	1,4	0,3
233783	Hs.136338	Interleukin 17 receptor B	*Il17rb*			x	1,2	0,1
Downregulated								
504763	Hs.252189	Syndecan 4 (amphiglycan, ryudocan)	*Sdc4*	x	x	x	0,6	0,2
668851	Hs.502182	Brain-derived neurotrophic factor	*Bdnf*	x	x	x	0,6	0,2
753211	Hs.445000	Prostaglandin E receptor 3 (subtype EP3)	*Ptger3*	x	x	x	0,5	0,1
504236	Hs.1905	Prolactin	*Prl*	x	x	x	0,7	0,1
167280	Hs.496255	Protein kinase C, zeta	*Prkcz*	x	x	x	0,8	0,2
363590	Hs.459070	Aryl-hydrocarbon receptor nuclear translocator 2	*Arnt2*	x	x		0,8	0,2
768370	Hs.502876	Ras homolog gene family, member B	*Rhob*	x	x		0,6	0,1
210887	Hs.546303	Suppression of tumorigenicity 13 (Hsp70-interacting protein)	*St13*	x	x		0,6	0,1

303048	Hs.408073	Ribosomal protein S6	*Rps6*	x	x		0,8	0,0
166195	Hs.530687	Ribonuclease/angiogenin inhibitor 1	*Rnh*	x	x		0,8	0,1
550355	Hs.440833	Protein kinase N2	*Pkn2*	x	x		0,8	0,1
79502	Hs.516157	Methionine adenosyltransferase II, alpha	*Mat2a*	x	x		0,8	0,1
714453	Hs.513457	Interleukin 4 receptor	*Il4r*	x		x	0,8	0,1
813552	Hs.446414	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	*Cd47*	x		x	0,8	0,2
188232	Hs.376206	Kruppel-like factor 4 (gut)	*Klf4*	x		x	0,7	0,2
470393	Hs.2256	Matrix metalloproteinase 7 (matrilysin, uterine)	*Mmp7*	x		x	0,8	0,1
136382	Hs.432993	Cylindromatosis (turban tumor syndrome)	*Cyld*	x		x	0,8	0,1
810131	Hs.514167	Keratin 19	*Krt19*	x		x	0,6	0,3
768561	Hs.303649	Chemokine (C-C motif) ligand 2	*Ccl2*	x		x	0,6	0,2
308588	Hs.173464	FK506-binding protein 8 (38kD)	*Fkbp8*	x			0,8	0,1
740554	Hs.263671	Radixin	*Rdx*	x			0,6	0,1
83120	Hs.401509	RNA binding motif protein 10	*Rbm10*	x			0,8	0,1
591653	Hs.165830	Ecotropic viral integration site 1	*Evi1*	x			0,7	0,2
138917	Hs.437922	V-myc myelocytomatosis viral oncogene homolog 1	*Mycl1*	x			0,7	0,1
898123	Hs.473648	Phosphoribosylglycinamide formyltransferase	*Gart*	x			0,6	0,2
261204	Hs.505924	High mobility group AT-hook 2	*Hmga2*	x			0,7	0,2

208940	Hs.284414	Hydroxysteroid (17-beta) dehydrogenase 13	*Scdr9*	x			0,8	0,1
770794	Hs.521482	Src homology 2 domain containing adaptor protein B	*Shb*	x			0,7	0,2
786048	Hs.108371	E2F transcription factor 4, p107/p130-binding	*E2f4*	x			0,9	0,1
842980	Hs.115242	Developmentally regulated GTP-binding protein 1	*Drg1*	x			0,9	0,1
280752	Hs.79362	Retinoblastoma-like 2 (p130)	*Rbl2*	x			0,6	0,1
548957	Hs.278589	General transcription factor II, i	*Gtf2i*		x		0,7	0,0
26021	Hs.463928	Discs, large (Drosophila) homolog 4	*Dlg4*		x		0,7	0,2
815781	Hs.36927	Heat shock 105kDa/110kDa protein 1	*Hsph1*		x		0,8	0,1
774036	Hs.190028	Glutathione S-transferase omega 1	*Gsto1*		x		0,8	0,1
752732	Hs.371914	Bleomycin hydrolase	*Blmh*		x		0,5	0,2
235155	Hs.433300	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	*Fcer1g*			x	0,9	0,0
813256	Hs.489033	ATP-binding cassette, sub-family B (MDR/TAP), member 1	*Abcb1*			x	0,6	0,2
897906	Hs.207459	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	*Siat1*			x	0,7	0,2
490368	Hs.34341	CD58 antigen, (lymphocyte function-associated antigen 3)	*Cd58*			x	0,7	0,1

Genes that are likely to be involved in proliferation and apoptosis, stress response, and immune and inflammatory responses are listed. A full account of all genes differentially regulated in esomeprazole-treated patients is given in Supplemental Table S1. Unigene Build 186 was used.

Table 2 Differentially expressed genes in esomeprazole-treated patients that are found to have rat orthologs previously shown to be similarily regulated in omeprazole-dosed rats.

CloneNo	***Unigene 186 Cluster***	***Gene name***	***Symbol***
Upregulated			
714106	Hs.77274	Plasminogen activator, urokinase	*Plau*
843287	Hs.532826	Myeloid cell leukemia sequence 1 (BCL2-related)	*Mcl1*
Downregulated			
504763	Hs.252189	Syndecan 4 (amphiglycan, ryudocan)	*Sdc4*
167280	Hs.496255	Protein kinase C, zeta	*Prkcz*
768370	Hs.502876	Ras homolog gene family, member B	*Rhob*
188232	Hs.376206	Kruppel-like factor 4 (gut)	*Klf4*
548957	Hs.278589	General transcription factor II, i	*Gtf2i*
752732	Hs.371914	Bleomycin hydrolase	*Blmh*

Paper V

Supplemental Table S1. A full list of differentially regulated genes in esomeprazole-treated patients.

