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NOVEL ASPECTS OF THE MOLECULAR BIOLOGY OF GASTROINTESTINAL STROMAL TUMORS

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NOVEL ASPECTS OF THE MOLECULAR BIOLOGY OF GASTROINTESTINAL STROMAL TUMORS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my family

Thesis defense

Rolf Lufts Auditorium

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Friday June 13th 2014 at 9 a.m.

ABSTRACT

The gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor in the gastrointestinal tract (GI). Historically, these tumors were commonly mistaken for myogenic and neurogenic masses, and then eventually came to be recognized as a distinct type of soft-tissue sarcoma through ultrastructural findings and specific immunomarkers. GISTs can arise anywhere along the GI tract, and are believed to originate from or share a common progenitor with the interstitial cell of Cajal. The majority of GISTs carry activating *KIT* or *PDGFRA* mutations, which form the molecular basis for the successful tyrosine kinase inhibitor therapy. Although genetic discoveries and treatment advances have greatly improved clinical outcomes, the significance of GIST neuroendocrine phenotype, the role of the relatively newly identified DOG1, and the impact of regional imatinib pharmacodynamics remain obscure. The aim of the overall thesis was to explore functional aspects of the human GIST biology.

Evaluation of the presence of functional GIST cell stimulus-secretion coupling demonstrated an intact intracellular Ca^{2+}-signaling pathway and an active ATP release that is dependent on $[Ca^{2+}]_e$ levels and is augmentable by pharmacological stimuli. **(Paper I)**

The existence and composition of a putative GIST secretome was assessed by shotgun proteomics. The findings demonstrate that GIST cells contain a secretome signature made up of classically and non-classically released proteins. The protein subsets and appurtenant functional clustering varied in the presence of drug stimulation. The types of released proteins, which significantly increased through cell stimulation, were consistent with the types of proteins found in other cancers. Moreover, the secretome overlapped extensively with exosomal proteins. **(Paper II)**

A protocol to measure intracellular imatinib levels was developed for use in both *in vitro* and *in vivo* systems of GIST cells. The liquid-liquid extraction LC-MS TOF-based protocol offered a reliable way to determine intracellular imatinib levels with high recovery, good linearity, and low limit of detection, in both the experimental and clinical settings. The imatinib uptake differed between imatinib-sensitive and imatinib-resistant cell lines, and accumulated in tumors from three patients, with large intra- and inter-tumoral variations. **(Paper III)**

The functional significance of DOG1 in GIST cells was addressed. DOG1 have different subcellular localizations in imatinib-sensitive and imatinib-resistant GIST cells. Specific inhibitors or activators modulated the DOG1 activity efficaciously. The overall effect on GIST cell viability and proliferation was small, but DOG1 inhibition induced late apoptosis among a small proportion of early apoptotic imatinib-resistant GIST cells. **(Paper IV)**

LIST OF SCIENTIFIC PAPERS

The thesis is based on the following papers, referred to in the text by their Roman numerals.

I. **Berglund E**, Ubhayasekera SJ, Karlsson F, Akçakaya P, Aluthgedara W, Åhlén J, Fröbom R, Nilsson IL, Lui WO, Larsson C, Zedenius J, Bergquist J, Bränström R.
Intracellular concentration of the tyrosine kinase inhibitor imatinib in gastro-intestinal stromal tumor cells.
Anticancer Drugs 2014;25(4):415-422.

II. **Berglund E**, Berglund D, Akçakaya P, Ghaderi M, Daré E, Berggren PO, Köhler M, Aspinwall CA, Lui WO, Zedenius J, Larsson C, Bränström R.
Evidence for Ca^{2+}-regulated ATP release in gastrointestinal stromal tumors.
Experimental Cell Research 2013;319(8):1229-1238.

III. **Berglund E**, Daré E, Branca R, Akçakaya P, Fröbom R, Berggren P-O, Lui W-O, Larsson C, Zedenius J, Orre L, Lehtiö J, Kim J, Bränström R.
Secretome protein signature of human gastrointestinal stromal tumor cells.
Manuscript.

IV. **Berglund E**, Akçakaya P, Berglund D, Karlsson F, Vukojević V, Lee L, Bogdanović D, Lui WO, Larsson C, Zedenius J, Fröbom R, Bränström R.
Functional role of the Ca^{2+}-activated Cl^- channel DOG1/TMEM16A in gastrointestinal stromal tumor cells.
Experimental Cell Research 2014. Epub ahead of print.

LIST OF OTHER PUBLICATIONS

1. Ma Z, Lavebratt C, Almgren M, Portwood N, Forsberg LE, Bränström R, **Berglund E**, Falkmer S, Sundler F, Wierup N, Björklund A.
   Evidence for presence and functional effects of Kv1.1 channels in β-cells: general survey and results from mceph/mceph mice.
   PLoS One 2011;6(4):e18213.

2. Berglund D, Bengtsson M, Biglarnia A, **Berglund E**, Yamamoto S, von Zur-Mühlen B, Lorant T, Tufveson G.
   Screening of mortality in transplant patients using an assay for immune function.
   Transpl Immunol 2011;24(4):246-250.

3. Lu M, **Berglund E**, Larsson C, Höög A, Farnebo LO, Bränström R.
   Calmodulin and calmodulin-dependent protein kinase II inhibit hormone secretion in human parathyroid adenoma.
   J Endocrinol 2011;208(1):31-39.

4. Bränström R, **Berglund E**, Curman P, Forsberg L, Höög A, Grimelius L, Berggren PO, Mattsson P, Hellman P, Juntti-Berggren L.
   Electrical short-circuit in β-cells from a patient with non-insulinoma pancreatogenous hypoglycemic syndrome (NIPHS): a case report.
   J Med Case Rep 2010;4:315.

5. Lu M, Bränström R, **Berglund E**, Höög A, Björklund P, Westin G, Larsson C, Farnebo LO, Forsberg L.
   Expression and association of TRPC subtypes with Orai1 and STIM1 in human parathyroid.
   J Mol Endocrinol 2010;44(5):285-294.

6. Akçakaya P, Caramuta S, Åhlén J, Ghaderi M, **Berglund E**, Östman A, Bränström R, Larsson C, Lui WO.
   Small RNA expression signatures of gastrointestinal stromal tumors: associations to imatinib resistance and patient outcome.
   Submitted manuscript.

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LIST OF ABBREVIATIONS

ACOSOG	American College of Surgeons Oncology Group
AKT	v-akt murine thymoma viral oncogene homolog
ANO1	Anoctamin-1
ATP	Adenosine triphosphate
BRAF	v-raf murine sarcoma viral oncogene homolog B1
Ca^{2+}	Calcium ion
$[Ca^{2+}]_i$	Cytoplasmic free Ca^{2+}
$[Ca^{2+}]_e$	Extracellular Ca^{2+} concentration
CaCC	Ca^{2+}-activated Cl^- channels
CD34	Hematopoietic progenitor cell antigen CD34
CD117	Stem cell factor receptor (c-kit protein)
CLSM	Confocal laser scanning microscopy
CML	Chronic myelogenous leukemia
CT	Computed tomography
DAVID	Database for Annotation, Visualization and Integrated Discovery
DNA	Deoxyribonucleic acid
DOG1	Discovered on GIST-1
ERK	Extracellular signal-regulated protein kinase
ESI	Electrospray ionization
ESMO	European Society for Medical Oncology
ETV1	ETS translocation variant 1
EUS	Endoscopic ultrasound
EV	Extracellular vesicle
fcDNA	Free circulating DNA
FDA	Food and Drug Administration
FDG	^{18}F-fluoro-2-deoxy-D-glucose
FNA	Fine-needle aspiration
GI	Gastrointestinal
GIST	Gastrointestinal stromal tumor
GLUT4	Glucose transporter 4
HBSS	Hanks buffered salt solution
HCD	Higher-energy collision dissociation
HIF1α	Hypoxia-inducible factor α
HPF	High power field
IC	Current-clamp
ICC	Interstitial cells of Cajal
IGF	Insulin-like growth factor
IHC	Immunohistochemistry

IP3	Inositol triphosphate
JAK-STAT	Janus kinase – Signal Transducer and Activator of Transcription
KIT	v-kit Hardy Zuckerman 4 feline sarcoma viral oncogene homolog
LC	Liquid chromatography
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MRI	Magnetic resonance imaging
MS	Mass spectrometry
m/z	Mass-to-charge ratio
mTOR	Mammalian target of rapamycin
MV	Microvesicle
NCCN	National Comprehensive Cancer Network
NF1	Neurofibromin 1
NIH	National Institutes of Health
OS	Overall survival
PCR	Polymerase chain reaction
PDGFRA	Platelet-derived growth factor receptor alpha
PET	Positron emission tomography
PFS	Progression-free survival
PI3K	Phosphoinositide-3-kinase
PSM	Peptide-spectrum match
RAF1	v-RAF-1 murine leukemia viral oncogene homolog
RFS	Recurrence-free survival
RNA	Ribonucleic acid
SCF	Stem cell factor
SCX-SPE	Strong cation exchange solid phase extraction
SDH	Succinate dehydrogenase
SSG	Scandinavian Sarcoma Group
STAT3	Signal transducer and activator of transcription 3
STS	Soft-tissue sarcoma
TDM	Therapeutic drug monitoring
TKI	Tyrosine kinase inhibitor
TMEM16A	Transmembrane member 16A
TRPC	Transient receptor potential canonical type ion channel
VC	Voltage-clamp
VEGF	Vascular endothelial growth factor
wtGIST	wild-type GIST

INTRODUCTION

"There is new ammunition in the war against cancer. These are the bullets."
- TIME Magazine on imatinib, May 28, 2001.

Gastrointestinal stromal tumor

Historical background

With roughly 7.6 million deaths annually, cancer is one of the leading causes of death worldwide (Jeman et al., 2011). Sarcomas are a rare and heterogeneous group of connective tissue tumors of mesenchymal origin. These tumors can arise virtually anywhere in the body and are classified as either skeletal sarcomas or soft-tissue sarcomas (STS). More than 50 different histological subtypes of STS have been distinguished in the classification of the World Health Organization (WHO) (Fletcher et al., 2002), and new entities are continuously recognized; each with varying clinical phenotypes and behavior (Fletcher et al., 2013; Jo and Fletcher, 2014). STS are reported to account for approximately 1% of all adult malignant tumors (Mastrangelo et al., 2012; Stiller et al., 2013), and the gastrointestinal stromal tumor (GIST) is the most common STS of the gastrointestinal (GI) tract. Before we came to know GISTs as we do today, almost all mesenchymal tumors of the GI tract were considered to be "GI smooth muscle tumors". Thus, GISTs were commonly misclassified as leiomyomas (if benign), leiomyosarcomas (if malignant), or leiomyoblastomas. It was also difficult to distinguish GIST from Schwannoma and other nerve sheath tumors. This was mainly due to the wide morphological spectrum of GISTs, which complicated the differential diagnostics (Corless, 2014). In 1969, Welsh and Meyer were the first to show cogent evidence supporting ultrastructural differences between "GI smooth muscle tumors" and classical smooth muscle tumors (Welsh and Meyer, 1969). In the 1980s, Welsh and Meyer's evidence were further supported by immunohistochemical findings: the smooth muscle antigen, desmin, is rarely expressed, actin staining is often focal or negative, and Schwann cell features are absent in "GI smooth muscle tumors" (Evans et al.,

1983; Mazur, Clark, 1983). As a result, the term "gastrointestinal stromal tumor" was introduced as a histogenetically noncommittal term for these tumors (Mazur, Clark, 1983; Chan, 1999). CD34 was the first clinically useful marker for distinguishing GISTs from true GI leiomyomas, leiomyosarcomas, and Schwannomas in clinical routine (Miettinen et al., 1995). During the 1980s and 1990s, several other acronyms still flourished within the relevant literature due to somewhat disparate results (Chan, 1999). However, this indiscriminate usage of acronyms abruptly changed when Kindblom et al. (1998) noticed similarities between GISTs and the interstitial cells of Cajal (ICC), as evidence of its mesenchymal origin. These GI pacemaker cells – ICCs – were discovered by the Spanish Nobel laureate Santiago Ramón y Cajal and are associated with the myenteric plexus in the intestinal wall. The ICC is responsible for the initiation and coordination of the slow-waves organizing the gut peristalsis. The following ICC characteristics provide strong support for the hypothesis that ICCs are the originating cells of GISTs: its pluripotent mesenchymal stem cell character (Kindblom et al., 1998; Torihashi et al., 1999), its absence in KIT-deficient mice, and development of ICC hyperplasia and GISTs when *KIT*-activating mutations are introduced (Kitamura, Hirotab, 2004; Sommer et al, 2004). The greatest landmark in the history of GISTs was the discovery of *KIT* proto-oncogene mutations, which distinguished GISTs as unique tumor entities, brought knowledge to etiology and pathogenesis at the molecular level, and formed the basis for successful molecular-targeted therapies (Hirota et al., 1998; Kindblom et al, 1998).

Epidemiology

GISTs are responsible for almost one-fifth of all STS, which makes GISTs the single most common type of sarcoma (Ducimetiére et al., 2011; Mastrangelo et al., 2012; Stiller et al., 2013). Population-based studies report an annual incidence of 11-20 cases per million inhabitants of clinically relevant GISTs (Chan et al., 2006; Goettsch et al., 2005; Nilsson et al., 2005; Tryggvason et al., 2005). GIST prevalence is around 130 per million inhabitants (Nilsson et al., 2005). As subclinical GISTs are much more common than the overt tumors, these estimates captures only the minimum prevalence of GISTs. Micro-GISTs –

referring to GISTs less than 1 cm in diameter – are incidental findings in 10-22.5% of stomachs thoroughly examined at autopsy or after surgical removal (Abraham et al., 2007; Agaimy et al., 2007). Even though micro-GISTs seem quiescent with low mitotic counts (Kawanowa et al., 2006), the type and frequency of *KIT* mutations found in micro-GISTs are essentially the same as in clinically significant GISTs (Agaimy et al., 2007, Rossi et al., 2010). This suggests that the pool of micro-GISTs among the general population can probably develop into more advanced lesions, but that additional molecular aberrations are required for malignant transformation. GISTs arise at any age, even in infancy, but show proclivity toward developing in the middle-aged and elderly, with a median age of 63 years at diagnosis (Miettinen et al., 2005). More than 80% of patients are older than 50 years (Ducimetiére et al., 2011). Men and women are affected almost equally (Tran et al., 2005), and there is no ethnical predilection. Patients younger than 20 are very few (Joensuu et al., 2012), and pediatric GISTs are usually clinically distinct with female predominance, lack of *KIT* or *PDGFRA* mutations, gastric location, and potential lymph node metastases (Pappo, Janeway, 2009). GISTs commonly present in the stomach (50-60%) and the small intestine (30-35%), and occur less frequently in the colorectal areas (5%) and the esophagus (<1%). Adult GISTs almost never spread via the lymph vessels, and metastases have a predilection to the liver, whereas locoregional recurrences appear in the omentum or peritoneum (DeMatteo et al., 2000). Extra-gastrointestinal tract tumors (E-GISTs) can occasionally (<5%) be found in the omentum, the mesentery, or the retroperitoneum. There is an ongoing debate to whether E-GISTs are metastases from undetected primary tumors or the primary lesions themselves (Joensuu et al., 2012).

Clinical presentation

The clinical spectrum of GISTs ranges from local lesions to highly aggressive and disseminated tumors. About 25% of GISTs are discovered *en passant* during imaging, endoscopy, or surgery for other GI-related diseases. The majority of patients present with vague and non-specific symptoms. Gastrointestinal bleeding, abdominal pain or discomfort, dyspepsia, dysphagia, satiety, nausea,

vomiting, constipation or diarrhea, and a palpable tumor account for some of the more common symptoms. GISTs may also produce symptoms that are secondary to obstruction, such as tumor rupture, hemorrhages and bowel perforation, of which some may require emergent care. Because GISTs are submucosal tumors, the overlying mucosa is susceptible to pressure-related necrosis and ulceration that creates hemorrhaging from the disrupted vessels. Patients with significant blood loss may experience anemia-related symptoms (Bumming et al., 2006; Muccariani et al., 2007; Caterino et al., 2011).