CloneID	*Unigene 186 Cluster*	*Name*	*Symbol*	*P value*	*Mean Ratio*	*SD*
Upregulated genes, P<0.01						
81599*	Hs.464416	ubiquitin specific protease 14 (tRNA-guanine transglycosylase)	*Usp14*	0,001	1,2	0,1
207370	Hs.80720	GRB2-associated binding protein 1	*Gab1*	0,001	1,2	0,1
725223	Hs.413801	Proteasome (prosome, macropain) activator subunit 4	*Psme4*	0,001	1,4	0,1
124795	Hs.461453	WW domain containing oxidoreductase	*Wwox*	0,001	1,9	0,5
843287*	Hs.532826	myeloid cell leukemia sequence 1 (BCL2-related)	*Mcl1*	0,001	1,6	0,4
809557	Hs.179565	minichromosome maintenance deficient (S. cerevisiae) 3	*Mcm3*	0,001	1,6	0,2
296094	Hs.50308	Huntingtin interacting protein 2	*Hip2*	0,001	1,3	0,1
235924	Hs.233552	Cell division cycle 2-like 5 (cholinesterase-related cell division controller)	*Cdc2l5*	0,002	1,3	0,2
783849	Hs.86724	GTP cyclohydrolase 1 (dopa-responsive dystonia)	*Gch1*	0,002	1,1	0,1
840702§	Hs.124027	Selenophosphate synthetase 1	*Sephs1*	0,002	1,6	0,4
840702§	Hs.528583	Similar to selenophosphate synthetase		0,002	1,6	0,4
681948	Hs.196981	Nasopharyngeal carcinoma, down-regulated 1	*Fhit*	0,003	1,5	0,4
81331§	Hs.530733	Syntaxin 3A	*Stx3a*	0,003	1,9	0,6
81331§	Hs.408061	Fatty acid binding protein 5 (psoriasis-associated)	*Fabp5*	0,003	1,9	0,6
136821†	Hs.1706	Interferon-stimulated transcription factor 3, gamma 48kDa	*Isgf3g*	0,003	1,5	0,3
753620*	Hs.274313	insulin-like growth factor binding protein 6	*Igfbp6*	0,003	1,5	0,3
810372	Hs.32942	phosphoinositide-3-kinase, catalytic, gamma polypeptide	*Pik3cg*	0,003	2,2	1,0
138745	Hs.28426	Transcribed locus		0,003	1,1	0,0
789011	Hs.83347	angio-associated, migratory cell protein	*Aamp*	0,003	1,5	0,4
45556	Hs.35828	MAP/microtubule affinity-regulating kinase 3	*Mark3*	0,004	1,8	0,4
544664	Hs.268939	matrin 3	*Matr3*	0,005	1,4	0,3
307882	Hs.291212	tubulin-specific chaperone a	*Tbca*	0,005	1,5	0,4
773236*	Hs.753	formyl peptide receptor 1	*Fpr1*	0,005	1,4	0,3
714106	Hs.77274	plasminogen activator, urokinase	*Plau*	0,005	1,7	0,6
144924	Hs.486228	TRAF3 interacting protein 2	*C6orf4*	0,005	1,6	0,4
70489	Hs.120950	Rhesus blood group-associated glycoprotein	*Rhag*	0,006	1,6	0,5
739993*	Hs.258314	brain and reproductive organ-expressed (TNFRSF1A modulator)	*Bre*	0,006	1,3	0,2
34773	Hs.506852	protein tyrosine phosphatase, non-receptor type 11	*Ptpn11*	0,006	1,5	0,4
727390*	Hs.3260	presenilin 1 (Alzheimer disease 3)	*Psen1*	0,006	1,5	0,4
133180	Hs.250648	Hypothetical LOC389188		0,006	1,4	0,3
128695	Hs.502100	Hypothetical protein FLJ90119	*Flj90119*	0,006	1,5	0,3
898281	Hs.195464	filamin A, alpha (actin-binding protein-280)	*Flna*	0,006	1,7	0,5
725274	Hs.519718	tetratricopeptide repeat domain 1	*Ttc1*	0,007	1,5	0,4
843312	Hs.528305	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 17 (72kD)	*Ddx17*	0,007	1,5	0,5
796253	Hs.533383	polymerase (RNA) II (DNA directed) polypeptide J (13.3kD)	*Polr2j*	0,007	1,4	0,3
137736	Hs.176854	Hypothetical LOC401320	*Loc401320*	0,007	1,3	0,3
343352	Hs.507805	Component of oligomeric golgi complex 6	*Cog6*	0,007	1,3	0,1
67033	Hs.170205	Transcribed locus, mod. sim. to NP_001432.1 fatty acid amide hydrolase		0,007	1,2	0,1
838676	Hs.496593	calpain 6	*Capn6*	0,007	1,8	0,6
79688	Hs.515472	Small nuclear ribonucleoprotein D2 polypeptide 16.5kDa	*Snrpd2*	0,008	1,6	0,5
824068	Hs.401903	cytochrome c oxidase subunit Va	*Cox5a*	0,008	1,4	0,3
788566*	Hs.80296	Purkinje cell protein 4	*Pcp4*	0,008	1,6	0,5
144802	Hs.49787	Latent transforming growth factor beta binding protein 1	*Ltbp1*	0,008	1,9	0,5
233783	Hs.136338	Interleukin 17 receptor B	*Il17rb*	0,008	1,2	0,1
796542	Hs.43697	ets variant gene 5 (ets-related molecule)	*Etv5*	0,008	1,7	0,5
293328*	Hs.432438	Echinoderm microtubule associated protein like 4	*Eml4*	0,008	1,2	0,1
151251	Hs.239459	regulatory solute carrier protein, family 1, member 1	*Rsc1a1*	0,008	1,5	0,4
544639	Hs.95612	Desmocollin 2	*Dsc2*	0,008	1,4	0,4
128143*	Hs.370995	paraoxonase 1	*Pon1*	0,008	1,1	0,1
24085	Hs.432424	tripeptidyl peptidase II	*Tpp2*	0,008	1,9	0,6
24415*	Hs.408312	tumor protein p53 (Li-Fraumeni syndrome)	*Tp53*	0,009	1,6	0,3
840658	Hs.515540	prostate tumor over expressed gene 1	*Ptov1*	0,009	1,2	0,2
75254	Hs.530904	cysteine and glycine-rich protein 2	*Csrp2*	0,009	1,2	0,2
240637	Hs.40094	Leucine rich repeat containing 42	*Mgc8974*	0,009	1,4	0,4
248688	Hs.530473	Transcribed locus		0,009	1,5	0,3
40017§	Hs.437060	Cytochrome c, somatic	*Cycs*	0,010	2,1	0,6
40017§	Hs.511873	Transcribed locus, strongly similar to NP_061820.1 cytochrome c		0,010	2,1	0,6
782587	Hs.75275	Ubiquitination factor E4A (UFD2 homolog, yeast)	*Ube4a*	0,010	1,7	0,6
814696	Hs.482497	Transportin 1	*Tnpo1*	0,010	1,4	0,4
Downregulated genes, P<0.01						
303048	Hs.408073	ribosomal protein S6	*Rps6*	<0,001	0,8	0,0
210887	Hs.546303	suppression of tumorigenicity 13 (Hsp70-interacting protein)	*St13*	<0,001	0,6	0,1
753211*	Hs.445000	prostaglandin E receptor 3 (subtype EP3)	*Ptger3*	<0,001	0,5	0,1
39127†	Hs.530199	Ankyrin repeat domain 46	*Loc157567*	<0,001	0,7	0,1
813742§	Hs.90572	PTK7 protein tyrosine kinase 7	*Ptk7*	<0,001	0,6	0,1
813742§	Hs.460468	Exportin 6	*Xpo6*	<0,001	0,6	0,1
470368	Hs.517731	hypothetical protein PP2447	*Pp2447*	<0,001	0,6	0,1
222181	Hs.506759	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	*Atp2a2*	<0,001	0,8	0,1

768370*§	Hs.502876	ras homolog gene family, member B	*Rhob*	<0,001	0,6	0,1
753430	Hs.533526	alpha thalassemia/mental retardation syndrome X-linked	*Atrx*	<0,001	0,7	0,1
752732*	Hs.371914	bleomycin hydrolase	*Blmh*	<0,001	0,5	0,2
280752*§	Hs.79362	retinoblastoma-like 2 (p130)	*Rbl2*	<0,001	0,6	0,1
80946	Hs.12013	ATP-binding cassette, sub-family E (OABP), member 1	*Abce1*	0,001	0,8	0,1
381166	Hs.35758	MYST histone acetyltransferase (monocytic leukemia) 4	*Myst4*	0,001	0,8	0,1
469952	Hs.508266	COMM domain containing 6	*Commd6*	0,001	0,7	0,1
666829	Hs.387207	sarcoglycan, delta (35kD dystrophin-associated glycoprotein)	*Sgcd*	0,001	0,5	0,2
293727	Hs.208912	Chromosome 22 open reading frame 18	*C22orf18*	0,001	0,7	0,1
282501	Hs.437174	solute carrier family 6 (neurotransmitter transporter), member 12	*Slc6a12*	0,001	0,8	0,1
823930	Hs.124126	actin related protein 2/3 complex, subunit 1A (41 kD)	*Arpc1a*	0,001	0,8	0,1
154138	Hs.443490	membrane-bound transcription factor protease, site 2	*Mbtps2*	0,001	0,7	0,1
234331	Hs.458499	Ubiquitin specific protease 3	*Usp3*	0,001	0,8	0,1
166195	Hs.530687	Ribonuclease/angiogenin inhibitor 1	*Rnh*	0,001	0,8	0,1
668851	Hs.502182	brain-derived neurotrophic factor	*Bdnf*	0,001	0,6	0,2
202535§	Hs.433391	metallothionein 1G	*Mt1g*	0,001	0,5	0,2
202535§	Hs.556040	H19, imprinted maternally expressed untranslated mRNA	*H19*	0,001	0,5	0,2
79502§	Hs.516157	methionine adenosyltransferase II, alpha	*Mat2A*	0,001	0,8	0,1
79502§	Hs.506309	Early endosome antigen 1, 162kD	*Eea1*	0,001	0,8	0,1
753215	Hs.134587	guanine nucleotide binding protein (G protein)	*Gnai1*	0,002	0,5	0,2
591653†	Hs.165830	Ecotropic viral integration site 1	*Evi1*	0,002	0,7	0,2
490368	Hs.34341	CD58 antigen, (lymphocyte function-associated antigen 3)	*Cd58*	0,002	0,7	0,1
261204	Hs.505924	High mobility group AT-hook 2	*Hmga2*	0,002	0,7	0,2
740554	Hs.263671	radixin	*Rdx*	0,002	0,6	0,1
504236	Hs.1905	prolactin	*Prl*	0,002	0,7	0,1
343443	Hs.334587	RNA-binding protein gene with multiple splicing	*Rbpms*	0,002	0,6	0,1
470393	Hs.2256	matrix metalloproteinase 7 (matrilysin, uterine)	*Mmp7*	0,002	0,8	0,1
768357	Hs.458423	cerebellin 1 precursor	*Cbln1*	0,002	0,6	0,2
548957*§	Hs.278589	general transcription factor II, i	*Gtf2i*	0,003	0,7	0,0
188232*	Hs.376206	Kruppel-like factor 4 (gut)	*Klf4*	0,003	0,7	0,2
281904	Hs.529925	Ubiquitin protein ligase E3 component n-recognin 2	*Ubr2*	0,003	0,7	0,1
550355	Hs.440833	Protein kinase N2	*Pkn2*	0,003	0,8	0,1
245484§	Hs.160561	Similar to KIF27C	*Loc389765*	0,003	0,9	0,1
245484§	Hs.545918	Transcribed locus		0,003	0,9	0,1
49344	Hs.35490	KIAA0350 protein	*Kiaa0350*	0,003	0,7	0,2
138917	Hs.437922	V-myc myelocytomatosis viral oncogene homolog 1	*Mycl1*	0,003	0,7	0,1
46897	Hs.30954	phosphomevalonate kinase	*Pmvk*	0,003	0,6	0,1
207255	Hs.361323	ATP-binding cassette, sub-family F (GCN20), member 3	*Abcf3*	0,003	0,6	0,2
714426	Hs.132370	cleavage stimulation factor, 3' pre-RNA, subunit 2, 64kD	*Cstf2*	0,003	0,8	0,1
714453	Hs.513457	interleukin 4 receptor	*Il4r*	0,004	0,8	0,1
592359	Hs.278721	Solute carrier family 39 (zinc transporter), member 7	*Slc39a7*	0,004	0,7	0,2
768561*	Hs.303649	Chemokine (C-C motif) ligand 2	*Ccl2*	0,004	0,6	0,2
840493	Hs.78224	ribonuclease, RNase A family, 1 (pancreatic)	*Rnase1*	0,004	0,7	0,2
156045	Hs.200600	Secretory carrier membrane protein 3	*Scamp3*	0,004	0,8	0,2
485858	Hs.391781	protocadherin 20	*Pcdh20*	0,004	0,8	0,1
824704	Hs.75694	mannose phosphate isomerase	*Mpi*	0,004	0,9	0,1
48614*	Hs.271940	E74-like factor 4 (ets domain transcription factor)	*Elf4*	0,004	0,6	0,2
296529	Hs.160976	SA hypertension-associated homolog (rat)	*Sah*	0,004	0,8	0,1
668182	Hs.100921	zinc finger protein 193	*Znf193*	0,005	0,8	0,1
136382	Hs.432993	Cylindromatosis (turban tumor syndrome)	*Cyld*	0,005	0,8	0,1
813256	Hs.489033	ATP-binding cassette, sub-family B (MDR/TAP), member 1	*Abcb1*	0,005	0,6	0,2
770794	Hs.521482	Src homology 2 domain containing adaptor protein B	*Shb*	0,005	0,7	0,2
26021	Hs.463928	discs, large (Drosophila) homolog 4	*Dlg4*	0,005	0,7	0,2
815781	Hs.36927	Heat shock 105kDa/110kDa protein 1	*Hsph1*	0,005	0,8	0,1
767069	Hs.368592	Chromosome 11 open reading frame 32	*Sorl1*	0,005	0,6	0,1
842980	Hs.115242	developmentally regulated GTP-binding protein 1	*Drg1*	0,006	0,9	0,1
42558	Hs.75335	glycine amidinotransferase (L-arginine:glycine amidinotransferase)	*Gatm*	0,006	0,7	0,2
470216	Hs.149387	myosin VI	*Myo6*	0,006	0,7	0,2
167280§	Hs.496255	protein kinase C, zeta	*Prkcz*	0,006	0,8	0,2
167280§	Hs.107101	Chromosome 1 open reading frame 86	*C1orf86*	0,006	0,8	0,2
363590	Hs.459070	aryl-hydrocarbon receptor nuclear translocator 2	*Arnt2*	0,006	0,8	0,2
32684	Hs.488171	NudC domain containing 3	*Kiaa1068*	0,006	0,8	0,1
28098	Hs.270869	Zinc finger protein 410	*Znf410*	0,006	0,8	0,1
262231	Hs.515162	Calreticulin	*Calr*	0,006	0,8	0,2
199610	Hs.516243	Thrombospondin repeat containing 1	*Tsrc1*	0,006	0,8	0,1
504763*	Hs.252189	syndecan 4 (amphiglycan, ryudocan)	*Sdc4*	0,006	0,6	0,2
328591	Hs.73923	pancreatic lipase-related protein 1	*Pnliprp1*	0,006	0,7	0,2
33941	Hs.89655	protein tyrosine phosphatase, receptor type, N	*Ptprn*	0,006	0,8	0,1
813552	Hs.446414	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	*Cd47*	0,006	0,8	0,2
46518	Hs.58919	dystrobrevin, alpha	*Dtna*	0,007	0,8	0,2
83120	Hs.401509	RNA binding motif protein 10	*Rbm10*	0,007	0,8	0,1
305606	Hs.89839	EphA1	*Epha1*	0,007	0,9	0,1
193481	Hs.490394	Single-stranded DNA binding protein 1	*Ssbp1*	0,007	0,8	0,1
897906	Hs.207459	ST6 beta-galactosamide alpha-2,6-sialyltranferase 1	*Siat1*	0,007	0,7	0,2
235155*	Hs.433300	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	*Fcer1g*	0,007	0,9	0,0
308588	Hs.173464	FK506-binding protein 8 (38kD)	*Fkbp8*	0,007	0,8	0,1
753313	Hs.371021	Lysosomal-associated multispanning membrane protein-5	*Laptm5*	0,007	0,9	0,1
814246	Hs.531089	proteasome (prosome, macropain) subunit, alpha type, 3	*Psma3*	0,007	0,8	0,1
774036*	Hs.190028	Glutathione S-transferase omega 1	*Gsto1*	0,008	0,8	0,1

208940	Hs.284414	Hydroxysteroid (17-beta) dehydrogenase 13	*Scdr9*	0,008	0,8	0,1
767769	Hs.334848	hypothetical protein FLJ13291	*Flj13291*	0,008	0,8	0,1
251135	Hs.523145	dolichyl-diphosphooligosaccharide-protein glycosyltransferase	*Ddost*	0,008	0,8	0,1
786048	Hs.108371	E2F transcription factor 4, p107/p130-binding	*E2f4*	0,008	0,9	0,1
127821	Hs.1211	acid phosphatase 5, tartrate resistant	*Acp5*	0,008	0,7	0,2
470846	Hs.528834	cleavage and polyadenylation specific factor 5, 25 kD subunit	*Cpsf5*	0,009	0,7	0,2
810131	Hs.514167	keratin 19	*Krt19*	0,009	0,6	0,3
502067	Hs.517761	Lupus brain antigen 1	*Lba1*	0,009	0,6	0,2
26711	Hs.240770	nuclear cap binding protein subunit 2, 20kD	*Ncbp2*	0,009	0,8	0,1
898123	Hs.473648	phosphoribosylglycinamide formyltransferase	*Gart*	0,009	0,6	0,2
898138	Hs.385986	ubiquitin-conjugating enzyme E2B (RAD6 homolog)	*Ube2b*	0,009	0,8	0,2

* Sequenced clone
§ Gene probe accociated with multiple clusters
† Gene information changed due to wrong sequence

The table lists all genes differentially regulated in esomeprazole-treated patients. Unigene build 186 was used.