Clinical workup

Expeditious management of GISTs at early stages is important for reducing the risk of progression. About 40% of patients with localized GISTs at the time of diagnosis eventually develop metastases, while 10-20% of patients present with metastases from the time of diagnosis (Joensuu, 2013). Consequently, a multidisciplinary approach to treatment involving surgeons, oncologists, pathologists, radiologists, among others, is advocated to optimize the outcome for a rare tumor such as GIST (Mullady, Tan, 2013). For these reasons the Scandinavian Sarcoma Group (SSG) recommends that any patient with a tumor larger than 5 cm in diameter, or with deep tissue tumors of any size, should be referred to an experienced sarcoma center for workup. A reliable diagnosis is crucial, since treatment options differ markedly between different abdominal tumors. At present, no test applicable to blood samples exists that can confirm or rule out GISTs: diagnosis is based on morphology and immunophenotyping, as well as mutation analysis at some centers. The standard approach to diagnose gastric, duodenal and rectal GISTs is by collecting endoscopic ultrasound-guided tumor material. Endoscopic ultrasound (EUS)-obtained fine-needle aspirations (FNAs) are preferable to transcutaneous approaches in order to minimize spillage of tumor cells into the abdominal cavity. EUS in itself also provides important diagnostic features, in addition to the location and size of tumors (Sepe et al., 2009). High-risk biopsies of cystic lesions should only be performed at experienced centers. Core-needle biopsies are indicated in neo-adjuvant settings, when mutation status is needed, or if FNA is insufficient. Considering that metastases are rare outside of the abdominal cavity, anatomic imaging of the

abdomen and pelvis, by contrast-enhanced computed tomography (CT) or magnetic resonance imaging (MRI), is usually adequate for detection, staging, and allows anatomical judgment. This is crucial for surgeon consideration of treatment options, avoidance of unmerited operations, optimizing the chances for adequate surgical margins without tumor rupture and spillage, evaluation of treatment responses in the neo-adjuvant setting, and monitoring of postoperative patients for recurrences (Figure 1) (Chourmouzi et al., 2009).

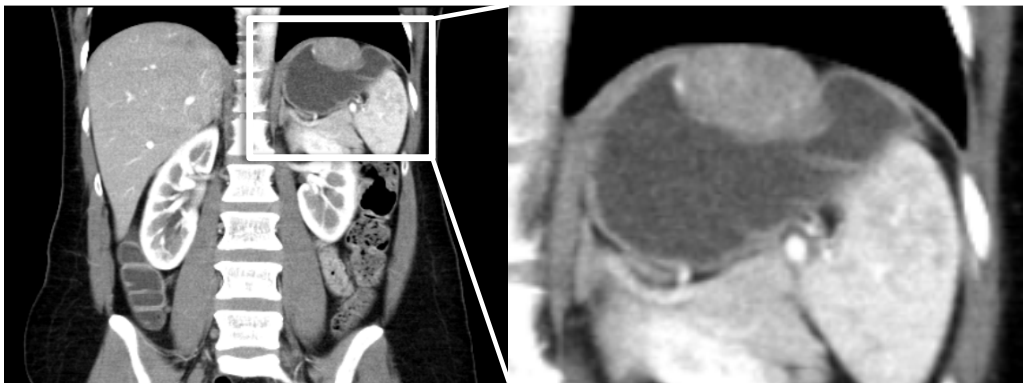

Figure 1. CT of the pelvis and abdomen is helpful to diagnose and stage GISTs: it provides information about size, location, and relationship to adjacent structures. The case above shows a local gastric GIST. CT can also detect multiple tumors and metastatic spread. Ghanem et al. (2003) described CT characteristics on histologically verified GISTs by dividing them into small (<5 cm), intermediate (5-10 cm), and large (>10 cm) tumors. Small masses usually appear as sharply demarcated and homogenous, with mainly intraluminal growth. Large masses more often feature irregular margins, varying densities, and aggressive behavior.

GISTs have an increased GLUT4 expression and glucose uptake (van den Abbeele et al., 2012). Based on these observations, dual modality ^{18}F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT has become increasingly common in the work-up of patients with GISTs to obtain both anatomic and functional information (Figure 2). It is useful in the imaging of initial disease evaluation, treatment response, and detection of recurrent tumors (Malle et al., 2012).

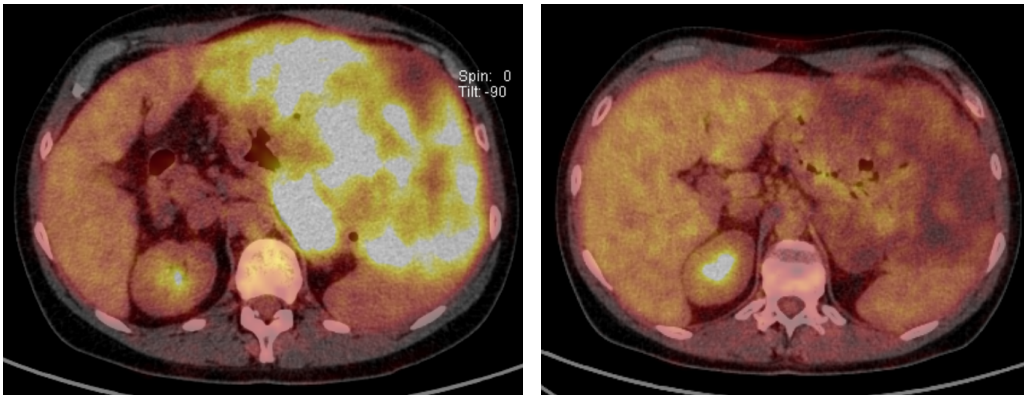

Figure 2. Example of a large, locally advanced, gastric GIST, scanned with ^{18}F-FDG PET/CT before (left) and one month after initiation of imatinib treatment (400 mg daily) (right). Different patient than in Figure 1.

Histopathology

GISTs are generally well demarcated with a fleshy pink or tan cut surface, and can contain hemorrhagic and cystic degenerative areas. Clinically significant GISTs range from 1 cm to more than 40 cm in diameter, with an average diameter of 5 cm (Nilsson et al., 2005; Miettinen et al., 2005). In addition to size, the pathologist should also always determine the mitotic count on a total area of 5 mm^2 (equivalent to 50 high power fields (HPFs)), as both are important prognostic variables (Joensuu, 2008). There are several different morphological patterns that overlap with various other GI tumors, which include three main patterns: 1) spindle cell (70%), 2) epitheloid (15%), or 3) mixed forms (15%) (Fletcher et al., 2002; Kindblom et al., 1998; Miettinen, 1988). Relying purely on morphology-based diagnostics makes the list of differential diagnoses long. Immunohistochemical analyses are therefore used for suspected GISTs for more accurate diagnosis (Figure 3). α-SMA is variably expressed in GIST, whereas desmin is usually absent. As previously mentioned, CD34 was the first marker that helped to distinguish between true GI smooth muscle tumors and GISTs (Miettinen et al., 1995) (Table 1). Three years later, two groups reported over-expression of the highly sensitive and specific marker KIT (CD117), which, for the first time, led to reliable diagnostics of GISTs (Hirota et al., 1998; Kindblom

et al., 1998). CD117 staining pattern can be membranous, diffusely cytoplasmic or perinuclear. Additional biological insight about STS and GIST was retrieved from microarray-based gene expression studies (Allander et al., 2001; Katoh and Katoh, 2003; Khan et al., 2001; Nielsen et al., 2002). In 2004, through guidance from these studies, West and co-workers generated an anti-serum against the protein FLJ10261, which turned out to be an even more reliable marker than CD117 (West et al., 2004). Today, this marker is known as DOG1 (discovered on GIST-1), but has aliases such as ANO1 (anoctamin 1), TMEM16A, and ORAOV2 (over-expressed in oral carcinoma). Almost 97% of GISTs are DOG1 positive, including some KIT-negative tumors. Together CD117 and DOG1 diagnose nearly 100% of GISTs, and are rarely expressed in other mesenchymal tumors (Miettinen et al., 2009). DOG1 is a Ca^{2+}-activated Cl^- channel protein (Yang et al., 2008). Since DOG1 is so abundant in GIST, we hypothesized that it is likely that it plays an important functional role. Very little data exist in this field, which was therefore explored in **paper IV**.

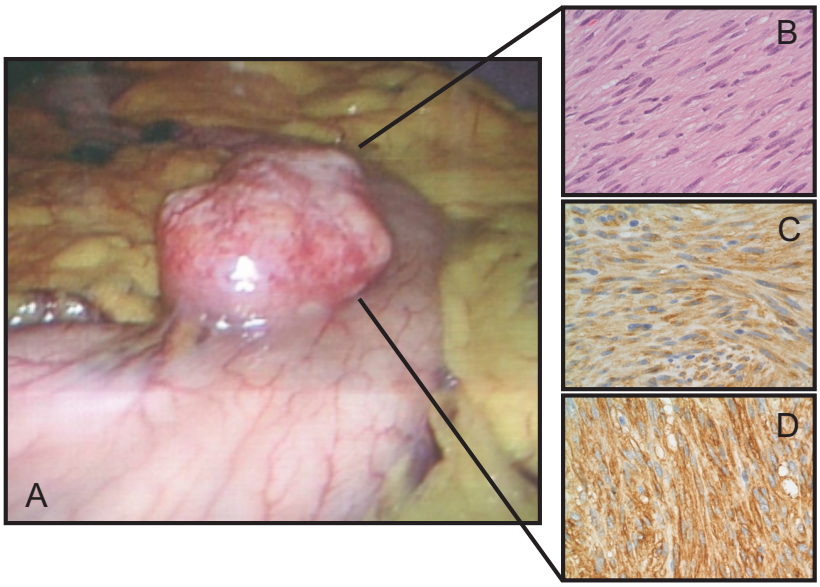

Figure 3. Macroscopic and microscopic appearance of a gastric GIST. (A) Intra-operative photograph. (B) Hematoxylin and eosin (HE) stained GIST cells with spindle cell morphology. Immunohistochemistry positive for (C) CD117 and (D) DOG1.

Table 1. Immunohistochemical markers for GIST.

Target protein	Proportion positive tumors (%)
DOG1 (discovered on GIST-1)	88-97
KIT (stem cell factor receptor)	88-95
CD34 (hematopoietic progenitor cell antigen)	60-70
α-SMA (alpha smooth muscle actin)	30-40
S-100 protein	5
Desmin	< 5

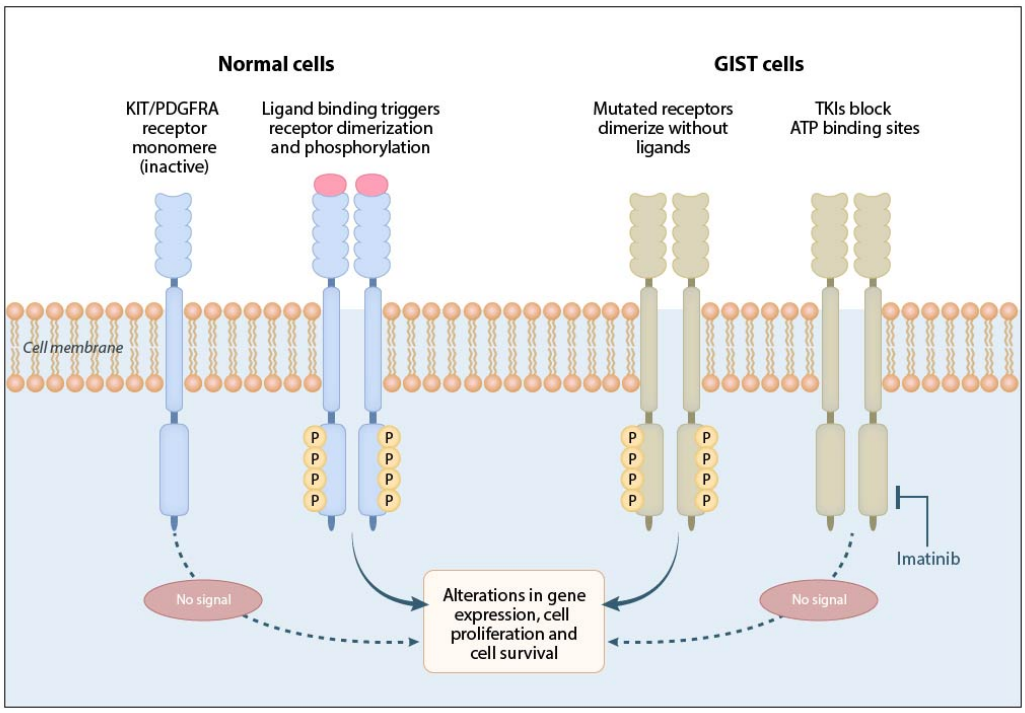

Figure 4. Tyrosine kinase receptor functions in health and disease. Left, Native KIT and PDGFRA are activated by their respective ligands, SCF and PDGF, which lead to receptor dimerization and subsequent activation. Right, in GIST cells, mutated receptors are constitutively activated independently of ligand binding. Tyrosine kinase inhibitors, like imatinib, fit into the ATP-binding pocket of the intracellular part of the receptor and hinder its activation.

Molecular pathology

Multicellular organisms regulate cell growth tightly. When a cell loses the ability to adjust itself to an organism's needs, it exhibits cancer hallmarks: constant proliferation, immortality, evasion of growth suppression, resistance to cell death, angiogenesis, invasion and metastasis, change in metabolism, and immune surveillance escape (Hanahan and Weinberg, 2011). The KIT molecule (stem cell factor receptor) is a growth factor receptor that belongs to the type III tyrosine kinase receptor family. The platelet-derived growth factor receptor types A and B (PDGFR-A and B), and colony stimulating factor 1 (CSF-1) also belong to the same group of receptors. The KIT protein is encoded by the *KIT* gene, which is located in chromosomal region 4q12 (www.ensembl.org); its extracellular domain consists of five immunoglobulin domains (Chan, 1999). The second and third loops bind the ligand, the stem-cell factor (SCF, also known as steel factor or mast cell growth factor). Once the receptor has bound the ligand, it undergoes homodimerization and autophosphorylation and activates downstream signaling (Blume-Jensen et al., 1991). The platelet-derived growth factor is the ligand for the PDGFRA receptor (Figure 4). The intracellular parts of the KIT and PDGFRA receptors consist of a juxtamembrane domain, a tyrosine kinase domain I (ATP-binding pocket), and a tyrosine kinase domain II (activation loop). The juxtamembrane region regulates dimerization, and mutations in this region disturb its normal function (Chan et al., 2003). Changes in the kinase II domain affect the activation loop that regulates the ATP-binding pocket of the KIT and PDGFRA receptors (Figure 5).

Hirota and co-workers published their breakthrough discovery of *KIT* mutations in GISTs in 1998. It is now well established that most GISTs (75-80%) have oncogenic *KIT* mutations (Hirota et al., 1998; Corless et al., 2011) that render the kinase constitutively active through ligand-independent self-phosphorylation (Figure 4). Oncogenic signals are passed on downstream by the active receptors mainly through the MAPK, PI3K/AKT, and STAT3 pathways, thereby promoting cell survival and proliferation (Figure 6) (Corless et al., 2011; Duensing et al., 2004; Rubin et al., 2007).

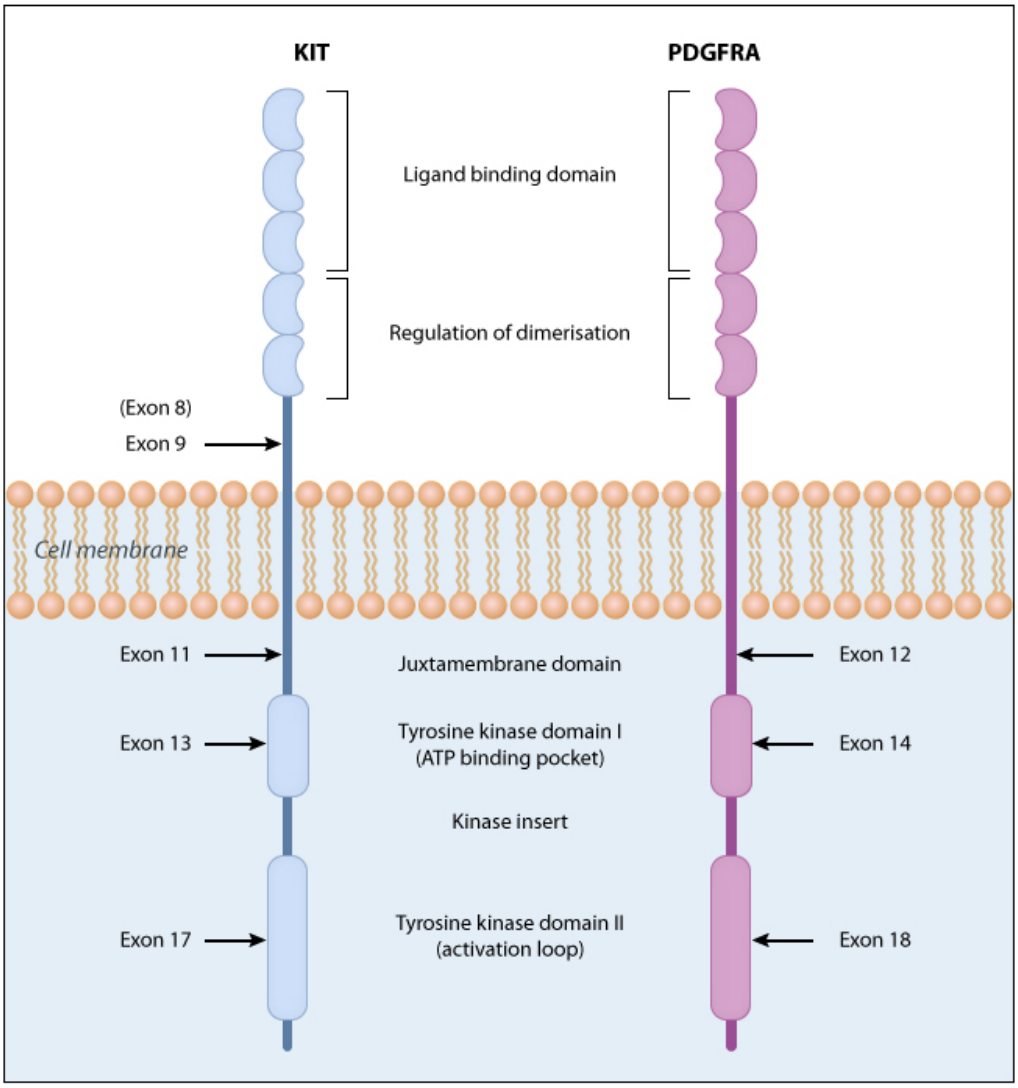

Figure 5. Topology and functional domains of the type III receptor tyrosine kinases KIT and PDGFRA. Corresponding exons with recurrent mutations in GISTs are indicated.

Mutation analysis is important in the clinical evaluation as a diagnostic tool, to predict the sensitivity to tyrosine kinase inhibitors, and for prognostic purposes (Figure 5, Table 2). Most *KIT* mutations affect the juxtamembrane domain encoded by exon 11 (65%) that promotes the activation loop to switch into its active conformation.

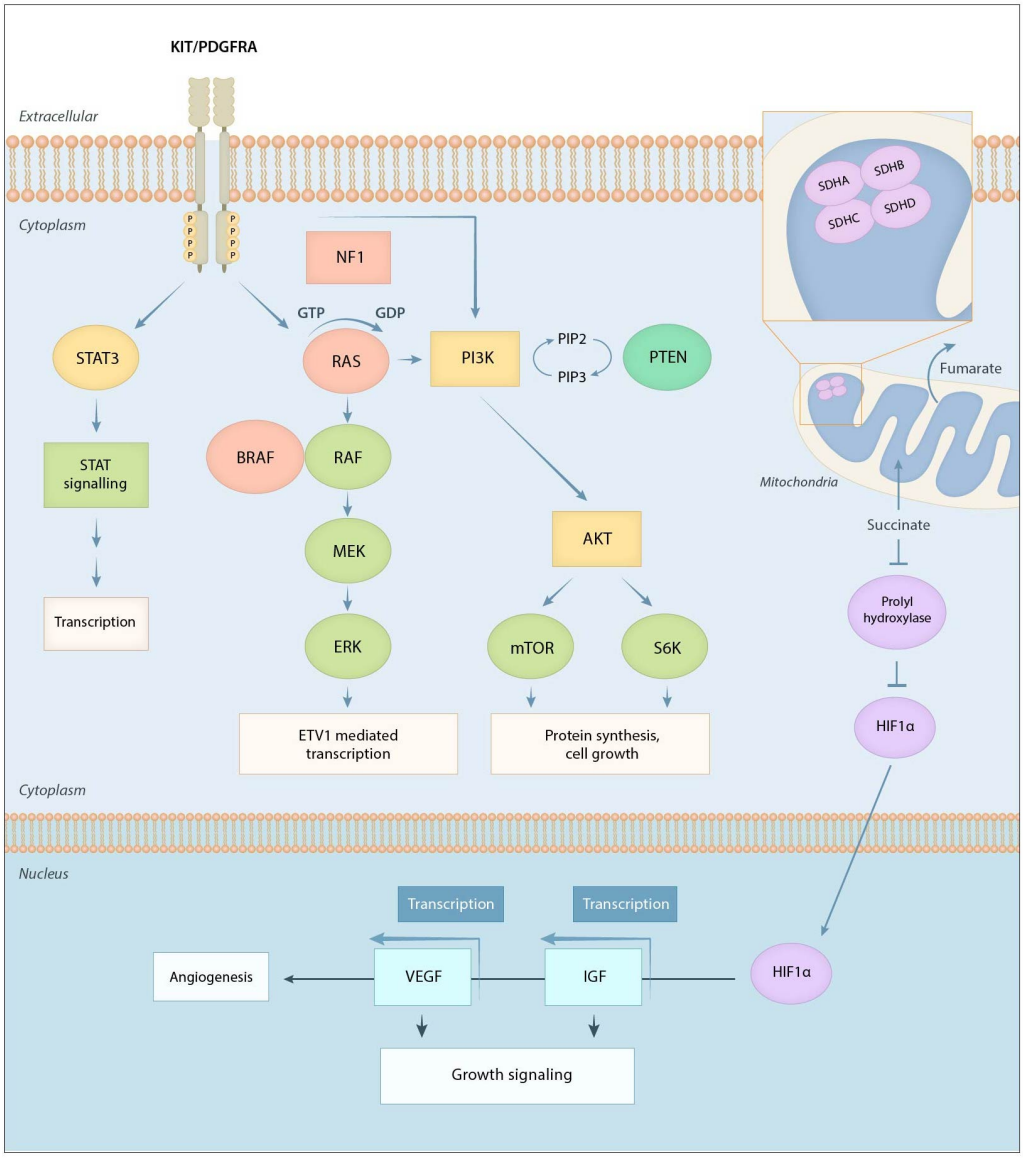

Figure 6. Signaling pathways in GIST. Typically, KIT or PDGFRA mutated receptors are constitutively active conferring signals through the MAPK (RAF, MEK, ERK), PI3K/AKT, and STAT3 pathways. Signaling through the MAPK pathway also maintains the ETV1 activity, a lineage survival factor regulating gene expression in GIST (Chi et al., 2010). A few GISTs, earlier classified as wtGISTs, contain mutations in *NF1*, *RAS*, and *BRAF*, resulting in MAPK activation downstream of the receptors. *SDHx* complex mutations result in higher HIF1α levels and increased *VEGF* and *IGF* transcription. Adapted and modified from Joensuu 2013 and Corless 2014.

Table 2. Molecular classification of GISTs.

Genetic type	Frequency (%)	Anatomical distribution
***KIT* mutation**	75-80	
Exon 8	Rare	Small bowel
Exon 9 (e.g. ins AY502-503)	8	Small bowel, colon
Exon 11 (deletions, insertions, substitutions)	65	All sites
Exon 13 (e.g. K642E)	1	All sites
Exon 17	1	All sites
***PDGFRA* mutation**	~10	
Exon 12 (e.g. V561D)	1	All sites
Exon 14 (e.g. N659K)	<1	Stomach
Exon 18 (D842V)	6	Stomach, mesentery, omentum
Exon 18 (other)	1	All sites
Wild-type *KIT* and *PDGFRA*	10-15	All sites
BRAF V600E	~2-7	
SDHA/B/C/D mutations	~6	Stomach and small bowel
HRAS, NRAS, and *PIK3CA*	<1	
Pediatric/Carney's triad	~1	Stomach
NF1-related	<1	Small bowel

(Adapted from Corless et al., 2011 and 2014).

Among the different types of exon 11 mutations, deletions stand out as conferring the poorest progression-free and overall likelihood of survival (Andersson et al., 2006). Mutations can also occur in the extracellular domain (encoded by exon 9), and they are believed to change the receptor conformation in a fashion similar to how the normal ligand binds (Lux et al., 2000). Interestingly, these mutations are almost exclusively restricted to GISTs in the small or large intestine, although there might be a population differences (Sakurai et al., 2001). Exon 9 mutations are critical because of their poor response to imatinib and higher tyrosine kinase inhibitor (TKI) dose requirements (Marrari et al., 2010). Exon 13 and exon 17 mutations are rare and possess variable imatinib sensitivity, but can be detected in recurrences and cases with imatinib resistance (Corless et al., 2011). *PDGFRA* mutations, homologous to those in *KIT*, have been discovered in about 30% of *KIT*-negative GISTs (*i.e.* 10% prevalence) (Hirota et al., 2003). *KIT* and *PDGFRA* mutations are mutually exclusive (Heinrich et al., 2003). Most *PDGFRA* mutant GISTs are indolent and

found in the stomach (Lasota et al., 2004). However, D842V substitutions are exceptions, which are known for primary imatinib resistance (Corless et al., 2005). Although the intracellular signaling pathways activated by PDGFRA are almost identical to those in *KIT*-mutant GISTs (Heinrich et al., 2003), the gene expression profile is distinct (Kang et al., 2005; Subramanian et al., 2004). The two tumor types are difficult to distinguish morphologically, but irrespective of mutation status they are DOG1 positive (West et al., 2004).

Approximately 10-15% of GISTs do not have mutations in either *KIT* or *PDGFRA*, and have been named wtGISTs. This heterogenous subgroup of GISTs still has phosphorylated active KIT and is clinically very similar to *KIT* and *PDGFRA*-mutated GISTs. Proposed mechanisms that drive these mutations downstream of the kinase receptors include: the *BRAF* V600E mutation, *NF1* mutations, *RAS*-family mutations (*HRAS, NRAS, KRAS*), or mutations in *SDH/A-D* (Agaram et al., 2008; Kinoshita et al., 2004; Janeway et al., 2011; Miranda et al., 2012). Mutations in *RAS, BRAF,* and *NF1* activate the MAPK pathway, while loss of the SDH complex function leads to accumulation of succinate, and subsequent upregulation of HIF1α transcription and its target genes (IGF1R and VEGF) (Figure 5). The majority of GISTs occur as sporadic tumors without known risk factors, but some are part of tumor syndromes. Germ-line autosomal dominant mutations in familial GISTs affect both *KIT* and *PDGFRA* exons, which leads to tumor development in the stomach or small intestine at early ages (Chompret et al., 2004; Nishida et al., 1998). These patients can also have altered skin pigmentation, ICC hyperplasia, and hematologic disorders. This underscores how constitutional mutations in the *KIT* gene also affect other cells where KIT is important (hematopoietic cells, melanocytes, and ICCs) (Chan, 1999). The non-hereditary syndrome, Carney's triad, is associated with gastric GISTs, paragangliomas, and pulmonary chondromas. GISTs associated with Carney's triad are wtGISTs, as they lack *KIT* and *PDGFRA* mutations but have *SDHx* mutations (Carney et al., 1977; Janeway et al., 2011). The germline mutation variant is called Carney-Stratakis syndrome with mutations in *SDHx* subunits (Carney and Stratakis, 2002; Dwight et al., 2013). This puts patients at increased risk for a dyad of GIST and paraganglioma. As already pointed out,

NF1 mutations lead to MAPK pathway activation. It is believed that almost one-tenth of patients with neurofibromatosis type 1 (von Recklinghausen's neurofibromatosis) develop KIT positive small intestine GISTs lacking *KIT* mutations (Takazawa et al., 2005). Furthermore, homozygous mutations and some chromosomal changes have been associated with GIST malignant progression and metastatic potential (Corless, 2011).

Risk stratification and prognosis

The clinical course of GISTs ranges from small indolent tumors to highly aggressive sarcomas. As in other malignancies, the key to choosing the correct treatment and follow-up strategies is to accurately determine disease grade, risk of recurrence and progression. Every GIST patient should be assessed by any of the commonly used risk stratification schemes (NIH consensus criteria, AFIP criteria, or modified NIH criteria), nomograms, or heat and contour maps, to determine which patients are likely to benefit from adjuvant therapy (Fletcher et al., 2002; Gold et al., 2009; Joensuu, 2008; Joensuu et al., 2012; Miettinen and Lasota, 2006; Rossi et al., 2011). At sarcoma center Karolinska, we use the modified NIH criteria to estimate the risk of recurrence after surgery (Table 3). The prognostic information is based on four factors: 1) tumor size, 2) mitotic count, 3) location, and 4) the presence of tumor rupture. In general, non-gastric large tumors with a high mitotic index have the poorest recurrence-free survival (RFS) (Joensuu, 2008). In the event of intraoperative tumor pseudo-capsule rupture, the patient is immediately at high risk of peritoneal relapse, and thus should be considered for adjuvant therapy. Other factors such as age, related diseases, and mutation status also influence the decision-making process for adjuvant therapy. Mutation status is not yet part of any risk stratification scheme, but it is important for choosing drugs and dosages. For example, *PDGFRA* D842V-mutated GISTs are not sensitive to imatinib and should not receive this adjuvant treatment, while exon 9 *KIT* mutated tumors should be treated with higher imatinib doses (800 mg daily), if tolerated.

Table 3. Criteria for risk-stratification of GIST recurrence after surgery.

Risk	Tumor characteristic			10-year RFS (%)*
	Diameter (cm)	Mitotic count (per 50 HPFs)	Location	
Very low	<2.0	≤5	Any	94.9
Low	2.1-5.0	≤5	Any	89.7
Intermediate	2.1-5.0	>5	Gastric	-
Intermediate	≤5.0	6-10	Any	86.9
Intermediate	5.1-10.0	≤5	Gastric	86.9
High	>10.0	Any	Any	36.2
High	Any	>10	Any	36.2
High	>5.0	>5	Any	36.2
High	2.1-5.0	>5	Non-gastric	36.2
High	5.1-10.0	≤5	Non-gastric	36.2

*Data from ten pooled population-based series.
HPF = high power microscope field. NIH = National Institutes of Health.
RFS = Recurrence-free survival.
If tumor rupture is present (regardless of size, count, location): High risk.
Modified NIH criteria. Adapted from Joensuu, 2008 and 2013.

Medical treatment

"I don't think we're going to hit home runs, but if we can get a series of line-drive singles going and put enough singles back to back, we can score runs."

- Dr. Leonard Saltz on imatinib treatment, Memorial Sloan-Kettering.

Considering that GIST is a rare tumor, which necessitates treatment planning by physicians from several specialties, it is important to organize the treatment around centers with multidisciplinary experience (Mullady and Tan, 2013). At our center, GIST treatment follows the Scandinavian Sarcoma Group (SSG) and the European Sarcoma Network Group guidelines (ESMO, 2012). Figure 7 summarizes the workflow basics.

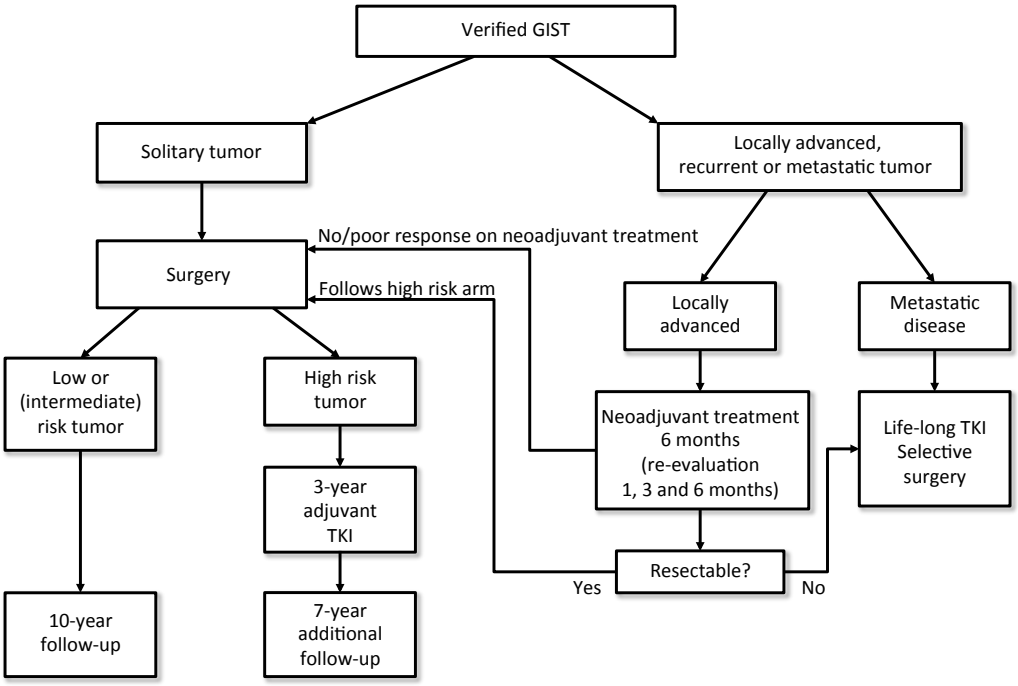

Figure 7. Schematic flow-chart for the treatment and follow-up of histologically verified GISTs.

GISTs emerged as a solid paradigm for receptor tyrosine kinase-induced tumors in 2002, when the Food and Drug Administration (FDA) approved the TKI imatinib for the treatment of advanced and metastatic GISTs (Dagher et al., 2002). In the pre-imatinib era, patients with advanced disease had a median survival rate of 10-20 months (Joensuu, 2002); imatinib treatment offers a prolonged survival of, on average, approximately 5 years, compared to historical controls. Imatinib was not designed for GISTs originally, but for chronic myelogenous leukemia (CML), which has the bcr-abl oncogene present in 95% of cases. The inhibitory effect by imatinib on bcr-abl colony formation was impressive, with a 92-98% response (Druker et al., 1996). Imatinib (also known as STI571, or Gleevec® in the US, Glivec® elsewhere) exerts its effect via competitive blockage of the ATP-binding site of bcr-abl, KIT, and PDGFRA receptors, which terminates downstream phosphorylation (Reichardt et al., 2011; Ricci et al., 2002).