Dissertations at the Faculty of Medicine, NTNU

1977

1. Knut Joachim Berg: EFFECT OF ACETYLSALICYLIC ACID ON RENAL FUNCTION
2. Karl Erik Viken and Arne Ødegaard: STUDIES ON HUMAN MONOCYTES CULTURED *IN VITRO*

1978

3. Karel Bjørn Cyvin: CONGENITAL DISLOCATION OF THE HIP JOINT.
4. Alf O. Brubakk: METHODS FOR STUDYING FLOW DYNAMICS IN THE LEFT VENTRICLE AND THE AORTA IN MAN.

1979

5. Geirmund Unsgaard: CYTOSTATIC AND IMMUNOREGULATORY ABILITIES OF HUMAN BLOOD MONOCYTES CULTURED IN VITRO

1980

6. Størker Jørstad: URAEMIC TOXINS
7. Arne Olav Jenssen: SOME RHEOLOGICAL, CHEMICAL AND STRUCTURAL PROPERTIES OF MUCOID SPUTUM FROM PATIENTS WITH CHRONIC OBSTRUCTIVE BRONCHITIS

1981

8. Jens Hammerstrøm: CYTOSTATIC AND CYTOLYTIC ACTIVITY OF HUMAN MONOCYTES AND EFFUSION MACROPHAGES AGAINST TUMOR CELLS *IN VITRO*

1983

9. Tore Syversen: EFFECTS OF METHYLMERCURY ON RAT BRAIN PROTEIN.
10. Torbjørn Iversen: SQUAMOUS CELL CARCINOMA OF THE VULVA.

1984

11. Tor-Erik Widerøe: ASPECTS OF CONTINUOUS AMBULATORY PERITONEAL DIALYSIS.
12. Anton Hole: ALTERATIONS OF MONOCYTE AND LYMPHOCYTE FUNCTIONS IN REALTION TO SURGERY UNDER EPIDURAL OR GENERAL ANAESTHESIA.
13. Terje Terjesen: FRACTURE HEALING AN STRESS-PROTECTION AFTER METAL PLATE FIXATION AND EXTERNAL FIXATION.
14. Carsten Saunte: CLUSTER HEADACHE SYNDROME.
15. Inggard Lereim: TRAFFIC ACCIDENTS AND THEIR CONSEQUENCES.
16. Bjørn Magne Eggen: STUDIES IN CYTOTOXICITY IN HUMAN ADHERENT MONONUCLEAR BLOOD CELLS.
17. Trond Haug: FACTORS REGULATING BEHAVIORAL EFFECTS OG DRUGS.

1985

18. Sven Erik Gisvold: RESUSCITATION AFTER COMPLETE GLOBAL BRAIN ISCHEMIA.
19. Terje Espevik: THE CYTOSKELETON OF HUMAN MONOCYTES.
20. Lars Bevanger: STUDIES OF THE Ibc (c) PROTEIN ANTIGENS OF GROUP B STREPTOCOCCI.
21. Ole-Jan Iversen: RETROVIRUS-LIKE PARTICLES IN THE PATHOGENESIS OF PSORIASIS.
22. Lasse Eriksen: EVALUATION AND TREATMENT OF ALCOHOL DEPENDENT BEHAVIOUR.
23. Per I. Lundmo: ANDROGEN METABOLISM IN THE PROSTATE.

1986

24. Dagfinn Berntzen: ANALYSIS AND MANAGEMENT OF EXPERIMENTAL AND CLINICAL PAIN.
25. Odd Arnold Kildahl-Andersen: PRODUCTION AND CHARACTERIZATION OF MONOCYTE-DERIVED CYTOTOXIN AND ITS ROLE IN MONOCYTE-MEDIATED CYTOTOXICITY.
26. Ola Dale: VOLATILE ANAESTHETICS.

1987

27. Per Martin Kleveland: STUDIES ON GASTRIN.
28. Audun N. Øksendal: THE CALCIUM PARADOX AND THE HEART.
29. Vilhjalmur R. Finsen: HIP FRACTURES

1988

30. Rigmor Austgulen: TUMOR NECROSIS FACTOR: A MONOCYTE-DERIVED REGULATOR OF CELLULAR GROWTH.
31. Tom-Harald Edna: HEAD INJURIES ADMITTED TO HOSPITAL.
32. Joseph D. Borsi: NEW ASPECTS OF THE CLINICAL PHARMACOKINETICS OF METHOTREXATE.

33. Olav F. M. Sellevold: GLUCOCORTICOIDS IN MYOCARDIAL PROTECTION.
34. Terje Skjærpe: NONINVASIVE QUANTITATION OF GLOBAL PARAMETERS ON LEFT VENTRICULAR FUNCTION: THE SYSTOLIC PULMONARY ARTERY PRESSURE AND CARDIAC OUTPUT.
35. Eyvind Rødahl: STUDIES OF IMMUNE COMPLEXES AND RETROVIRUS-LIKE ANTIGENS IN PATIENTS WITH ANKYLOSING SPONDYLITIS.
36. Ketil Thorstensen: STUDIES ON THE MECHANISMS OF CELLULAR UPTAKE OF IRON FROM TRANSFERRIN.
37. Anna Midelfart: STUDIES OF THE MECHANISMS OF ION AND FLUID TRANSPORT IN THE BOVINE CORNEA.
38. Eirik Helseth: GROWTH AND PLASMINOGEN ACTIVATOR ACTIVITY OF HUMAN GLIOMAS AND BRAIN METASTASES - WITH SPECIAL REFERENCE TO TRANSFORMING GROWTH FACTOR BETA AND THE EPIDERMAL GROWTH FACTOR RECEPTOR.
39. Petter C. Borchgrevink: MAGNESIUM AND THE ISCHEMIC HEART.
40. Kjell-Arne Rein: THE EFFECT OF EXTRACORPOREAL CIRCULATION ON SUBCUTANEOUS TRANSCAPILLARY FLUID BALANCE.
41. Arne Kristian Sandvik: RAT GASTRIC HISTAMINE.
42. Carl Bredo Dahl: ANIMAL MODELS IN PSYCHIATRY.

1989

43. Torbjørn A. Fredriksen: CERVICOGENIC HEADACHE.
44. Rolf A. Walstad: CEFTAZIDIME.
45. Rolf Salvesen: THE PUPIL IN CLUSTER HEADACHE.
46. Nils Petter Jørgensen: DRUG EXPOSURE IN EARLY PREGNANCY.
47. Johan C. Ræder: PREMEDICATION AND GENERAL ANAESTHESIA IN OUTPATIENT GYNECOLOGICAL SURGERY.
48. M. R. Shalaby: IMMUNOREGULATORY PROPERTIES OF TNF-α AND THE RELATED CYTOKINES.
49. Anders Waage: THE COMPLEX PATTERN OF CYTOKINES IN SEPTIC SHOCK.
50. Bjarne Christian Eriksen: ELECTROSTIMULATION OF THE PELVIC FLOOR IN FEMALE URINARY INCONTINENCE.
51. Tore B. Halvorsen: PROGNOSTIC FACTORS IN COLORECTAL CANCER.