Adjuvant therapy

The use of adjuvant treatment should be considered in all operable high-risk primary GISTs. A one-year adjuvant daily regimen of 400 mg imatinib was embraced for imatinib-sensitive, high-risk GISTs (categorized by for example modified NIH criteria), as the standard of care when the first randomized ACOSOG Z9001 placebo-controlled phase III trial showed convincing prolongation of RFS in comparison to placebo (DeMatteo et al., 2009). Three years later, a second randomized controlled trial on high-risk operable GISTs proved superiority of a 3-year adjuvant imatinib treatment over one year, with improved RFS and OS (Joensuu et al., 2012). The current adjuvant standard of care duration is therefore three years. However, the optimal adjuvant treatment duration is still unknown. Imatinib is generally well accepted by patients, but some develop more severe side-effects. The most common adverse effects, of any grade, include anemia, periorbital swelling, diarrhea, nausea, muscle cramps, fatigue, and low white blood cell count (DeMatteo et al., 2009; Joensuu et al., 2012). Most of these side-effects can be managed by symptomatic treatment

(Joensuu et al., 2011). If the recurrence risk is predicted to be low or very low, adjuvant therapy is generally not indicated. In patients with intermediate risk, there is still some ambiguity and individual assessments are necessary. Future studies are needed to clarify the best treatment options for this group of patients. About one-third of patients suffer from tumor relapse within two years of treatment completion. Most of them are still imatinib-sensitive, and imatinib re-challenge is therefore an option (Reid, 2013).

Mutation analysis

Mutational analysis has proven to be an important tool in clinical decision-making among primary GISTs, and is required for adjuvant therapy decisions. Information regarding mutational status provides prognostic information and knowledge about a GIST's sensitivity to TKIs. Imatinib is most efficient in exon 11 mutated tumors (Heinrich et al., 2003), but other mutations are also responsive (Corless et al., 2011). Oppositely, *PDGFRA* exon 18 D842V substitution mutated GISTs are usually imatinib-insensitive and do not benefit from adjuvant therapy, regardless of risk classification (Prenen et al., 2006). Moreover, the available randomized clinical trials have been too small for subgroup analysis to bring consensus among the true wtGISTs. WtGISTs are often more indolent tumors with lower sensitivity to imatinib. However, the sensitivity to imatinib may vary, and the benefit to high-risk wtGISTs has been difficult to foretell, which is why individualized clinical decision-making has been proposed on a case-to-case basis (Reichardt et al., 2012). Interestingly, clinical trials have only been conducted in the adjuvant setting with imatinib 400 mg/day, and exon 9-mutated GIST responses were suboptimal with this dosing. In the advanced disease setting, on the other hand, studies support an imatinib dose increase to 800 mg/day to induce a significantly higher response rate and prolonged progression-free survival (PFS) (Joensuu et al., 2012; MetaGIST, 2010). Some clinicians believe that this evidence is sufficient to use the same dose also in the adjuvant setting for exon 9 mutations, though this treatment strategy is not yet supported by any controlled trials (Heinrich et al., 2008).

Obtaining such evidence can be somewhat troublesome given the rarity of exon 9 mutations and the potential side-effects inflicted by the higher imatinib doses. Other tumor genotypes such as *SDHx* deficiency and *NF1* patients often have poor responses to imatinib (Chou et al., 2012; Yantiss et al., 2005). Primary imatinib resistance is seen in about 15% of GISTs (defined as progression within the first 6 months of treatment).

Advanced/metastatic GIST and imatinib resistance

The first-line treatment of locally advanced or metastatic GISTs is imatinib 400 mg/daily unless the tumor expresses imatinib-resistant gene mutations. The first imatinib-treated patient was reported in 2001, and had a complete metabolic response within one month (Joensuu et al., 2001). It is now known that tumor regression, durable stable disease, or both can be achieved in more than 80% of patients, and that tumor burden decreases in about 50% (Blanke et al., 2008; Demetri et al., 2002; Verweij et al., 2004). The long-term outcome seems to be similar between patients with stable disease and those with an objective response (Blanke et al., 2008). The use of imatinib should never be discontinued in advanced GISTs due to the high risk of progression within one year of cessation, excepting for those patients that experience serious/toxic side-effects (Le Cesne et al., 2010; Patrikidou et al., 2013). Unfortunately, even if the patients do follow the prescriptions carefully, the vast majority will still have disease-progression within two years while on therapy (Blanke et al., 2008; Verweij et al., 2004). The most common cause of TKI resistance is acquired secondary mutations in the *KIT* or *PDGFRA* receptors, which lead to interference with drug binding and subsequent overgrowth of mutated tumor clones. These secondary mutations develop almost solely in the same gene and allele as the initiating oncogenic mutation (Antonescu et al., 2005; Corless et al., 2011). Other studies have observed a significant intra- and inter-lesional heterogeneity of secondary mutations in progressing GISTs, which underscore the challenges faced in trying to treat patients when first-line imatinib fails (Liegl et al., 2008; Wardelmann et al., 2005). Sunitinib has approval as a second-line therapy for advanced GIST

patients presenting with imatinib-resistance or intolerance (Demetri et al., 2012). This is a small-molecule TKI that possesses multitargeted properties with inhibiting effect on *KIT*, *PDGFRA*, and *VEGFR*s. Median time to disease progression is prolonged from 1.5 months to 6.3 months compared to placebo (Demetri et al., 2012). More recently, the FDA approved the multikinase inhibitor Regorafenib for the third-line treatment of advanced GISTs with failure or intolerance to both imatinib and sunitinib, with a significantly increased median PFS from 0.9 months in the placebo group to 4.8 months in Regorafenib treated patients (Demetri et al., 2013).

As with imatinib treatment, GISTs commonly develop resistance over time also against these drugs. Several additional TKIs (e.g. Nilotinib, Sorafenib, Dasatenib) have been tested, but none have proven activity or results sufficient for clinical implementation. Masitinib is an oral TKI with encouraging results from a phase II study (Le Cesne et al., 2010), and is currently under investigation in the first-line setting in a randomized phase III trial. Another alternative in selected advanced GISTs progressing on imatinib is dose-escalation (Hislop et al., 2012).

Most imatinib-resistant GISTs are still dependent on tyrosine kinase signaling, as supported by preclinical studies (Heinrich et al., 2006). Based on the identified intracellular signaling pathways in GISTs (Figure 5) several new strategies targeting pathways downstream of the receptors are therefore under evaluation in clinical trials. According to ClinicalTrials.gov (a service of the U.S. National Institutes of Health), accessed April 12 2014, 19 agents are under investigation, including: a selective cyclin D inhibitor (PD-0332991), a Hsp90 inhibitor (AUY922), a MEK inhibitor (MEK162), multikinase inhibitors (masitinib, dasitinib, regorafenib, pazopanib, nilotinib, sorafenib, vandetanib), an immunomodulating agent (ipilimumab), a hypoxia-activated prodrug (TH-302), a hedgehog signaling pathway targeting agent (vismodegib), a gamma-secretion/notch signaling pathway inhibitor (R04929097), oral angiogenesis inhibitors, PI3K inhibitors (BKM120, BYL719), and BRAFV600E inhibitors (dabrafenib, trametinib).

Therapeutic drug monitoring and drug transporters

Another interesting aspect of imatinib resistance is imatinib trough concentrations. Plasma levels below 1100 μg/L four weeks after treatment initiation, are associated with shorter time to GIST progression than as compared to concentrations >1100 μg/L (Demetri et al., 2009). Eechoute and co-workers reported that the plasma imatinib concentration drops with up to 30% from baseline within three months on treatment (Eechoute et al., 2012). The mechanism by which this pharmacokinetic phenomenon happens is unknown, but the authors consider drug transporter expression as one possible explanation. Current evidence supports that imatinib therapeutic drug monitoring (TDM) may provide complementing information to clinical evaluation regarding efficacy, safety, and compliance. In patients with poor treatment response, major drug interactions, or unexpected observed toxicities, a dose adjustment may be needed (Judson, 2012; Teng et al., 2012).

A well-documented efflux pump is the p-glycoprotein (ABCB1 or MDR1), a member of the ATP-binding cassette transport superfamily, and is associated with multidrug resistance (Gottesman et al., 2002). Both ABCB1 and ABCC1 are expressed in roughly 75% of GISTs (Perez-Gutierrez et al., 2007; Plaat et al., 2002; Theou et al., 2005), which imply a possible role in drug transport. Although preclinical data suggest that the cellular over-expression of ABCB1 cause decreased intracellular levels of imatinib (Hamada et al., 2003; Widmer et al., 2003), it is not known whether this has functional consequences in GIST cells. It is also not known whether imatinib treatment induces over-expression of drug transporters. However, clinical observations indicate that imatinib dose-escalation to 600 mg or 800 mg/day can sometimes control advanced GISTs progressing on imatinib (Hislop et al., 2012). Imatinib is likely to be transported into cells by the OCT-1 influx protein, a member of the solute carrier superfamily (SLC) (Thomas et al., 2004). This was shown in CML cell lines and the tumoral expression of OCT-1 in CML patients and has been correlated with patient outcome. Similar drug transporter correlations are limited in GIST patients. We therefore set out to develop a protocol to measure the intracellular

imatinib concentration so that concentration measurements can be correlated with the drug transporter expression in GIST patients (**paper I**).

Neo-adjuvant treatment

An area in GIST treatment that is under rigorous investigation is whether selected patients can benefit from neo-adjuvant imatinib treatment. Several studies have shown promising cytoreductive effects on locally advanced GIST patients treated 2-6 months with imatinib before surgery, inducing tumor regression and a decrease in tumor vascularity. These effects have provided useful help to the surgeon during tumor resection, allowing for a less mutilating procedure (Eisenberg and Trent, 2011; Fiore et al., 2009; McAuliffe et al., 2009; Rutkowski et al., 2013). Knowledge about mutation status and tumor response early after neo-adjuvant treatment initiation is important for identification of patients likely to benefit from the treatment and for timely planning of surgical excision. We apply a six months long neo-adjuvant treatment at our center to minimize the risk of disease-progression during therapy.

Surgery of GIST

"After reviewing all facts, I am pleased to say that the operation is needed, that it shall provide excellent results."

- Dr. Michael DeBakey, surgical pioneer.

Even though targeted therapy has revolutionized the oncological treatment of GISTs it cannot clear the disease completely, perhaps because of persistent mature GIST cells or due to GIST stem cells (Agaram et al., 2007). To date, the only chance to be cured from a non-metastatic operable GIST is by complete surgical resection without tumor rupture (R0, *i.e.* surgical margins without any tumor cells) (Joensuu et al., 2012). Radical surgery can be achieved by open or minimally invasive surgery (Novitsky et al., 2006). Based on pooled cohorts of 2459 patients, about 60% of patients with operable GISTs are cured, with few recurrences after 10 years (Joensuu et al., 2012). Wedge resection is particularly common for small to medium-sized gastric lesions, and segmental resection for localized intestinal tumors. Resection of clinically negative lymph nodes is not needed in adults since the prevalence of lymph node metastasis is less than 1% (Everet and Gutman, 2008). Of note, in young and pediatric patients lymph node metastasis rate is higher (Agaimy and Wunsch, 2009). Tumor resection is commonly considered for tumors larger than 2 cm in diameter (ESMO, 2012; Joensuu, 2013). Colorectal GISTs, however, should only be operated on after having considered neo-adjuvant down-staging, which can allow more structure-preserving surgery and less technical challenges (Jakob et al., 2013). In metastatic disease, surgery might still fill an important role among selected patients, provided they are stable or have limited disease progression on TKI treatment. By removing TKI-resistant tumor clones, resistance to TKI therapy may be delayed or possibly even prevented (Bamboat and DeMatteo, 2014; Gronchi et al., 2007; Raut et al., 2006).

Monitoring

As previously emphasized, GIST patients should preferably be followed at experienced sarcoma centers, especially since there is not yet enough data to support one optimal routine follow-up policy. Institutions therefore have different monitoring preferences. Regardless of protocol used, all patients should be informed about the importance of therapy adherence, potential drug and food interactions, and how to manage side-effects, when put on TKI treatment.

Follow-up schedules for GIST usually include physical examination (PE), blood chemistry analysis, blood cell count, and imaging. In the randomized trials for adjuvant imatinib therapy, PE and blood biochemistry were monitored every 1-3 months while on therapy (DeMatteo et al., 2003; Joensuu et al., 2012). Imaging is usually performed of the pelvis and abdomen, since most relapses are found in the liver and/or peritoneum (infrequently in bone or lungs). Different imaging modalities can be used (e.g. CT/MRI/FDG-PET). CT is considered the gold standard of imaging in GIST, but FDG-PET represents the most sensitive technique for tumor staging and evaluating therapy response. At our center, we currently follow the ESMO imaging guidelines (2012) for high-risk patients (Table 4), with the highest propensity to recur within the first two years after adjuvant therapy discontinuation. Depending on the presence of side-effects and/or toxic effects, clinical monitoring can be motivated more frequently. The benefit of following low-risk GISTs is not established, but can be carried out at 6-12 month intervals for five years (ESMO, 2012). Very-low risk patients have so low risk for recurrence that routine follow-up is not automatically merited.

The follow-up schedules are based partly on expert opinions, and vary between studies and guidelines (Table 4). They may be replaced by more tailored approaches: for example, Joensuu and co-workers (2014) recently developed a mathematical model where they adjusted the timing of CT scans with the hazard of cancer recurrence with time. Their result showed that such adjustment could detect tumor recurrences earlier compared to current guidelines, and without using increased imaging frequencies (Joensuu et al., 2014). Routine MRI instead of CT is seldom performed due to limited access, but can be considered

especially in young patients to lower the accumulated radiation dosages. MRI at the same frequency as CT is considered to be equally efficient (Reichardt et al., 2012). Since most GISTs recur within the first two years after imatinib cessation it is justified to re-intensify the follow-up imaging during this period (Joensuu et al., 2013; ESMO, 2012). Recurrences occur only sporadically after more than 10 years of monitoring (Joensuu et al., 2012).

Free circulating DNA (fcDNA) has been possible to detect and quantify in plasma from GIST patients (Maier et al., 2013). However, currently, there are no known protein biomarkers used in the follow-up for detection of disease relapse, therapy responses, or disease progression in GIST patients, even though GIST cells have a neuroendocrine phenotype (Bumming et al., 2007; Erlandsson et al., 1996). In **paper II** and **paper III** the presence of functional cell secretion and protein secretome in GIST cells have therefore been evaluated.

Table 4. CT monitoring of GIST patients after surgery.

		# of CT scans[1]	
Guideline/study	Follow-up with abdominal and pelvic CT[3]	Year 1-5	Year 1-10
SSG XVII guidelines	6 m intervals for 5 y, then annually	10	15
ACOSOG Z9001	3 m intervals for 2 y, then 6 m intervals for 5 y	14	NA
ESMO 2012 guidelines[2]	3-6 m for 3 y, then 3 m intervals for 2 y, then 6 m intervals for 3 y, then annually	14-20	22-28
NCCN 2012 guidelines	3-6 m intervals for 3-5 y, then annually	8-20	13-25

Abbreviations: ACOSOG, American College of Surgeons Oncology Group; ESMO, European Society for Medical Oncology; NA, not available; NCCN, National Comprehensive Cancer Network; SSG, Scandinavian Sarcoma Group.

[1] One abdominal CT scan is considered to deliver an effective radiation dose of 8 mSv on average, corresponding to 3.3 years of natural background radiation (Davies et al., 2011).

[2] The ESMO guidelines acknowledge that the optimal monitoring schedule is unknown and exemplify how some departments choose to follow their patients.

[3] 0-3 years = during adjuvant imatinib therapy. >3 years = after imatinib cessation.

(Adapted and modified from Joensuu et al., 2014).

AIMS OF THE STUDY

The overall aims of the thesis were to elucidate the presence of a functional GIST cell stimulus-secretion mechanism, to determine the existence of a GIST secretome and its constituents, to measure intracellular imatinib concentrations, and to investigate the role of DOG1 in GIST cells.

The specific aims were:

- To develop a novel protocol for the measurement of intracellular imatinib concentration in *in-vitro* and *in-vivo* systems of GIST cells.
- To characterize stimulus-release coupling in GIST cells by confocal microscopy of cytoplasmic free $[Ca^{2+}]_i$ and luminometric measurements of extracellular ATP levels.
- To determine the existence of a GIST secretome and its protein composition from imatinib-sensitive GIST cells by applying shotgun proteomics.
- To examine the functional role of DOG1 pharmacological modulation on apoptosis, proliferation, and viability in GIST cells.

Below is a general introduction to the materials and methods used in **papers I-IV**. More detailed methodological descriptions are outlined in each original paper.

Patients and clinical material

The thesis includes imatinib concentration determinations on human plasma and tumor tissue specimens from three patients **(paper I)**. Plasma and tissue samples were snap-frozen to -80°C in conjunction with surgery and stored until further use. All samples were collected with informed consent and ethically-obtained approval from patients undergoing surgery for GISTs. Approval of the study was obtained from the regional ethical review board in Stockholm.

Established cell lines

Two established human GIST cell lines were used for functional studies: 1) the imatinib-sensitive GIST882 **(paper I-IV)**, and 2) the imatinib-resistant GIST48 **(paper I, IV)**. For method validation purposes in **paper II** and **paper III**, we used the murine insulin-secreting pancreatic β-cell line MIN6m9. We used HEK-293 (human embryonic kidney) cells for flow cytometric control experiments in **paper I** and **paper II**.

Experimental methods

Mutation detection

The British biochemist and Nobel Prize laureate Frederick Sanger developed the "dideoxy" chain-termination method for DNA sequencing, also known as the "Sanger method", in the 1970s (Sanger et al., 1977). By mixing chain-terminating fluorescent dideoxynucleotides (ddNTP) with deoxynucleotides (dNTP), polymerase chain reaction (PCR) creates DNA fragments of varying lengths that can be read as a chromatogram. Each curve then depicts a nucleotide in the DNA sequence. DNA sequencing can be used to search for genetic mutations, such as substitutions, insertions, and deletions, in selected genome regions. The Sanger method was used in **paper I** and **paper II** to identify and confirm the *KIT* mutation status of GIST882 and GIST48 cell lines. In **paper I**, tumor tissue specimens from three patients were searched for mutations in hot spot exons of the *KIT* and *PDGFRA* genes, using previously described methodology (Sihto et al., 2005). If no mutation was found in the *KIT* or *PDGFRA* genes, the tumor was considered wtGIST.

Luciferase-based detection of ATP release

Several techniques have been developed for analysis of cellular response and release events, for example electrophysiology and fluorescence imaging (Smith, Betz, 1996). Although powerful, most currently available methods are either cell-type dependent, invasive or restricted to being low-throughput. A commercially available assay to detect ATP release as an indicator of cell secretion, from large numbers of GIST cells *in vitro*, was therefore used **(paper II)**. The detection is based on luminescence and measures extracellular ATP in the 10^{-11} to 10^{-16} molar range within which the ATP release is the limiting component in the luciferase reaction (Figure 8).

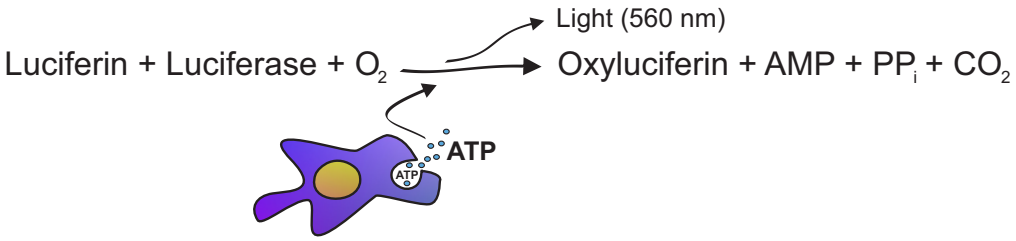

Figure 8. Schematic illustration of assay principle. Recombinant luciferase catalyzes the above reaction in the presence of ATP and oxygen.

Measurements of the emitted light enabled quantitation of extracellular ATP. Standard curves and usage of "blank" samples ensured a good calibration quality and low background to signal ratio. Several steps were taken to avoid biased measurements. To minimize biased ATP release from dying cells, the GIST cells were cultured sub-confluent. Cells were carefully washed to remove any loose cells or particles prior to static incubation in triplicates with seven different conditioned media. Supernatant was collected from flasks kept upside down, to avoid detaching of cells, and then centrifuged to obtain cell-free samples. The supernatant was analyzed within 5 min after collection with a multi-well plate reader at the 560 nm emission wavelength. To avoid technical variations the samples were measured twice. Stimulation of MIN6m9 β-cells with glucose increased insulin release and ATP accumulation, and served as method validation.

Lactate dehydrogenase (LDH) concentrations

The UniCel DxC 800 clinical system (Beckman Coulter) was used together with a LDH reagent in an enzymatic method to measure the reversible conversion of lactate into pyruvate by LDH. It monitors changes in absorbance at 340 nm during a certain time interval, which is correlated to the LDH activity. LDH concentrations were utilized as an indicator of possible cell lysis in **paper II**. The method, with a 0.1-45 μkat/L detection range, was performed at the routine clinical chemistry laboratory at the Karolinska University Hospital.

Flow cytometry

Flow cytometry is a biophysical method used for concurrent measurements of numerous physical characteristics at the single-particle level, commonly cells, as they flow in liquid suspension through one or several laser beams of light (Normal, 1980). The technique is routinely used today in both basic research and clinical practice. Cell properties that can be evaluated include relative size (detected by forward scattered light), relative granularity or internal complexity (detected by side scattered light), and relative fluorescence emission intensity. Every flow cytometer contains three main modules: the fluidic system that brings the particles to the interrogation point in the stream of fluid, the laser optics system which focuses the laser beams and directs the scattered and emitted light to appropriate detectors, and the electronic system that converts the detected light into electronic signals. Multiple software solutions exist for the computerized data processing. Flow cytometry was used to verify CD117 expression on the cell surface of GIST cells (**paper I**), and to assess single-cells stained with 7-aminoactinomycin D (7-AAD) (**paper II** and **IV**) and Annexin V-PE (**paper IV**).

Cytoplasmic free Ca^{2+} measurements

The bioimager BD Pathway^{TM} 855 (Becton Dickinson Biosciences, CA, USA) is an advanced optical system with motionless stage and movable optics for cell imaging in multi-well plates and high-content analysis. The system provides illumination possibilities across the spectrum from λ 340-750 nm wavelengths and 16 different excitation filters. Liquid handling, environmental control, and laser focusing, are all automated. Transmitted light enables the capture of bright field images. Fluorescence images can be acquired in both wide-field and spinning disc confocal modes, and the latter together with a long single-wavelength Ca^{2+} dye (X-rhod-1 AM) was used to detect cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) changes in response to different basal and stimulatory conditions in GIST cells (**paper II**).

Discovery proteomics

The proteome consist of all proteins expressed by the genome (Wilkins et al., 1996). Proteomics refers to the large-scale in-depth investigation of proteins, their expression, functions, and structures. More than 100.000 protein isoforms are expectedly expressed in human cells, and the human proteome is dynamic and variable at certain times, in different cell types and conditions (Gstaiger and Aebersold, 2009). Mass spectrometry (MS) is a commonly used analytical technique to identify and quantify proteins in biological samples (Domon and Aebersold, 2006; Nilsson et al., 2010; Geiger et al., 2012). There are two main spectrometry-based proteomics approaches: bottom-up and top-down. A bottom-up label-free discovery (also known as shotgun) proteomics strategy was used to examine the existence and composition of a GIST cell secretome (Figure 9) (**paper III**). Isolated proteins were first broken down into peptides by *in solution* tryptic digestion, which produces peptides amenable to ionization in the electrospray (ESI) source. Peptides can subsequently be analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which combines the physical separation properties of LC with the mass analysis capabilities of MS (Yates, 2009).

In the first part, the reversed-phase liquid chromatography column separates peptides based on their hydrophobicity, thereby reducing the sample complexity. Peptide separation also increases the chances of detecting low-abundant proteins that would otherwise be masked by high-abundant signals. The MS instrument usually contains three main components: an ion source, a mass analyzer and a detector (Figure 10). The mass analyzer in **paper III** was a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Scientific), which was coupled to a nano-electrospray ionization (ESI) source that ionized peptides eluted from the LC column. The Orbitrap separated ions based on their *m/z* (mass-to-charge ratios) resonance frequencies. This generated a mass spectrum; *i.e.* an abundance plot of all separated ions with ion counts (intensity) on the y-axis and *m/z* on the x-axis. Since mass spectra were recorded continuously over time during LC-separation, there was an indexation of each time point in the LC chromatogram to the single mass spectrum of that time point.

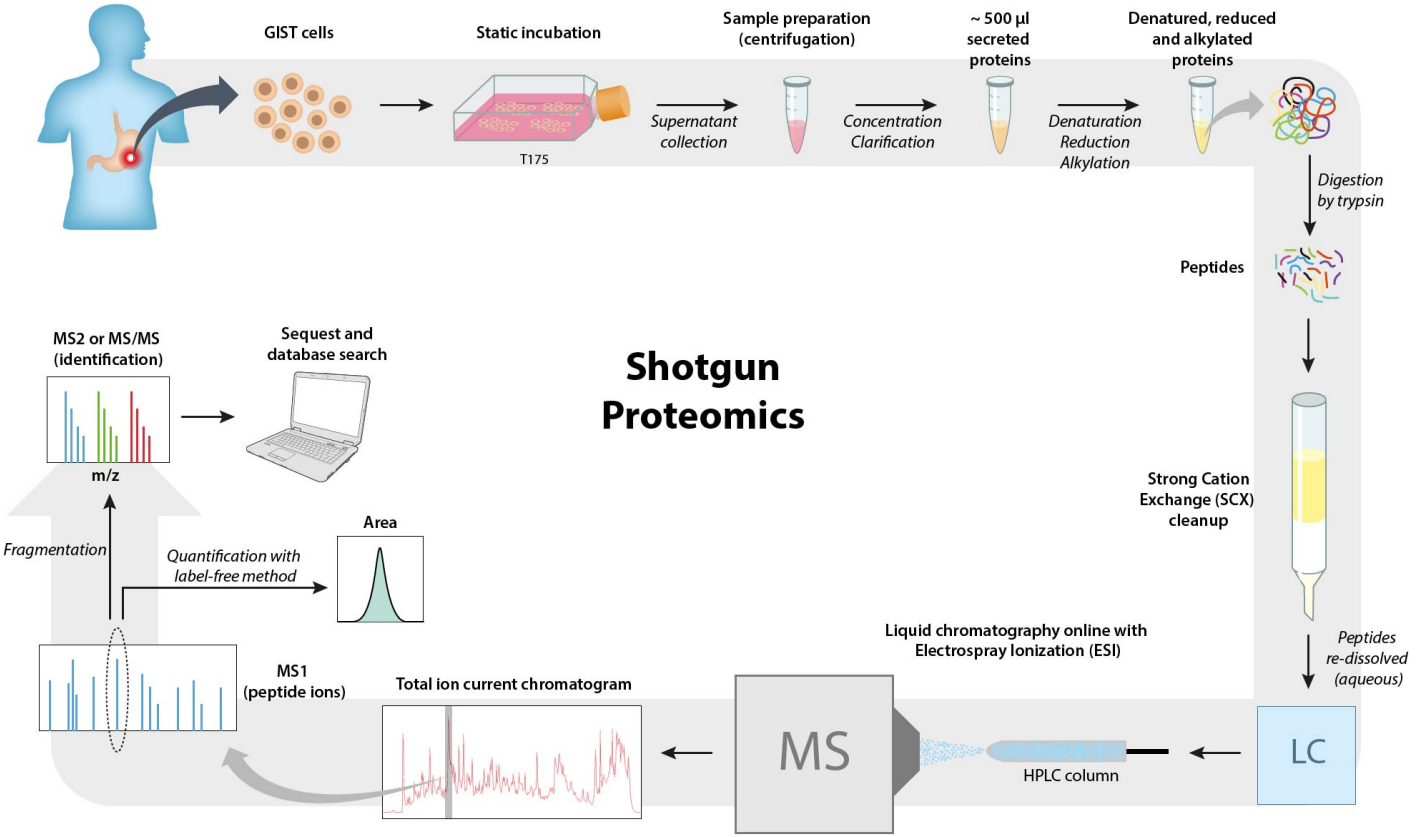

Figure 9. Workflow for label-free discovery proteomics. Conditioned media was processed from *in vitro* un-treated and treated samples to obtain a peptide mixture. Peptide fractions were cleaned up with SCX-SPE. LC-eluted peptides were coupled online to tandem MS-analysis, and the MS2 generated peptide spectra were used to infer proteins using the Proteome discoverer software. MS1 precursor peak areas were used for relative protein quantification. Data were normalized and quantitatively expressed as Log_2ratio. Results were validated by orthogonal methods.

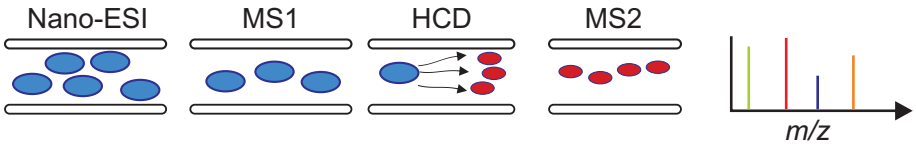

Figure 10. Illustration of tandem mass spectrometry modules. The nano-ESI ionizes LC-eluted peptides, and the mass analyzer MS1 separate the precursor ions before they were fragmented by higher-energy collision dissociation (HCD). The second mass analyzer (MS2) analyzes fragmented ions, and mass spectra were ultimately generated through detector measurements of ions, based on *m/z* ratios.

By matching the fragment ion spectra (MS2) against established databases of *in-silico* generated peptide sequences, the peptides were identified, and from there inferred the originating protein. This was carried out with the Sequest search algorithm and scoring scheme, which matched the theoretical peptide spectrum with the experimentally observed spectrum (Sadygov et al., 2004). The outputs were peptide-spectrum matches (PSMs) and associated scores, which reflected the degree of similarity between measured and predicted spectra. A 1% false discovery rate (FDR) at peptide level was accepted. Peptide quantification was obtained from measurements of precursor ion intensities in the first MS spectrum (MS1), which is considered to accurately determine low abundant protein quantities (Wiener et al., 2004; Old et al., 2005). To express quantitative changes each protein area was normalized to the total intensity of all identified proteins. Only proteins present in at least two biological replicates were included in the quantitative analysis, and expressed as Log_2 ratio to make the data normally distributed. Algorithms for zero values were avoided by setting an arbitrary value equal to half the minimum value among all normalized proteins if no peptide was identified in an experimental group.

To assess secretory properties among identified proteins classical secretory proteins (SP^+, endoplasmic reticulum/Golgi-dependent pathway) were predicted with a N-terminal signal sequence using SignalP 4.1 software. Proteins not predicted as classically secreted with SignalP 4.1 were assessed for non-classical secretion (SP^-) with SecretomeP 2.0 and Gene Ontology (GO). Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to see if

the identified proteins clustered into biological processes of interest. Proteins were also compared to the exosomal proteins reported in Exocarta.

Imatinib quantification

Imatinib concentration in two human GIST cell lines, in plasma and tumor samples from three GIST patients were determined as described in **paper I** in two steps: liquid-liquid extraction (LLE) and LC-MS (TOF) analysis (Figure 11). In brief, GIST cells cultured in monolayers were statically incubated for three hours with different imatinib concentrations. They were subsequently trypsinized, centrifuged, and stored as a cell pellet in -80°C. The clinical samples (plasma, adipose, and tumor tissues) were collected and prepared in conjunction to surgery, and stored at -80°C until further use.

Sample preparation prior to LC-MS analysis

Preparation of cell culture and tissue samples involved homogenization prior to the extraction. Cell culture samples were homogenized using sonication (3 mm probe (Sonics Vibra Cell, Chemical instrument AB, Sweden) at 30% amplitude) where tissue samples were frozen in liquid nitrogen and homogenized with mortar and pestle. Cell culture, plasma (100 µL) and tissue (50 mg) samples were spiked with a known amount of trazodone as an internal standard (IS). Imatinib was extracted into methanol. Plasma samples were thoroughly vortexed, and the tissue samples were disrupted by blending (Polyron PT 1200, Kinematica) to facilitate the extraction. Samples were centrifuged, and the resulting supernatants were transferred into new vials and dried under a gentle stream of nitrogen. The residues were re-dissolved in methanol:water (20:80, v/v) prior to the LC-MS analysis.

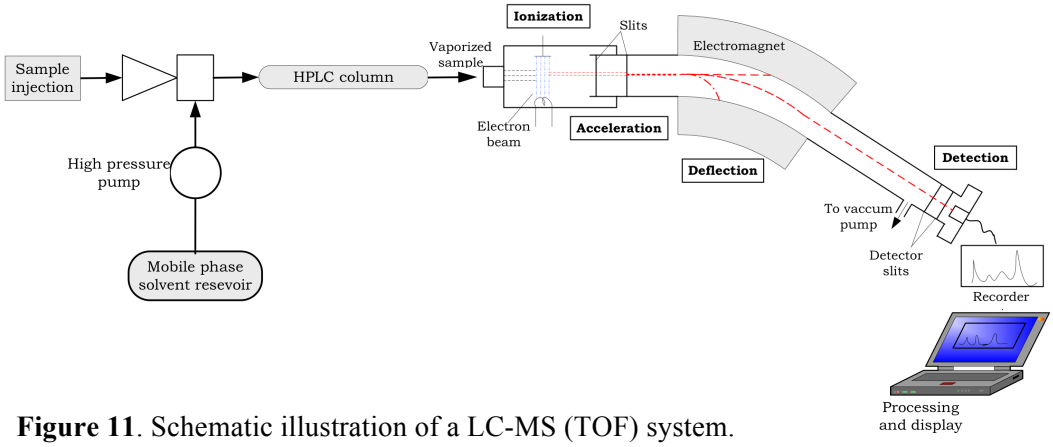

Figure 11. Schematic illustration of a LC-MS (TOF) system.