1990

52. Asbjørn Nordby: CELLULAR TOXICITY OF ROENTGEN CONTRAST MEDIA.
53. Kåre E. Tvedt: X-RAY MICROANALYSIS OF BIOLOGICAL MATERIAL.
54. Tore C. Stiles: COGNITIVE VULNERABILITY FACTORS IN THE DEVELOPMENT AND MAINTENANCE OF DEPRESSION.
55. Eva Hofsli: TUMOR NECROSIS FACTOR AND MULTIDRUG RESISTANCE.
56. Helge S. Haarstad: TROPHIC EFFECTS OF CHOLECYSTOKININ AND SECRETIN ON THE RAT PANCREAS.
57. Lars Engebretsen: TREATMENT OF ACUTE ANTERIOR CRUCIATE LIGAMENT INJURIES.
58. Tarjei Rygnestad: DELIBERATE SELF-POISONING IN TRONDHEIM.
59. Arne Z. Henriksen: STUDIES ON CONSERVED ANTIGENIC DOMAINS ON MAJOR OUTER MEMBRANE PROTEINS FROM ENTEROBACTERIA.
60. Steinar Westin: UNEMPLOYMENT AND HEALTH: Medical and social consequences of a factory closure in a ten-year controlled follow-up study.
61. Ylva Sahlin: INJURY REGISTRATION, a tool for accident preventive work.
62. Helge Bjørnstad Pettersen: BIOSYNTHESIS OF COMPLEMENT BY HUMAN ALVEOLAR MACROPHAGES WITH SPECIAL REFERENCE TO SARCOIDOSIS.
63. Berit Schei: TRAPPED IN PAINFUL LOVE.
64. Lars J. Vatten: PROSPECTIVE STUDIES OF THE RISK OF BREAST CANCER IN A COHORT OF NORWEGIAN WOMAN.

1991

65. Kåre Bergh: APPLICATIONS OF ANTI-C5a SPECIFIC MONOCLONAL ANTIBODIES FOR THE ASSESSMENT OF COMPLEMENT ACTIVATION.
66. Svein Svenningsen: THE CLINICAL SIGNIFICANCE OF INCREASED FEMORAL ANTEVERSION.
67. Olbjørn Klepp: NONSEMINOMATOUS GERM CELL TESTIS CANCER: THERAPEUTIC OUTCOME AND PROGNOSTIC FACTORS.

68. Trond Sand: THE EFFECTS OF CLICK POLARITY ON BRAINSTEM AUDITORY EVOKED POTENTIALS AMPLITUDE, DISPERSION, AND LATENCY VARIABLES.
69. Kjetil B. Åsbakk: STUDIES OF A PROTEIN FROM PSORIATIC SCALE, PSO P27, WITH RESPECT TO ITS POTENTIAL ROLE IN IMMUNE REACTIONS IN PSORIASIS.
70. Arnulf Hestnes: STUDIES ON DOWN´S SYNDROME.
71. Randi Nygaard: LONG-TERM SURVIVAL IN CHILDHOOD LEUKEMIA.
72. Bjørn Hagen: THIO-TEPA.
73. Svein Anda: EVALUATION OF THE HIP JOINT BY COMPUTED TOMOGRAMPHY AND ULTRASONOGRAPHY.

1992

74. Martin Svartberg: AN INVESTIGATION OF PROCESS AND OUTCOME OF SHORT-TERM PSYCHODYNAMIC PSYCHOTHERAPY.
75. Stig Arild Slørdahl: AORTIC REGURGITATION.
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77. Maurice B. Vincent: VASOACTIVE PEPTIDES IN THE OCULAR/FOREHEAD AREA.
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79. Turid Nilsen: PYROPHOSPHATE IN HEPATOCYTE IRON METABOLISM.
80. Olav Haraldseth: NMR SPECTROSCOPY OF CEREBRAL ISCHEMIA AND REPERFUSION IN RAT.
81. Eiliv Brenna: REGULATION OF FUNCTION AND GROWTH OF THE OXYNTIC MUCOSA.

1993

82. Gunnar Bovim: CERVICOGENIC HEADACHE.
83. Jarl Arne Kahn: ASSISTED PROCREATION.
84. Bjørn Naume: IMMUNOREGULATORY EFFECTS OF CYTOKINES ON NK CELLS.
85. Rune Wiseth: AORTIC VALVE REPLACEMENT.
86. Jie Ming Shen: BLOOD FLOW VELOCITY AND RESPIRATORY STUDIES.
87. Piotr Kruszewski: SUNCT SYNDROME WITH SPECIAL REFERENCE TO THE AUTONOMIC NERVOUS SYSTEM.
88. Mette Haase Moen: ENDOMETRIOSIS.
89. Anne Vik: VASCULAR GAS EMBOLISM DURING AIR INFUSION AND AFTER DECOMPRESSION IN PIGS.
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91. Kjell Å. Salvesen: ROUTINE ULTRASONOGRAPHY IN UTERO AND DEVELOPMENT IN CHILDHOOD.

1994

92. Nina-Beate Liabakk: DEVELOPMENT OF IMMUNOASSAYS FOR TNF AND ITS SOLUBLE RECEPTORS.
93. Sverre Helge Torp: *erb*B ONCOGENES IN HUMAN GLIOMAS AND MENINGIOMAS.
94. Olav M. Linaker: MENTAL RETARDATION AND PSYCHIATRY. Past and present.
95. Per Oscar Feet: INCREASED ANTIDEPRESSANT AND ANTIPANIC EFFECT IN COMBINED TREATMENT WITH DIXYRAZINE AND TRICYCLIC ANTIDEPRESSANTS.
96. Stein Olav Samstad: CROSS SECTIONAL FLOW VELOCITY PROFILES FROM TWO-DIMENSIONAL DOPPLER ULTRASOUND: Studies on early mitral blood flow.
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99. Torvid Kiserud: THE DUCTUS VENOSUS IN THE HUMAN FETUS.
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103. Unni Syversen: CHROMOGRANIN A. Phsysiological and Clinical Role.

1995

104. Odd Gunnar Brakstad: THERMOSTABLE NUCLEASE AND THE *nuc* GENE IN THE DIAGNOSIS OF *Staphylococcus aureus* INFECTIONS.
105. Terje Engan: NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY OF PLASMA IN MALIGNANT DISEASE.
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108. Roar Stenseth: THORACIC EPIDURAL ANALGESIA IN AORTOCORONARY BYPASS SURGERY.

109. Arild Faxvaag: STUDIES OF IMMUNE CELL FUNCTION *in mice infected with* MURINE RETROVIRUS.

1996

110. Svend Aakhus: NONINVASIVE COMPUTERIZED ASSESSMENT OF LEFT VENTRICULAR FUNCTION AND SYSTEMIC ARTERIAL PROPERTIES. Methodology and some clinical applications.
111. Klaus-Dieter Bolz: INTRAVASCULAR ULTRASONOGRAPHY.
112. Petter Aadahl: CARDIOVASCULAR EFFECTS OF THORACIC AORTIC CROSS-CLAMPING.
113. Sigurd Steinshamn: CYTOKINE MEDIATORS DURING GRANULOCYTOPENIC INFECTIONS.
114. Hans Stifoss-Hanssen: SEEKING MEANING OR HAPPINESS?
115. Anne Kvikstad: LIFE CHANGE EVENTS AND MARITAL STATUS IN RELATION TO RISK AND PROGNOSIS OF CANSER.
116. Torbjørn Grøntvedt: TREATMENT OF ACUTE AND CHRONIC ANTERIOR CRUCIATE LIGAMENT INJURIES. A clinical and biomechanical study.
117. Sigrid Hørven Wigers: CLINICAL STUDIES OF FIBROMYALGIA WITH FOCUS ON ETIOLOGY, TREATMENT AND OUTCOME.
118. Jan Schjøtt: MYOCARDIAL PROTECTION: Functional and Metabolic Characteristics of Two Endogenous Protective Principles.
119. Marit Martinussen: STUDIES OF INTESTINAL BLOOD FLOW AND ITS RELATION TO TRANSITIONAL CIRCULATORY ADAPATION IN NEWBORN INFANTS.
120. Tomm B. Müller: MAGNETIC RESONANCE IMAGING IN FOCAL CEREBRAL ISCHEMIA.
121. Rune Haaverstad: OEDEMA FORMATION OF THE LOWER EXTREMITIES.
122. Magne Børset: THE ROLE OF CYTOKINES IN MULTIPLE MYELOMA, WITH SPECIAL REFERENCE TO HEPATOCYTE GROWTH FACTOR.
123. Geir Smedslund: A THEORETICAL AND EMPIRICAL INVESTIGATION OF SMOKING, STRESS AND DISEASE: RESULTS FROM A POPULATION SURVEY.

1997

124. Torstein Vik: GROWTH, MORBIDITY, AND PSYCHOMOTOR DEVELOPMENT IN INFANTS WHO WERE GROWTH RETARDED *IN UTERO*.
125. Siri Forsmo: ASPECTS AND CONSEQUENCES OF OPPORTUNISTIC SCREENING FOR CERVICAL CANCER. Results based on data from three Norwegian counties.
126. Jon S. Skranes: CEREBRAL MRI AND NEURODEVELOPMENTAL OUTCOME IN VERY LOW BIRTH WEIGHT (VLBW) CHILDREN. A follow-up study of a geographically based year cohort of VLBW children at ages one and six years.
127. Knut Bjørnstad: COMPUTERIZED ECHOCARDIOGRAPHY FOR EVALUTION OF CORONARY ARTERY DISEASE.
128. Grethe Elisabeth Borchgrevink: DIAGNOSIS AND TREATMENT OF WHIPLASH/NECK SPRAIN INJURIES CAUSED BY CAR ACCIDENTS.
129. Tor Elsås: NEUROPEPTIDES AND NITRIC OXIDE SYNTHASE IN OCULAR AUTONOMIC AND SENSORY NERVES.
130. Rolf W. Gråwe: EPIDEMIOLOGICAL AND NEUROPSYCHOLOGICAL PERSPECTIVES ON SCHIZOPHRENIA.
131. Tonje Strømholm: CEREBRAL HAEMODYNAMICS DURING THORACIC AORTIC CROSSCLAMPING. An experimental study in pigs.

1998

132. Martinus Bråten: STUDIES ON SOME PROBLEMS REALTED TO INTRAMEDULLARY NAILING OF FEMORAL FRACTURES.
133. Ståle Nordgård: PROLIFERATIVE ACTIVITY AND DNA CONTENT AS PROGNOSTIC INDICATORS IN ADENOID CYSTIC CARCINOMA OF THE HEAD AND NECK.
134. Egil Lien: SOLUBLE RECEPTORS FOR **TNF** AND **LPS**: RELEASE PATTERN AND POSSIBLE SIGNIFICANCE IN DISEASE.
135. Marit Bjørgaas: HYPOGLYCAEMIA IN CHILDREN WITH DIABETES MELLITUS
136. Frank Skorpen: GENETIC AND FUNCTIONAL ANALYSES OF DNA REPAIR IN HUMAN CELLS.
137. Juan A. Pareja: SUNCT SYNDROME. ON THE CLINICAL PICTURE. ITS DISTINCTION FROM OTHER, SIMILAR HEADACHES.
138. Anders Angelsen: NEUROENDOCRINE CELLS IN HUMAN PROSTATIC CARCINOMAS AND THE PROSTATIC COMPLEX OF RAT, GUINEA PIG, CAT AND DOG.

139. Fabio Antonaci: CHRONIC PAROXYSMAL HEMICRANIA AND HEMICRANIA CONTINUA: TWO DIFFERENT ENTITIES?
140. Sven M. Carlsen: ENDOCRINE AND METABOLIC EFFECTS OF METFORMIN WITH SPECIAL EMPHASIS ON CARDIOVASCULAR RISK FACTORES.

1999

141. Terje A. Murberg: DEPRESSIVE SYMPTOMS AND COPING AMONG PATIENTS WITH CONGESTIVE HEART FAILURE.
142. Harm-Gerd Karl Blaas: THE EMBRYONIC EXAMINATION. Ultrasound studies on the development of the human embryo.
143. Noèmi Becser Andersen:THE CEPHALIC SENSORY NERVES IN UNILATERAL HEADACHES. Anatomical background and neurophysiological evaluation.
144. Eli-Janne Fiskerstrand: LASER TREATMENT OF PORT WINE STAINS. A study of the efficacy and limitations of the pulsed dye laser. Clinical and morfological analyses aimed at improving the therapeutic outcome.
145. Bård Kulseng: A STUDY OF ALGINATE CAPSULE PROPERTIES AND CYTOKINES IN RELATION TO INSULIN DEPENDENT DIABETES MELLITUS.
146. Terje Haug: STRUCTURE AND REGULATION OF THE HUMAN UNG GENE ENCODING URACIL-DNA GLYCOSYLASE.
147. Heidi Brurok: MANGANESE AND THE HEART. A Magic Metal with Diagnostic and Therapeutic Possibilites.
148. Agnes Kathrine Lie: DIAGNOSIS AND PREVALENCE OF HUMAN PAPILLOMAVIRUS INFECTION IN CERVICAL INTRAEPITELIAL NEOPLASIA. Relationship to Cell Cycle Regulatory Proteins and HLA DQBI Genes.
149. Ronald Mårvik: PHARMACOLOGICAL, PHYSIOLOGICAL AND PATHOPHYSIOLOGICAL STUDIES ON ISOLATED STOMACS.
150. Ketil Jarl Holen: THE ROLE OF ULTRASONOGRAPHY IN THE DIAGNOSIS AND TREATMENT OF HIP DYSPLASIA IN NEWBORNS.
151. Irene Hetlevik: THE ROLE OF CLINICAL GUIDELINES IN CARDIOVASCULAR RISK INTERVENTION IN GENERAL PRACTICE.
152. Katarina Tunòn: ULTRASOUND AND PREDICTION OF GESTATIONAL AGE.
153. Johannes Soma: INTERACTION BETWEEN THE LEFT VENTRICLE AND THE SYSTEMIC ARTERIES.
154. Arild Aamodt: DEVELOPMENT AND PRE-CLINICAL EVALUATION OF A CUSTOM-MADE FEMORAL STEM.
155. Agnar Tegnander: DIAGNOSIS AND FOLLOW-UP OF CHILDREN WITH SUSPECTED OR KNOWN HIP DYSPLASIA.
156. Bent Indredavik: STROKE UNIT TREATMENT: SHORT AND LONG-TERM EFFECTS
157. Jolanta Vanagaite Vingen: PHOTOPHOBIA AND PHONOPHOBIA IN PRIMARY HEADACHES

2000

158. Ola Dalsegg Sæther: PATHOPHYSIOLOGY DURING PROXIMAL AORTIC CROSS-CLAMPING CLINICAL AND EXPERIMENTAL STUDIES
159. xxxxxxxxx (blind number)
160. Christina Vogt Isaksen: PRENATAL ULTRASOUND AND POSTMORTEM FINDINGS – A TEN YEAR CORRELATIVE STUDY OF FETUSES AND INFANTS WITH DEVELOPMENTAL ANOMALIES.
161. Holger Seidel: HIGH-DOSE METHOTREXATE THERAPY IN CHILDREN WITH ACUTE LYMPHOCYTIC LEUKEMIA: DOSE, CONCENTRATION, AND EFFECT CONSIDERATIONS.
162. Stein Hallan: IMPLEMENTATION OF MODERN MEDICAL DECISION ANALYSIS INTO CLINICAL DIAGNOSIS AND TREATMENT.
163. Malcolm Sue-Chu: INVASIVE AND NON-INVASIVE STUDIES IN CROSS-COUNTRY SKIERS WITH ASTHMA-LIKE SYMPTOMS.
164. Ole-Lars Brekke: EFFECTS OF ANTIOXIDANTS AND FATTY ACIDS ON TUMOR NECROSIS FACTOR-INDUCED CYTOTOXICITY.
165. Jan Lundbom: AORTOCORONARY BYPASS SURGERY: CLINICAL ASPECTS, COST CONSIDERATIONS AND WORKING ABILITY.
166. John-Anker Zwart: LUMBAR NERVE ROOT COMPRESSION, BIOCHEMICAL AND NEUROPHYSIOLOGICAL ASPECTS.
167. Geir Falck: HYPEROSMOLALITY AND THE HEART.