Determination of imatinib by LC-MS (TOF)

The LC-MS system contained the following parts: an Agilent model 1100 Auto-sampler, an Agilent 1100 Quaternary pump and an Agilent ZORBAX C18 (5 µm, 2.1x50 mm) reversed phase analytical column controlled by Agilent ChemStation software (Agilent Technologies, Palo Alto, CA, USA). The mobile phase ran at linear gradient containing 0.1% formic acid in both methanol and water. The chromatographic system was coupled to a time-of-flight (TOF) mass spectrometer (Agilent Technologies, CA, USA), equipped with electrospray ionization (ESI). The detection was performed in positive ion mode. The TOF mass analyzer used in this study is based on the principle that particles with different masses require different time to travel a given distance, under the assumption that they are charged with equal kinetic energy by the ESI. The ionization potential was 3800 V and ion source temperature was at 300 ºC. The nebulising gas was used for ESI at the rate of 15 psi. The voltages fixed at fragmentor, skimmer, and octopole guides were 225, 60, and 250 V, respectively. The ion pulser at the TOF analyzer was set at the measurement frequency of two cycles/s, and peak lists were achieved using the molecular feature extractor software "MassHunter" (Agilent Technologies, Santa, CA, USA). Extraction of total ion chromatogram for imatinib and trazodone were done with *m/z* ranges 494.15 – 494.3 and 372.1 – 372.2, respectively.

Quantification was inferred by comparing imatinib and IS peak areas. The results were obtained by mean value of triplicate analysis, and results were normalized to protein content.

Protein concentration determination

Protein concentrations were determined in GIST cell line samples and tissue specimens to enable imatinib normalization in **paper I**. Protein precipitates were homogenized, clarified and assessed by the Dot-it-Spot-it protein assay (Maple Stone AB) according to the manufacturer's instructions. The blackness intensity was quantified in each dedicated spot on the image with Image J (http://rsbweb.nih.gov/ij/). Protein concentrations were estimated by comparing the sample results with the results from a calibration curve using Rodbard curve fitting in Image J.

Insulin concentration determination

In **paper II** and **paper III** the ArcDia 2-photon fluorescence excitation microparticle fluorometry (TPX) assay for insulin content (Arc-Dia Group, Turku, Finland) was used as an orthogonal method to validate data obtained from ATP measurements and discovery proteomics experiments in MIN6m9 cells. The assay was performed according to the manufacturer's instructions, using a recombinant human insulin standard to determine the insulin concentration.

Western blot

Western blot, also known as immunoblot, is an analytical antigen-antibody mediated reaction method used for the detection of specific proteins of interest. Protein-containing samples are first electrophoretically separated in polyacrylamide gels based on their respective molecular weights, where smaller polypeptides travel faster than large through the gel. Next, the proteins are transferred to nitrocellulose membranes, and non-specific protein binding is blocked by the addition of non-fat milk. At this point, the protein of interest is targeted by a primary antibody, which in turn binds an enzyme-linked

horseradish peroxidase secondary antibody (used in this thesis). The resulting chemiluminescence signal is detected visually on a film or by a scanner. We used Western blot as an orthogonal method in **paper III** to verify the shotgun proteomics data.

Immunocytochemistry

Immunocytochemistry is an analytical method dependent on a specific antigen-antibody reaction at the protein level. Before conducting functional experiments on DOG1 in **paper IV**, stable expression of DOG1 and CD117 was verified in both the GIST882 and GIST48 cell lines. Cultured cells were harvested, washed, spun onto microscope slides, air-dried, and fixated in paraformaldehyde. After peroxidase blocking, the cells were incubated with CD117 and DOG1 antibodies. Next, the EnVision™ Detection Systems Peroxidase/DAB was applied on the cells. Cell nuclei were counter-stained with Mayer's Haematoxylin, and replacement of primary antibody with antibody diluent was used as negative control.

Confocal laser scanning microscopy

In contrast to conventional microscopy that illuminates the whole specimen, a confocal laser-scanning microscope (CLSM) illuminates one spot in the focal plane of interest, and images are acquired by sample raster-scanning with the focused laser beam. An optical arrangement of the objective, tube lens, and pinhole diaphragm essentially allows only light from one spot in the focal plane of interest to pass and be detected by the detector (Figure 12). Light from out-of-focus regions of the cells is thereby prevented, creating high-resolution images of reflected or fluorescence light. In **paper IV**, GIST cells cultured on coverslips were washed, fixated, permeabilized, incubated with primary antibodies against DOG1 or CD117 and with fluorescently labeled secondary antibodies, to investigate their respective expression and subcellular locations. DAPI (4,6-Diamidino-2-Phenylindole) was used for cell nuclei visualization. CLSM imaging was performed using a LSM510 ConfoCor3 instrument.

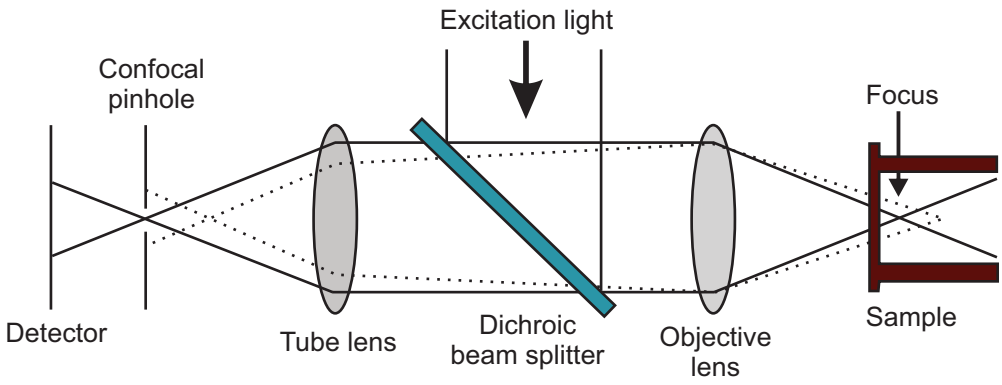

Figure 12. Confocal microscopy principles (adapted and modified from the LSM510 ConfoCor manual, Carl Zeiss).

Fluorescence of TRITC and Cy3 tags on the secondary antibodies was excited at 543 nm and emitted light was separated, spectrally selected, and recorded with an avalanche photodiode. DAPI fluorescence was excited using the 365 nm line of a mercury lamp, spectrally selected and detected in a semi-confocal arrangement using a photomultiplier tube (PMT).

Cell proliferation studies

In **paper IV**, the proliferation, viability, and cytotoxicity of exponentially growing GIST cells were assessed in the presence of different pharmacological agents, by the commercially available colorimetric WST-1 assay. WST-1 is a stable tetrazolium salt that is cleaved by mitochondrial succinate-tetrazolium reductase into a water-soluble formazan, which can be quantitated spectrophotometrically in a multi-well plate reader format. The amount of formed formazan is directly correlated to the number of viable, proliferating cells.

Electrophysiology

Patch-clamp is an electrophysiological method for the study of ion fluxes across biological membranes in single cells. The technique was first described in current recordings from denervated frog muscle fibers (Neher, Sakmann, 1976), and the inventors Erwin Neher and Bert Sakmann were awarded the Nobel Prize

in Physiology or Medicine fifteen years after the publication of their original work. The technique was improved in 1981, enabling high-resolution current recordings from single ion channels (Hamill et al., 1981). A central step to obtain such high-resolution recordings is an electrical tightseal, typically exceeding 10^9 Ω, between the glass pipettes and the cell membranes. Patch-clamp can be carried out in one of two modes: voltage-clamp (VC) or current-clamp (IC). In the VC mode, the voltage of cells or membrane patches is clamped while measuring the ion-channel currents, and *vice versa* in IC. All configurations that can be obtained originate from the "cell-attached" tightseal mode (Figure 13), and the configuration of choice is dependent on the research question to be answered. In cell-attached configuration, channel recordings are possible from ion channels encircled by the pipette tip, with the alternative to alter the extracellular solution while not affecting the internal cell milieu. Glass pipette withdrawal at this stage creates an excised patch with the cytosolic side facing the bath solution ("inside-out"), which can be modulated during the experiments. Applying mechanical suction through the pipette in cell-attached mode ruptures the plasma membrane only beneath its tip, and creates the necessary connection between the pipette and intracellular compartment for "whole-cell" recordings.

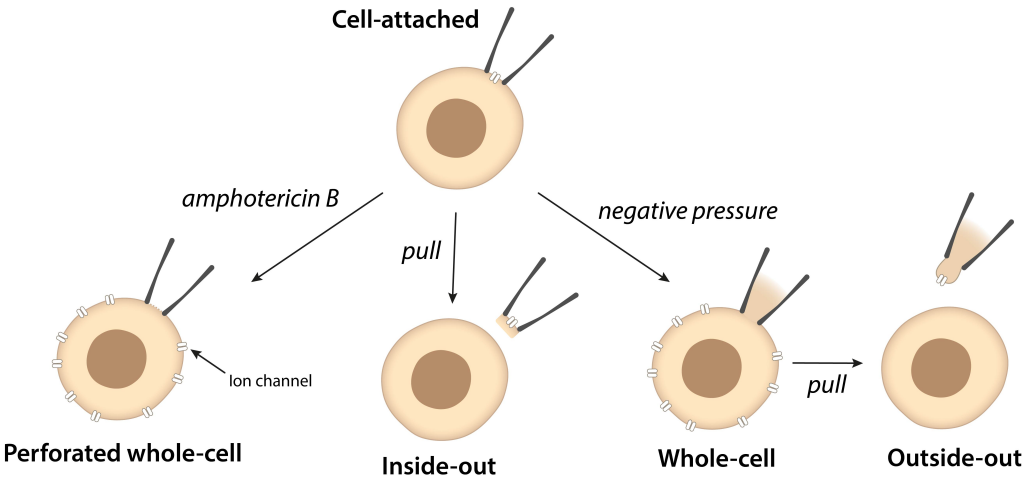

Figure 13. Patch-clamp configurations.

One disadvantage with this configuration is a second-fast dilution of cytoplasmic constituents. By using pore-forming antibiotics in the pipette, like amphotericin B or nystatin, electrical access to the whole cell can be obtained while preserving the intracellular milieu ("perforated whole-cell"). Lastly, the "outside-out" mode is the most difficult but can be obtained from the "whole-cell" configuration, and is suitable for the study of receptor-operated ion channels. In **paper IV**, GIST cells were voltage-clamped at -60 mV in a whole-cell configuration and depolarized for 600 ms in +20 mV increments until +100 mV, to study Ca^{2+}-activated Cl^- channels (DOG1) in GIST cells. Ion currents were measured at every step and the channel recordings were displayed according to the convention that upward deflection denotes inward current. Desired free Ca^{2+} concentrations were calculated with the MaxChelator software (Stanford, USA). All experiments were performed in room temperature. By fitting the data to the logistic regression *Hill* model the dose-response relation of a chloride channel blocker could be assessed in detail:

$$\frac{I}{I_c} = 1 - \frac{L}{1 + \left(\frac{[X]}{IC_{50}}\right)^{-h}}$$

where [X] is the concentration of the compound, L is the maximal inhibition caused by the compound, IC_{50} is the concentration of [X] causing half-maximal inhibition and h is the slope parameter (Hill coefficient). The I/I_c represents the relative whole-cell current at +100 mV, expressed as the ratio of mean current found during (I) and prior to (I_c) the addition of the test drug.

Paper I - Intracellular concentration of the tyrosine kinase inhibitor imatinib in gastrointestinal stromal tumor cells.

The imatinib success story in the treatment of GIST has not only relied on its ability to block the KIT and PDGFRA receptors, but also on its favorable pharmacokinetic properties. Imatinib has a high bioavailability irrespective of dose and route of administration (~98%) (Peng et al., 2004). The $T_{1/2}$ is around 18 hours, which makes once daily dosing appropriate. Imatinib is metabolized by the cytochrome P450 system in the liver and CGP-74588 is an active metabolite that is mostly secreted in the bile (urinary elimination is low). Age, ethnicity, gender and weight do not seem to impact the pharmacokinetics significantly (Peng at al., 2005). However, Eechoute and co-workers (2012) have reported that the plasma imatinib concentration drops for unknown reasons with up to 30% within three months after treatment initiation. They proposed changes in drug transporter expression as one possible explanation for this phenomenon, which raises the question if the drug distribution and possibly drug response can also vary within GIST tumors. To date there has been no readily accessible way to obtain regional pharmacokinetic/pharmacodynamic information from GIST tumors. Thus, it is not known if a similar pharmacokinetic phenomenon, as the one observed in plasma, occurs within the tumor. Neither is it known if the imatinib plasma concentration correlates to the intracellular concentration of the drug, and if the risk of local recurrences, metastasis or tumor resistance could be partly explained by differences in intracellular imatinib levels. It is important to keep in mind that imatinib requires access to its intracellular target, the ATP-binding site of the tyrosine kinase receptors, probably through active uptake (White et al., 2006), to exert its inhibitory effects. Also, some multidrug

transporter (efflux) proteins have high affinity for TKIs (Ozvegy-Laczka et al., 2005).

We have developed a liquid-liquid extraction (LLE) LC-MS (TOF)-based method for intracellular imatinib concentration measurements in *in vitro* and *in vivo* GIST cell systems. The analytical protocol was validated using cell lines, and extended to measurements of imatinib in plasma, tumor, and adipose (reference) tissues from patients. Calibration curves were created by plotting the peak area of imatinib normalized to internal standard (IS) peak area versus the nominal concentrations of imatinib. The linearity was determined by linear regression analysis and the correlation coefficient (r^2) of imatinib (30-7000 ng/mL). R^2 was 0.994. Quality control (QC) samples were prepared in three concentrations for intra- and inter-assay imatinib validation (100, 1000, 2 000 ng/mL). The variation coefficients were less than 8% with an accuracy ranging from 93 to 110%. Patient samples were analyzed in triplicates and imatinib peak area was normalized to IS peak area and all the measurements were calculated from the calibration curve. The limit of detection was set three times above the baseline separation (single to noise >3). The limit of quantification was calculated from the standard curve where single to noise >6. Calculated imatinib recovery was > 90% (unpublished data).

Clinically relevant imatinib incubation doses were chosen for the *in vitro* experiments, and significant differences were detected between the imatinib-sensitive and resistant cells. Imatinib was also determined in plasma, tumor tissue specimens, and fat (reference tissue) in clinical samples from three patients. There appeared to be a high degree of drug accumulation in the tumors, which cohere well with the volume of distribution (V_z/f) of imatinib in previous pharmacokinetic studies (Gschwind et al., 2005). As expected, the imatinib levels were lowest in the reference tissue (fat), but showed regional accumulation in the tumors, with highest levels in the periphery. It is plausible that disease-related factors, such as drug transporters and intratumoral heterogeneity can result in the observed variable drug distribution (Saunders et al., 2012; Wardelmann 2006). Our analytical method also encompasses any possible influence on drug levels mediated by the tumor stroma (Davids et al.,

2013), since both stromal cells and tumor cells are analyzed in the tumor specimen. This study was performed as a methodological proof-of-concept study and transporter expression was thus not studied. However, multidrug transporter expression can be detected in the majority of GISTs (Plaat et al., 2000; Perez-Guierrez et al., 2007; Theou et al., 2005). A correlation between imatinib drug transporters and patient outcome has already been established in CML (Thomas et al., 2004). Whether such correlations exist for tumor cells from GIST patients is not known. If a correlation will be found, no current straightforward approach exist to overcome multidrug resistance, and Higgins (2007) therefore suggested that implementation of strategies to avoid multidrug resistance may be more efficient than trying to overcome the resistance once it has occurred.

Based on these findings and current literature larger clinical studies are encouraged to correlate drug transporter expression over time with intracellular imatinib levels in tumor tissues to understand if drug transporters impact pharmacodynamics in GIST. At our center we have already started to include patients for a larger trial. From a clinical perspective, intracellular imatinib measurements may also be useful in selected patients. Trough imatinib plasma levels have been correlated with PFS (Demetri et al., 2009), and *KIT* exon 9 mutated GISTs with higher imatinib dose requirements. TDM can be used to assess compliance, drug interactions, and to reduce adverse effect rates (Teng et al., 2012). If our technique could be adapted for measuring imatinib concentrations from FNAs, analysis of drug concentration in close vicinity to its actual point-of-action may be more useful than plasma-level measurements, especially as a tumor- and location-dependent imatinib uptake is shown. Moreover, patients who do not respond to imatinib, while pharmacogenetic interpretation expects a sensitive tumor, may have altered uptake and/or efflux mechanisms. In these selected cases an early dose increase or switch to another TKI could be beneficial. Intracellular imatinib concentration determinations could in this way be used as a tool to complement other follow-up strategies, TDM, and imaging modalities, to better reflect tumor biology.

Paper II - Evidence for Ca^{2+}-regulated ATP release in gastrointestinal stromal tumors.