168. Eirik Skogvoll: CARDIAC ARREST Incidence, Intervention and Outcome.
169. Dalius Bansevicius: SHOULDER-NECK REGION IN CERTAIN HEADACHES AND CHRONIC PAIN SYNDROMES.
170. Bettina Kinge: REFRACTIVE ERRORS AND BIOMETRIC CHANGES AMONG UNIVERSITY STUDENTS IN NORWAY.
171. Gunnar Qvigstad: CONSEQUENCES OF HYPERGASTRINEMIA IN MAN
172. Hanne Ellekjær: EPIDEMIOLOGICAL STUDIES OF STROKE IN A NORWEGIAN POPULATION. INCIDENCE, RISK FACTORS AND PROGNOSIS
173. Hilde Grimstad: VIOLENCE AGAINST WOMEN AND PREGNANCY OUTCOME.
174. Astrid Hjelde: SURFACE TENSION AND COMPLEMENT ACTIVATION: Factors influencing bubble formation and bubble effects after decompression.
175. Kjell A. Kvistad: MR IN BREAST CANCER – A CLINICAL STUDY.
176. Ivar Rossvoll: ELECTIVE ORTHOPAEDIC SURGERY IN A DEFINED POPULATION. Studies on demand, waiting time for treatment and incapacity for work.
177. Carina Seidel: PROGNOSTIC VALUE AND BIOLOGICAL EFFECTS OF HEPATOCYTE GROWTH FACTOR AND SYNDECAN-1 IN MULTIPLE MYELOMA.

2001

178. Alexander Wahba: THE INFLUENCE OF CARDIOPULMONARY BYPASS ON PLATELET FUNCTION AND BLOOD COAGULATION – DETERMINANTS AND CLINICAL CONSEQUENSES
179. Marcus Schmitt-Egenolf: THE RELEVANCE OF THE MAJOR hISTOCOMPATIBILITY COMPLEX FOR THE GENETICS OF PSORIASIS
180. Odrun Arna Gederaas: BIOLOGICAL MECHANISMS INVOLVED IN 5-AMINOLEVULINIC ACID BASED PHOTODYNAMIC THERAPY
181. Pål Richard Romundstad: CANCER INCIDENCE AMONG NORWEGIAN ALUMINIUM WORKERS
182. Henrik Hjorth-Hansen: NOVEL CYTOKINES IN GROWTH CONTROL AND BONE DISEASE OF MULTIPLE MYELOMA
183. Gunnar Morken: SEASONAL VARIATION OF HUMAN MOOD AND BEHAVIOUR
184. Bjørn Olav Haugen: MEASUREMENT OF CARDIAC OUTPUT AND STUDIES OF VELOCITY PROFILES IN AORTIC AND MITRAL FLOW USING TWO- AND THREE-DIMENSIONAL COLOUR FLOW IMAGING
185. Geir Bråthen: THE CLASSIFICATION AND CLINICAL DIAGNOSIS OF ALCOHOL-RELATED SEIZURES
186. Knut Ivar Aasarød: RENAL INVOLVEMENT IN INFLAMMATORY RHEUMATIC DISEASE. A Study of Renal Disease in Wegener's Granulomatosis and in Primary Sjögren's Syndrome
187. Trude Helen Flo: RESEPTORS INVOLVED IN CELL ACTIVATION BY DEFINED URONIC ACID POLYMERS AND BACTERIAL COMPONENTS
188. Bodil Kavli: HUMAN URACIL-DNA GLYCOSYLASES FROM THE UNG GENE: STRUCTRUAL BASIS FOR SUBSTRATE SPECIFICITY AND REPAIR
189. Liv Thommesen: MOLECULAR MECHANISMS INVOLVED IN TNF- AND GASTRIN-MEDIATED GENE REGULATION
190. Turid Lingaas Holmen: SMOKING AND HEALTH IN ADOLESCENCE; THE NORD-TRØNDELAG HEALTH STUDY, 1995-97
191. Øyvind Hjertner: MULTIPLE MYELOMA: INTERACTIONS BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MICROENVIRONMENT
192. Asbjørn Støylen: STRAIN RATE IMAGING OF THE LEFT VENTRICLE BY ULTRASOUND. FEASIBILITY, CLINICAL VALIDATION AND PHYSIOLOGICAL ASPECTS
193. Kristian Midthjell: DIABETES IN ADULTS IN NORD-TRØNDELAG. PUBLIC HEALTH ASPECTS OF DIABETES MELLITUS IN A LARGE, NON-SELECTED NORWEGIAN POPULATION.
194. Guanglin Cui: FUNCTIONAL ASPECTS OF THE ECL CELL IN RODENTS
195. Ulrik Wisløff: CARDIAC EFFECTS OF AEROBIC ENDURANCE TRAINING: HYPERTROPHY, CONTRACTILITY AND CALCUIM HANDLING IN NORMAL AND FAILING HEART
196. Øyvind Halaas: MECHANISMS OF IMMUNOMODULATION AND CELL-MEDIATED CYTOTOXICITY INDUCED BY BACTERIAL PRODUCTS
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2002

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2003

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226. Torstein Hole: DOPPLER ECHOCARDIOGRAPHIC EVALUATION OF LEFT VENTRICULAR FUNCTION IN PATIENTS WITH ACUTE MYOCARDIAL INFARCTION
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230. Geir Torheim: PROCESSING OF DYNAMIC DATA SETS IN MAGNETIC RESONANCE IMAGING
231. Catrine Ahlén: SKIN INFECTIONS IN OCCUPATIONAL SATURATION DIVERS IN THE NORTH SEA AND THE IMPACT OF THE ENVIRONMENT
232. Arnulf Langhammer: RESPIRATORY SYMPTOMS, LUNG FUNCTION AND BONE MINERAL DENSITY IN A COMPREHENSIVE POPULATION SURVEY. THE NORD-TRØNDELAG HEALTH STUDY 1995-97. THE BRONCHIAL OBSTRUCTION IN NORD-TRØNDELAG STUDY
233. Einar Kjelsås: EATING DISORDERS AND PHYSICAL ACTIVITY IN NON-CLINICAL SAMPLES
234. Arne Wibe: RECTAL CANCER TREATMENT IN NORWAY – STANDARDISATION OF SURGERY AND QUALITY ASSURANCE

2004

235. Eivind Witsø: BONE GRAFT AS AN ANTIBIOTIC CARRIER
236. Anne Mari Sund: DEVELOPMENT OF DEPRESSIVE SYMPTOMS IN EARLY ADOLESCENCE
237. Hallvard Lærum: EVALUATION OF ELECTRONIC MEDICAL RECORDS – A CLINICAL TASK PERSPECTIVE
238. Gustav Mikkelsen: ACCESSIBILITY OF INFORMATION IN ELECTRONIC PATIENT RECORDS; AN EVALUATION OF THE ROLE OF DATA QUALITY
239. Steinar Krokstad: SOCIOECONOMIC INEQUALITIES IN HEALTH AND DISABILITY. SOCIAL EPIDEMIOLOGY IN THE NORD-TRØNDELAG HEALTH STUDY (HUNT), NORWAY
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241. Ingunn Dybedal: NEGATIVE REGULATORS OF HEMATOPOIETEC STEM AND PROGENITOR CELLS
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244. Anna Bofin: FINE NEEDLE ASPIRATION CYTOLOGY IN THE PRIMARY INVESTIGATION OF BREAST TUMOURS AND IN THE DETERMINATION OF TREATMENT STRATEGIES
245. Jim Aage Nøttestad: DEINSTITUTIONALIZATION AND MENTAL HEALTH CHANGES AMONG PEOPLE WITH MENTAL RETARDATION
246. Reidar Fossmark: GASTRIC CANCER IN JAPANESE COTTON RATS
247. Wibeke Nordhøy: MANGANESE AND THE HEART, INTRACELLULAR MR RELAXATION AND WATER EXCHANGE ACROSS THE CARDIAC CELL MEMBRANE

2005

248. Sturla Molden: QUANTITATIVE ANALYSES OF SINGLE UNITS RECORDED FROM THE HIPPOCAMPUS AND ENTORHINAL CORTEX OF BEHAVING RATS
249. Wenche Brenne Drøyvold: EPIDEMIOLOGICAL STUDIES ON WEIGHT CHANGE AND HEALTH IN A LARGE POPULATION. THE NORD-TRØNDELAG HEALTH STUDY (HUNT)
250. Ragnhild Støen: ENDOTHELIUM-DEPENDENT VASODILATION IN THE FEMORAL ARTERY OF DEVELOPING PIGLETS

251. Aslak Steinsbekk: HOMEOPATHY IN THE PREVENTION OF UPPER RESPIRATORY TRACT INFECTIONS IN CHILDREN
252. Hill-Aina Steffenach: MEMORY IN HIPPOCAMPAL AND CORTICO-HIPPOCAMPAL CIRCUITS
253. Eystein Stordal: ASPECTS OF THE EPIDEMIOLOGY OF DEPRESSIONS BASED ON SELF-RATING IN A LARGE GENERAL HEALTH STUDY (THE HUNT-2 STUDY)
254. Viggo Pettersen: FROM MUSCLES TO SINGING: THE ACTIVITY OF ACCESSORY BREATHING MUSCLES AND THORAX MOVEMENT IN CLASSICAL SINGING
255. Marianne Fyhn: SPATIAL MAPS IN THE HIPPOCAMPUS AND ENTORHINAL CORTEX
256. Robert Valderhaug: OBSESSIVE-COMPULSIVE DISORDER AMONG CHILDREN AND ADOLESCENTS: CHARACTERISTICS AND PSYCHOLOGICAL MANAGEMENT OF PATIENTS IN OUTPATIENT PSYCHIATRIC CLINICS
257. Erik Skaaheim Haug: INFRARENAL ABDOMINAL AORTIC ANEURYSMS – COMORBIDITY AND RESULTS FOLLOWING OPEN SURGERY
258. Daniel Kondziella: GLIAL-NEURONAL INTERACTIONS IN EXPERIMENTAL BRAIN DISORDERS
259. Vegard Heimly Brun: ROUTES TO SPATIAL MEMORY IN HIPPOCAMPAL PLACE CELLS
260. Kenneth McMillan: PHYSIOLOGICAL ASSESSMENT AND TRAINING OF ENDURANCE AND STRENGTH IN PROFESSIONAL YOUTH SOCCER PLAYERS
261. Marit Sæbø Indredavik: MENTAL HEALTH AND CEREBRAL MAGNETIC RESONANCE IMAGING IN ADOLESCENTS WITH LOW BIRTH WEIGHT
262. Ole Johan Kemi: ON THE CELLULAR BASIS OF AEROBIC FITNESS, INTENSITY-DEPENDENCE AND TIME-COURSE OF CARDIOMYOCYTE AND ENDOTHELIAL ADAPTATIONS TO EXERCISE TRAINING
263. Eszter Vanky: POLYCYSTIC OVARY SYNDROME – METFORMIN TREATMENT IN PREGNANCY
264. Hild Fjærtoft: EXTENDED STROKE UNIT SERVICE AND EARLY SUPPORTED DISCHARGE. SHORT AND LONG-TERM EFFECTS
265. Grete Dyb: POSTTRAUMATIC STRESS REACTIONS IN CHILDREN AND ADOLESCENTS
266. Vidar Fykse: SOMATOSTATIN AND THE STOMACH
267. Kirsti Berg: OXIDATIVE STRESS AND THE ISCHEMIC HEART: A STUDY IN PATIENTS UNDERGOING CORONARY REVASCULARIZATION
268. Björn Inge Gustafsson: THE SEROTONIN PRODUCING ENTEROCHROMAFFIN CELL, AND EFFECTS OF HYPERSEROTONINEMIA ON HEART AND BONE

2006

269. Torstein Baade Rø: EFFECTS OF BONE MORPHOGENETIC PROTEINS, HEPATOCYTE GROWTH FACTOR AND INTERLEUKIN-21 IN MULTIPLE MYELOMA
270. May-Britt Tessem: METABOLIC EFFECTS OF ULTRAVIOLET RADIATION ON THE ANTERIOR PART OF THE EYE
271. Anne-Sofie Helvik: COPING AND EVERYDAY LIFE IN A POPULATION OF ADULTS WITH HEARING IMPAIRMENT
272. Therese Standal: MULTIPLE MYELOMA: THE INTERPLAY BETWEEN MALIGNANT PLASMA CELLS AND THE BONE MARROW MICROENVIRONMENT
273. Ingvild Saltvedt: TREATMENT OF ACUTELY SICK, FRAIL ELDERLY PATIENTS IN A GERIATRIC EVALUATION AND MANAGEMENT UNIT – RESULTS FROM A PROSPECTIVE RANDOMISED TRIAL
274. Birger Henning Endreseth: STRATEGIES IN RECTAL CANCER TREATMENT – FOCUS ON EARLY RECTAL CANCER AND THE INFLUENCE OF AGE ON PROGNOSIS
275. Anne Mari Aukan Rokstad: ALGINATE CAPSULES AS BIOREACTORS FOR CELL THERAPY
276. Mansour Akbari: HUMAN BASE EXCISION REPAIR FOR PRESERVATION OF GENOMIC STABILITY
277. Stein Sundstrøm: IMPROVING TREATMENT IN PATIENTS WITH LUNG CANCER – RESULTS FROM TWO MULITCENTRE RANDOMISED STUDIES
278. Hilde Pleym: BLEEDING AFTER CORONARY ARTERY BYPASS SURGERY - STUDIES ON HEMOSTATIC MECHANISMS, PROPHYLACTIC DRUG TREATMENT AND EFFECTS OF AUTOTRANSFUSION
279. Line Merethe Oldervoll: PHYSICAL ACTIVITY AND EXERCISE INTERVENTIONS IN CANCER PATIENTS

280. Boye Welde: THE SIGNIFICANCE OF ENDURANCE TRAINING, RESISTANCE TRAINING AND MOTIVATIONAL STYLES IN ATHLETIC PERFORMANCE AMONG ELITE JUNIOR CROSS-COUNTRY SKIERS
281. Per Olav Vandvik: IRRITABLE BOWEL SYNDROME IN NORWAY, STUDIES OF PREVALENCE, DIAGNOSIS AND CHARACTERISTICS IN GENERAL PRACTICE AND IN THE POPULATION
282. Idar Kirkeby-Garstad: CLINICAL PHYSIOLOGY OF EARLY MOBILIZATION AFTER CARDIAC SURGERY
283. Linn Getz: SUSTAINABLE AND RESPONSIBLE PREVENTIVE MEDICINE. CONCEPTUALISING ETHICAL DILEMMAS ARISING FROM CLINICAL IMPLEMENTATION OF ADVANCING MEDICAL TECHNOLOGY
284. Eva Tegnander: DETECTION OF CONGENITAL HEART DEFECTS IN A NON-SELECTED POPULATION OF 42,381 FETUSES
285. Kristin Gabestad Nørsett: GENE EXPRESSION STUDIES IN GASTROINTESTINAL PATHOPHYSIOLOGY AND NEOPLASIA