ICCs are believed to be the progenitor cells of GIST, and they share several features, such as high KIT and DOG1 expressions. The spontaneous rhythmic oscillations responsible for motor activities in the GI tract are Ca^{2+}-dependent, initiated by the ICCs, and then propagated to the surrounding smooth muscle cells. High conductance Ca^{2+}-activated chloride (Cl^-) (TMEM16A) currents have been suggested as one important generator of slow wave activity in ICC. The slow wave amplitude and frequency is regulated partly by inhibitory and excitatory enteric neurons utilizing purines, nitric oxide, acetylcholine, substance-P, and TRPCs, among others (Sanders et al., 2011). ICCs also have cell surface receptors for serotonin (5-HT) that can modulate the pacemaker activity (Liu et al., 2011; Shahi et al., 2011).

A functional similarity between GIST and ICC was proposed when oscillations and ion channels necessary for pacemaker activity were found also in GIST (Furuzuno et al., 2006). GISTs show indication of a neuroendocrine phenotype through the expression of exocytotic proteins required for cell secretion and stores of synaptic-like vesicles (Bümming et al., 2007; Erlandsson et al., 1996). However, it is not known if GISTs contain a functional regulated secretory pathway. In many cell systems, ATP is stored at high concentrations within secretory vesicles and co-released during exocytosis (Aspinwall and Yeung, 2005; Leitner et al., 1975). The identification of signaling pathways plays an important role in understanding the mechanism of cellular response. The key oncogenic mutations of *KIT* and *PDGFRA* in GIST cause dysregulation of PI3K-AKT, MEK-MAPK, and STAT signaling pathways (Duensing et al., 2004; Rossi et al., 2006; Rubin et al., 2001), involved in cell cycle, protein translation, metabolism, apoptosis (Liegl-Atzwanger et al., 2010), and potentially cellular secretion (Jiang and Wong, 2013; Yano et al., 1993). In **paper II**, ATP was used as a universal tracer to evaluate cellular response and secretion events in GIST cells during stimulation with KCl, thapsigargin, acetylcholine, serotonin, substance-P, and low $[Ca^{2+}]_e$ levels. The method of measuring cell response and

secretion was validated in MIN6m9 cells, showing co-secretion of insulin and ATP upon glucose stimulation.

In many cell types, $[Ca^{2+}]_i$ have a pivotal role in triggering cell secretion (Jahn and Fasshauer, 2012). In GIST882, modulators of $[Ca^{2+}]_i$, thapsigargin, charbachol, and KCl, had a strong influence on ATP release. Serotonin and substance-P, on the other hand, was not able to augment the ATP response. By choosing the two compounds with the most profound effect on ATP release, thapsigargin and KCl, we confirmed their ability to increase $[Ca^{2+}]_i$. Thapsigargin's and Carbachol's effects can partly be explained by the TRPC channels expressed in GIST (Furuzuno et al., 2006). Carbachol has an ability to activate non-selective cation channels (like TRPCs) via muscarinic receptors (So et al., 2009; Tsvilovskyy et al., 2009), and thapsigargin has an ability to block the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPas (SERCA) pump and activate store-operated calcium entry (SOCE) via TRPCs. To confirm that the source of ATP was not related to cell lysis or compromised cell membrane integrity, trypan blue exclusion staining, lactate dehydrogenase measurements, and flow cytometric analysis of 7-AAD in the control and thapsigargin treated groups were performed. No differences were found, indicating preserved cell integrity and active ATP release. To strengthen the hypothesis that the increase in ATP release was indeed Ca^{2+}-regulated we excluded extracellular Ca^{2+}, which markedly reduced the thapsigargin stimulatory effect. Furthermore, the effects on ATP and $[Ca^{2+}]_i$ by high doses of KCl supported an electrically active cell secretion, indicating that the previously found K^+ and Ca^{2+} channels are probably functional in GIST (Furuzuno et al., 2006; Suehara et al., 2008; Yamaguchi et al., 2008). The expression of substance-P and serotonin receptors in ICC has been found to have modulatory effects on pacemaker currents. So far no study has demonstrated the existence of substance-P and serotonin receptors in GIST, and no effect of serotonin and substance-P analogues on ATP release could be detected.

From these findings it is concluded that GIST cells have, as ICCs, a functional intracellular Ca^{2+} signal transduction pathway. The signal transduction leading to ATP release is, at least partly, dependent on $[Ca^{2+}]_i$ since several modulators of

$[Ca^{2+}]_i$ affect the release in GIST. Understanding the release of ATP in GIST cells may be useful for dissecting the signaling network, mapping exocytotic components, and possibly for drug development. Besides being a useful marker for cellular response, release of ATP from GIST cells may have importance for tumor tissue homeostasis and immune surveillance escape (Burnstock and Lavin, 2002; Pellegatti et al., 2008).

Paper III. Secretome protein signature of human gastrointestinal stromal tumor cells.

In spite of major improvements in clinical management, GISTs do still have a significant mortality due to metastasis and recurrences. There is an unmet need for reliable follow-up modalities that can detect disease relapse, therapy responses, and disease progression. Tumor-specific secreted, shed or leaked proteins (collectively known as secretome) are considered promising sources for biomarkers, and suitable for detection in biofluids. MS-based proteomics analyses of conditioned media (CM) collected under well-defined conditions have aided biomarker discovery significantly, and generated several candidates that have also been validated in clinical samples (Dowling and Clynes, 2011). Cell cultures are *in vitro* systems that differ at the molecular level from *in vivo* situations. Although this makes direct correlations between CM and clinical samples difficult, it enhances the chances of identifying secretory proteins compared to complex clinical specimens by lowering the protein dynamic range and protein diversity. Also, GISTs are rare tumors (Nilsson et al., 2005; Zahm and Fraumeni, 1997), which make biomarker validation complex. Biomarker discovery can therefore be facilitated by analyzing proteins (secretome) released into CM.

As previously discussed, GIST cells possess a neuroendocrine phenotype. In **paper II** we found an intracellular Ca^{2+}-dependent ATP release from GIST cells, reflecting functional cell release. The purpose of this study was to extend the hypothesis regarding the presence of cell secretion and determine if a cancer

protein secretome exists in GIST cells. Experiments were carried out *in vitro*, and *KIT*-mutated imatinib-sensitive GIST882 cells were kept shortly in one of four conditions (non-stimulated, KCl, thapsigargin, or imatinib pre-treatment followed by thapsigargin stimulation) before the supernatants were collected for purification and LC-MS/MS analysis.

The methodological approach was first validated in a murine insulin-secreting pancreatic β-cell line (MIN6m9) with a known secretory signature in response to glucose stimulation. Following static incubation for 30 minutes, 656 proteins were identified in total by LC-MS/MS analysis. The proteins were classified on the basis of quantitative changes ($Log_2R \geq 1.0$ or $Log_2R \leq -1.0$) and statistical significance. By comparing results from low (0.1 mM) and high (25 mM) stimulatory glucose conditions an increased release of a subset of 15 proteins was identified. The majority of proteins in this subset were secreted through the classical secretory pathway, known for being localized in the insulin granules and to be co-secreted with insulin. The results were confirmed with the orthogonal ArcDia TPX insulin assay method, verifying increased extracellular insulin levels upon glucose stimulation. Based on these findings it was concluded that the shotgun proteomics experimental approach was suitable for exploring the composition of the putative protein secretome from statically incubated GIST cells.

The GIST882 cell secretome was investigated following similar principles as for MIN6m9. In total, LC-MS/MS identified 764 proteins in the conditioned media from all groups combined. 555 proteins were left for quantitative analysis after excluding proteins not found in at least two biological replicates. These proteins were spread into the following groups, according to secretory properties: 9.9% classically secreted (SP^+), 39.8% non-classically released (SP^-), and 64.9% of exosomal origin. There was a major overlap between non-released, SP^+, and SP^- proteins with the proteins of exosomal origin. Exosomes have been assigned a potential source for biomarkers (Thery et al., 2002). They have also been shown to impact tumor physiology, pathogenesis, intercellular communication and disease progression in several cancers, including tumors of the GI tract (Ji et al., 2013) and sarcomas other than GIST (Camussi et al., 2013; Kim et al., 2002;

Miller et al., 2013). The possibility of extracellular vesicle existence in GIST adds another complexity to the analysis warranting further investigations. We have therefore initiated a project to specifically isolate, purify, and analyze exosomes and microvesicles (MVs) (collectively known as extracellular vesicles, EV) from GIST cells, with respect to their protein and RNA contents.

In un-stimulated samples (culture medium), 375 proteins were found. The secretory proteins (9.4% classically and 40.9% non-classically released) clustered according to DAVID into functions responsible for *e.g.* regulation of apoptosis, cell growth, MAP kinase activity, immune response, development and response to wounding, organic substance, hormone stimulus, oxidative stress, endogenous and external stimuli. By comparing the stimulated groups with the un-stimulated group, subsets of proteins being significantly released upon stimulation were revealed. In **paper II**, KCl and thapsigargin effectively triggered a brisk $[Ca^{2+}]_i$ increase and concurrent ATP release. Since the secretion is dependent on $[Ca^{2+}]_i$ (Jahn, Fasshauer, 2012) in a number of tissues we therefore hypothesized that stimulation of the GIST cells with the same conditions used in **paper II** would maximize the chances of analyzing secretory proteins. KCl-stimulated samples contained 398 proteins, whereof 50.3% were secretory (9.8% classically and 40.5% non-classically released). The induced quantitative changes were minor with only five proteins significantly increased compared to un-stimulated samples. The majority of these have previously been described in cancers of abdominal origin.

In the secretome analysis using thapsigargin as stimulation, 432 proteins were identified in all replicate experiments, whereof 50.2% were secretory by either classical (10%) or non-classical (40.3%) mechanisms. The majority of significantly elevated proteins in this group have already been acknowledged in other tumor types. The greater effect observed with thapsigargin than with KCl treatment can possibly be explained by an irreversible block of the SERCA pump which prolongs Ca^{2+} signaling and consequently also cell secretion. Depolarization of the plasma membrane by high KCl causes only a transient Ca^{2+} increase (Pan, Kao, 1997).

Since KIT manipulation can alter cell secretion activity (Chen et al., 2010) we sought to also investigate if pre-incubation blockage of the mutated KIT protein with 1100 ng/ml imatinib before thapsigargin stimulation could change the GIST cell secretion pattern. In this group, 526 proteins were detected, whereof 49% were part of either classical (9.5%) or non-classical (39.5%) secretory pathways. Indeed, compared to thapsigargin stimulation alone there was a radical change of quantitatively released proteins, and among the significantly increased proteins, only three were shared between the two groups. This could potentially have clinical implications when searching for biomarkers in imatinib-treated versus non-treated GIST patients. Furthermore, DAVID functional annotation of proteins exclusive to imatinib showed enrichment of proteins with translational and transcriptional properties, which were vastly different to the functions of secreted proteins in un-stimulated samples (see above). Interestingly, the most increased protein following imatinib treatment was Y-box-binding protein 1 which is known for its involvement in the acquisition of global drug resistance through increased ABCB1 expression (Basaki et al., 2007). Proteins at high levels in each group were validated performing Western blot analysis.

It is important to keep in mind that measurements of secreted proteins *in vivo* might reveal other protein subsets. There are several factors, such as stromal cells in the tumor microenvironment, which can potentially influence tumor cell secretion (Zhong et al., 2008).

To conclude, these findings demonstrate that GIST cells contain a secretome signature made up by classically and non-classically released proteins. The subset compositions were dependent of stimulatory condition. Proteins having a significant increase in the release were associated with proteins already found in other cancer types. This study therefore motivates further quantitation of differentially expressed proteins in sera from GIST patients and characterization of the expression in GIST tissues.

Paper IV. Functional role of the Ca^{2+}-activated Cl^- channel DOG1/TMEM16A in gastrointestinal stromal tumor cells.

Ca^{2+}-activated Cl^- channels (CaCCs) have important regulative functions in normal cellular physiology (Ferrera et al., 2010; Hwang et al., 2009), and can be involved in tumorigenesis, cancer progression, metastasis, cell proliferation, and migration (Duvvuri et al., 2012; Elble and Pauli, 2001; Habela et al., 2009; Sontheimer, 2008; Spitzner et al., 2008). Clinically they have been suggested as potential drug targets in the treatment of asthma, secretory diarrhea, and hypertension (Verkman and Galietta, 2009). Even though CaCCs are omnipresent and play important roles both in normal physiology and disease, awareness of their full biological role has been limited due to the lack of specific CaCC modulatory drugs and inadequate insight in Cl^- conductance and regulation (Hartzell et al., 2005). In 2004, DOG1 (a CaCC also known as TMEM16A or ANO1) over-expression was discovered in almost all GISTs (West et al., 2004). DOG1 was rapidly included as a tumor marker in routine diagnostics (Espinosa et al., 2008). However, its functional role in GIST has remained largely unknown, while in, for example, head and neck squamous cell carcinomas, DOG1 is known to cause cell migration and is correlated with poor prognosis (Ruiz et al., 2012). Advances in pharmacological high throughput screenings have recently identified specific blockers and activators of DOG1 (Namkung et al., 2011). In this study, evaluation of DOG1's functional role in GIST cells by specific activation and inhibition of DOG1, and measurements of cell viability, apoptosis, and proliferation *in vitro* was conducted.

The expression of DOG1 and CD117 in the imatinib-sensitive cell line GIST882 and the imatinib-resistant GIST48 was first verified by immunocytochemistry. Detailed analysis with CLSM showed that DOG1 was localized at the cell periphery in GIST882 cells, including the plasma membrane, whereas perinuclear localization was observed in GIST48 cells. Next, the electrophysiological technique patch-clamp was used to characterize DOG1-currents in GIST882 cells. This cell line was chosen based on its plasma membrane predominance of DOG1. CaCCs are usually activated by cytosolic Ca^{2+} concentrations between 0.2 - 1.0 μM, and by modulation of membrane

potential (Frings et al., 2000; Hartzell and Putzier, 2005). To verify that DOG1 was functional GIST882 cells were therefore voltage-clamped (V_h) at -60 mV and depolarized for 600 ms in +20 mV increments until +100 mV, while keeping $[Ca^{2+}]_{pip}$ at either activating (305 nM) or inhibitory (90 nM) levels. Whole-cell currents increased from 3.8±0.6 pA/pF to 13.5±3.2 pA/pF at V_m +100 mV by elevating $[Ca^{2+}]_{pip}$ from 90 nM to 305 nM, verifying that DOG1 is indeed Ca^{2+}- and voltage-dependent. Next, the effects of the specific DOG1 activator Eact and inhibitor $T16A_{inh}$-A01 on whole-cell CaCC currents in GIST882 cells were evaluated using the same voltage-protocol as described above. Whole-cell currents were measured when $[Ca^{2+}]_{pip}$ was kept at 305 nM and V_m at +100 mV. When the effect of 1, 10, and 30 μM $T16A_{inh}$-A01 were plotted in a dose-response diagram, and fitted to a four-parameter logistic *Hill* regression model it was found that IC_{50} was 4.3 μM, and the *Hill's*-slope (h) 0.66, which refers to the steepness of the curve. A coefficient less than 1 indicates negative comparability of $T16A_{inh}$-A01 binding to the DOG1 protein. The inhibitory effect of 30 μM $T16A_{inh}$-A01 on DOG1-current was approximately 60%, whereas the DOG1 activator Eact (30 μM) elicited a 70% DOG1-current increase when $[Ca^{2+}]_{pip}$ was 305 nM. Eact could not increase the DOG1-current when $[Ca^{2+}]_{pip}$ was 90 nM, and an increase in Eact concentration to 60 μM did not induce any further response in the low $[Ca^{2+}]_{pip}$. In other cells expressing DOG1, $T16A_{inh}$-A01 has proven more potent by an almost complete channel block (Namkung et al., 2011). A possible explanation to this is that several Cl^- channels, including DOG1, with different pharmacological properties, make up the Cl^- currents in GIST882 cells.

PE-labeled Annexin V/7-AAD 2-color flow cytometric analysis was used to examine the effect of $T16A_{inh}$-A01 and Eact on the induction of apoptosis in GIST882 and GIST48 cells after 48 h incubation. The combination of these two antibodies can discriminate between viable cells, early apoptotic cells, and late apoptotic/necrotic cells. Only adherent cells were analyzed to increase the sensitivity for early apoptotic events. Flow cytometric analysis showed that GIST882 cells incubated with $T16A_{inh}$-A01 presented with significantly fewer early apoptotic cells than un-stimulated cells, while Eact (3 μM) treated cells

were more viable than the control ($P < 0.05$). Although these changes were statistically significant, it is important to stress that the absolute changes were small. A possible explanation to why the effect was seen by 3 μM Eact, but not by 30 μM, is that the dose-response curve does not follow a linear relationship. Instead, Eact may have a dose-optimum centered around half its maximum activating effect (EC_{50}), which is approximately 3 μM. In GIST48, no effect of DOG1 modulation could be seen among cells with high (>92%) viability in controls. In contrast, when a small proportion of untreated control GIST48 cells showed signs of early apoptosis by Annexin V positivity, likely due to natural cell culture variation, there was a marked effect by $T16A_{inh}$-A01, shifting early apoptotic cells to late apoptotic stages (evident by 7-AAD positivity).

To further test whether DOG1 activity affects GIST cell proliferation and survival formazan formation was measured with the colorimetric assay WST-1. GIST48 and GIST882 cells were incubated with either Eact (3 μM, 10 μM, 30 μM), or $T16A_{inh}$-A01 (1 μM, 10 μM, 30 μM) for 0 h, 24 h, 48 h, and 72 h. Growth rates were compared to untreated cells grown in fully supplemented medium and to imatinib treated cells. Proliferation analysis demonstrated that both cell lines remained relatively stable throughout all time points during DOG1 modulation. GIST882 cells take longer to passage than GIST48 cells, which was verified by comparing the onset of cell growth in controls. Minor pro-proliferative changes were detected in GIST882 cells after 72 h incubation with 30 μM Eact and 1 μM and 10 μM $T16A_{inh}$-A01. These absolute changes were small, especially when compared to the imatinib effect. In GIST48, all Eact and $T16A_{inh}$-A01 concentrations except 30 μM $T16A_{inh}$-A01, evoked significant pro-proliferative effects. As for GIST882, these changes were negligible compared to the large imatinib effect. Furthermore, it is not expected for both activators and inhibitors of DOG1 to possess pro-proliferative properties. These results are in accordance with the recent study by Simon et al. (2013); DOG1 has partial/minor effects on the viability of unstressed GIST cells. In addition, we also show that DOG1 may have pro-apoptotic effects on some early apoptotic GIST cells.

The detection of small *in vitro* effects by pharmacological DOG1 modulation raises the question of other potential explanations to DOG1 over-expression in GIST. For example, the TMEM16A gene encoding DOG1 is located in the chromosomal region 11q13.3 (Katoh and Katoh, 2003), which is commonly amplified in cancers (Akervall et al., 1995; Sugahara et al., 2011), and may include other cancer-relevant genes (Gibcus et al., 2007). High DOG1 expression could also simply be a trait inherited from the ICCs from which GIST cells are thought to originate or share common progenitor with (Chen et al., 2007; Gomez-Pinilla et al., 2009).

The two human cell lines used in this paper harbor mutations in exon 11, exon 17 (GIST48), and exon 13 (GIST882), and are commonly used in functional studies of GIST. However, they do not represent the entire mutational spectrum. Studies including additional mutations may provide a more complete understanding of DOG1's functional role in GIST. Furthermore, there was an incomplete block and activation of Cl^- currents with the DOG1-specific modulators. Less specific CaCC activators and inhibitors may therefore induce more profound effects.

From this study it is concluded that the pattern of the highly expressed DOG1 varies on a cellular level in imatinib-sensitive and imatinib-resistant GIST cells. Generated DOG1 Cl^--currents are voltage- and Ca^{2+}-dependent, and can be regulated pharmacologically by DOG1-specific agents. DOG1 modulation has minor effects on cell viability and proliferation *in vitro,* but may impact early apoptotic GIST cells to undergo late apoptosis. Even though this study does not support DOG1 as a therapeutic target in GIST, further studies are warranted by reason of DOG1's strong expression in GIST and potential impact on other not herein investigated cell functions, such as cell migration and metastasis.

CONCLUDING REMARKS

Within one and a half decades, GISTs have emerged from historical obscurity to provide a model for molecularly targeted therapies. GIST is the most prevalent STS in the GI-tract and was the first solid tumor targeted by TKI therapy. Along with the development of more sophisticated molecular methods, the understanding of GIST molecular pathology, new treatment options, and follow-up strategies, is continuously growing. This thesis has focused on further functional characterization of certain aspects of the human GIST biology, with emphasis on GIST cell response and secretion, DOG1 function, and regional imatinib pharmacodynamics. The main findings include:

- The development of an analytical method to measure intracellular imatinib levels in experimental and clinical settings. Imatinib uptake varied between imatinib-sensitive and imatinib-resistant cell lines, reflective of potential differences in uptake mechanisms. Imatinib accumulated in clinical samples, showed intratumoral regional differences, and unequal intertumoral levels.
- The demonstration of an intact intracellular Ca^{2+}-signaling pathway in GIST cells, and a Ca^{2+}-dependent active ATP release that is modifiable by various stimuli.
- The recognition of a GIST secretome signature made up by classically and non-classically released proteins. The subset compositions were stimuli-dependent, and released proteins clustered differently according to function in untreated and imatinib-treated samples. Several of the proteins with a significant increase upon thapsigargin or KCl stimulation had already been acknowledged in other cancer types. There was a considerable overlap between secreted proteins and proteins of exosomal origin.
- The identification of different subcellular DOG1 localizations in imatinib-sensitive and imatinib-resistant GIST cells. DOG1 generated voltage- and Ca^{2+}_i-dependent currents that were regulated by a specific DOG1 inhibitor and activator. DOG1 activity had small effects on cell viability and proliferation *in vitro*, but impacted early apoptotic GIST cells to undergo late apoptosis.

Populärvetenskaplig sammanfattning

Sarkom är sällsynta och heterogena bindvävstumörer som kan uppstå var som helst i kroppens mjukdels- och stödjevävnad. De utgör 1-2% av alla elakartade (maligna) tumörer. I magtarmkanalen är gastrointestinala stromacellstumörer (GIST) den helt dominerande sarkomtypen med en nyinsjuknandefrekvens på ungefär femton fall per miljon invånare och år. De vanligaste lokalisationerna för GIST är magsäck och tunntarm, men de kan även förekomma i matstrupe, tjocktarm, ändtarm, tarmkäx (ooment) eller bakom den bakre bukhinne-begränsningen (retroperitonealt). Oftast är de personer som drabbas av GIST äldre än 60 år. Vanligaste platserna för spridd (metastaserad) tumörsjukdom är i levern eller i bukhålan, däremot sällan i lymfsystem eller utanför bukhålan (extraabdominellt), såsom exempelvis skelett och lungor. Det kliniska spektrumet är brett med alltifrån lokalt växande tumörer med närmast godartat (benignt) beteende (även om GIST aldrig helt kan klassas som benigna) till högmaligna tumörer med snabb spridning och dålig prognos. Ungefär en fjärdedel av GIST upptäcks *en passant* genom bildgivande diagnostik vid utredning av annan sjukdom. Majoriteten av patienter upplever diffusa och ospecifika symtom, medan vissa kan vara helt besvärsfria vid diagnos. Som tumörbegrepp beskrevs GIST första gången i slutet av 1990-talet. Innan dess medförde den diagnostiska osäkerheten att GIST uppfattades vara nerv- eller muskeltumör. För närvarande definieras GIST som en spolcellig, epiteloid eller ibland pleomorf mesenkymal tumör som uttrycker tyrosinkinasreceptorn KIT, vanligtvis detekterad med CD117-antikropp, som en del i ett diagnostiskt mönster. GIST anses utgå ifrån stamceller som under normala betingelser är programmerade att utvecklas till Cajals interstitiella celler (Cajalceller), vilka fungerar som magtarmkanalens pacemakerceller och därmed utgör en länk mellan den parasympatiska innervationen och tarmens glatta muskulatur. Signalvägarna inuti Cajalcellen är relativt välstuderade, men om signalvägarna likt de som ses i Cajalcellen existerar i GIST-cellen är till stora delar oklart. Tyrosinkinasreceptorn KIT utgör en hörnsten i den normala utvecklingen och funktionen hos Cajalcellen. Både Cajalcellen och GIST uttrycker KIT, och genetiska förändringar (s.k. mutationer) i KIT anses vara en tidig händelse vid tumörutvecklingen. Mutationerna leder till okontrollerat överaktiva KIT-receptorer, vilka i sin tur stimulerar signaleringsvägar vilket leder till ökad

cellproliferation och förlängd cellöverlevnad. På motsvarande sätt leder även mutationer i PDGFRA-genen till tumöruppkomst. Cirka 10-15% av GIST saknar mutationer i dessa gener och de flesta har då istället mutationer i andra delar av signalvägarna. På senare år har man identifierat en kalcium- och spänningskänslig kloridkanal (DOG1) i GIST. DOG1s funktion är till stor del okänd i GIST, men har visat sig vara en utmärkt markör för sjukdomen. Använda tillsammans verifierar CD117 och DOG1 i praktiken samtliga GIST. Radikal tumörborttagning (kirurgisk resektion) av lokalt växande sjukdom kan bota upp emot 60% av GIST-patienterna och det finns idag ett relativt tillförlitligt riskklassifikationssystem som uppskattar återfallsrisken efter operation. Generellt sett har stora tumörer med hög tillväxthastighet högst återfallsrisk. Medelöverlevnaden för patienter med obehandlad avancerad/ metastatisk sjukdom är mellan 10-20 månader. För patienter med GIST har det dock skett en dramatisk förbättring av överlevnaden efter introduktionen av tyrosinkinashämmare (däribland imatinib, Glivec®), som har förlängt medel-överlevnaden med ungefär 5 år jämfört med historiskt obehandlade kontroller. Tyrosinkinashämmare är inget klassiskt cytostatikum utan utgör den nya generationens antitumoral behandling som specifikt blockerar ett nyckelprotein i en signalväg. Trots detta medicinska genombrott tenderar majoriteten av de avancerade fallen att utveckla resistens mot tyrosinkinashämmare och progrediera inom några år. Utan möjlighet till specifik uppföljning av tumörbördan förlitar man sig idag på upprepade bildgivande och fysikaliska undersökningar för att övervaka tumöråterfall eller kvarvarande sjukdom. Detaljstudier har tidigare visat att GIST möjligen skulle kunna tillhöra en klass av s.k. neuroendokrina tumörer. Denna grupp av tumörer utmärker sig bland annat genom frisättning av hormoner och andra ämnen. Vilka ämnen eller hormoner som eventuellt utsöndras från GIST är däremot oklart, likaså vilka signalvägar som i så fall är verksamma. Denna avhandling har detaljstuderat molekylära aspekter på humana GIST-celler i syfte att öka kunskapsläget kring dess neuroendokrina fenotyp, betydelsen av DOG1 för cellöverlevnad och celldelning samt utvärdering av imatinib inuti GIST-cellen.

Avhandlingen visar att GIST-celler har en intakt intracellulär kalciumsignalering och en aktiv frisättning av ATP som är beroende av extracellulära kalciumnivåer. Graden av ATP-frisättning är möjlig att modulera med olika farmakologiska stimuli (Artikel I). Detaljstudier med en avancerad metod för att detektera proteiner i ett prov (s.k. proteomik) visar att klassiska och icke-klassiska proteiner frisätts utöver ATP. Betydelsen av proteinerna går inte direkt att överföra till den kliniska situationen då de dels identifierats i cellodling (*in vitro*), dels hittats ifrån GIST-celler från en enskild patient vilket inte representerar hela sjukdomsspektrumet. Flera av de ämnen vars utsöndring

ökade vid stimulering har dock påträffats i andra cancertyper. Funktionerna bland de ämnen som frisattes förändrades markant vid behandling med tyrosinkinashämmare. Detta kan ha betydelse vid analys av ämnen från behandlade respektive obehandlade patienter. I samtliga analyserade grupper förelåg även stor överlappning med proteiner som ingår i så kallade exosomer, vilka utgör en annan potentiell källa för cell-till-cell-signalering och sjukdoms-specifika biomarkörer (Artikel II).

Därefter utvecklades ett protokoll för att kunna mäta nivåerna av imatinib inuti själva tumörcellen. Mätningar i cellinjer visade att upptaget av imatinib är lägre i celler med motstånd mot läkemedlet (imatinib-resistenta) jämfört med imatinib-känsliga celler. I tumörmateriel från tre GIST-patienter påvisade tekniken en ansamling av läkemedlet i tumören, med påtagliga variationer inom och mellan tumörerna. Denna metod har potential att kunna användas kliniskt för att genomföra imatinibmätningar i olika tumöromården vilka kan korreleras till de i GIST funna imatinibtransportörerna (Artikel III).

I den sista studien undersöktes funktionen av DOG1. På cellnivå återfanns DOG1 antingen runt om cellkärnan (perinukleärt) eller i cellens yttre membran (plasmamembran) beroende på om cellen var känslig eller okänslig för imatinib. Specifika DOG1-hämmare och -aktiverare påverkade kanalaktiviteten i stor utsträckning, men hade relativt modest effekt på cellöverlevnad och celldelning bland välmående celler. Däremot inducerade DOG1-inhibition sen celldöd hos imatinib-resistenta celler som uppvisade tecken till tidig programmerad celldöd (Artikel IV).

Sammanfattningsvis utgör studierna som ingår i denna avhandling en grund för att påbörja kliniska studier för att studera förekomsten av GIST-specifika ämnen i exempelvis blod från patienter. Sådana specifika tumörmarkörer skulle kunna användas vid uppföljning och kontroll av sjukdomen. En uppföljande studie har redan påbörjats för att utvärdera imatinibs lokala farmakodynamik/farmako-kinetik i relation till transportöruttryck. Ytterligare studier kring DOG1s roll i GIST är även motiverade, då dess effekt på flera cellfunktioner, såsom migration och metastaseringsförmåga, ännu inte kartlagts helt. Förhoppningen är att resultaten från denna avhandling ska kunna bidra till utveckling av bättre verktyg i omhändertagandet av patienter med GIST, samt peka mot nya tänkbara mål för en mer individanpassad terapi och uppföljning.

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REFERENCES

van den Abbeele AD, Gatsonis C, de Vries DJ, et al. ACRIN 6665/RTOG 0132 phase II trial of neoadjuvant imatinib mesylate for operable malignant gastrointestinal stromal tumor: monitoring with 18F-FDG PET and correlation with genotype and GLUT4 expression. J Nucl Med. 2012 Apr;53(4):567-74.

Abraham SC, Krasinskas AM, Hofstetter WL, et al. "Seedling" mesenchymal tumors (gastrointestinal stromal tumors and leiomyomas) are common incidental tumors of the esophagogastric junction. Am J Surg Pathol. 2007 Nov;31(11):1629-35.

Agaimy A, Wünsch PH. Lymph node metastasis in gastrointestinal stromal tumours (GIST) occurs preferentially in young patients < or = 40 years: an overview based on our case material and the literature. Langenbecks Arch Surg. 2009 Mar;394(2):375-81.

Agaimy A, Wünsch PH, Hofstaedter F, et al. Minute gastric sclerosing stromal tumors (GIST tumorlets) are common in adults and frequently show c-KIT mutations. Am J Surg Pathol. 2007 Jan;31(1):113-20.

Agaram NP, Besmer P, Wong GC, et al. Pathologic and molecular heterogeneity in imatinib-stable or imatinib-responsive gastrointestinal stromal tumors. Clin Cancer Res. 2007 Jan 1;13(1):170-81.

Agaram NP, Wong GC, Guo T, et al. Novel V600E BRAF mutations in imatinib-naive and imatinib-resistant gastrointestinal stromal tumors. Genes Chromosomes Cancer. 2008 Oct;47(10):853-9.

Akervall JA, Jin Y, Wennerberg JP, et al. Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma of the head and neck. Cancer. 1995 Sep 1;76(5):853-9.

Allander SV, Nupponen NN, Ringnér M, et al. Gastrointestinal stromal tumors with KIT mutations exhibit a remarkably homogeneous gene expression profile. Cancer Res. 2001 Dec 15;61(24):8624-8.

Andersson J, Bümming P, Meis-Kindblom JM, et al. Gastrointestinal stromal tumors with KIT exon 11 deletions are associated with poor prognosis. Gastroenterology. 2006 May;130(6):1573-81.

Antonescu CR, Besmer P, Guo T, et al. Acquired resistance to imatinib in gastrointestinal stromal tumor occurs through secondary gene mutation. Clin Cancer Res. 2005 Jun 1;11(11):4182-90.

Aspinwall CA, Yeung ES. Screening populations of individual cells for secretory heterogeneity. Anal Bioanal Chem. 2005 Feb;381(3):660-6.

Bamboat ZM, DeMatteo RP. Metastasectomy for gastrointestinal stromal tumors. J Surg Oncol. 2014 Jan;109(1):23-7.

Basaki Y, Hosoi F, Oda Y, et al. Akt-dependent nuclear localization of Y-box-binding protein 1 in acquisition of malignant characteristics by human ovarian cancer cells. Oncogene. 2007 Apr 26;26(19):2736-46.

Blanke CD, Demetri GD, von Mehren M, e al. Long-term results from a randomized phase II trial of standard- versus higher-dose imatinib mesylate for patients with unresectable or metastatic gastrointestinal stromal tumors expressing KIT. J Clin Oncol. 2008 Feb 1;26(4):620-5.

Blanke CD, Rankin C, Demetri GD, et al. Phase III randomized, intergroup trial assessing imatinib mesylate at two dose levels in patients with unresectable or metastatic gastrointestinal stromal tumors expressing the kit receptor tyrosine kinase: S0033. J Clin Oncol. 2008 Feb 1;26(4):626-32.

Blume-Jensen P, Claesson-Welsh L, Siegbahn A, et al. Activation of the human c-kit product by ligand-induced dimerization mediates circular actin reorganization and chemotaxis. EMBO J. 1991 Dec;10(13):4121-8.

Burnstock G, Lavin S. Interstitial cells of Cajal and purinergic signalling. Auton Neurosci. 2002 Apr 18;97(1):68-72.

Bümming P, Ahlman H, Andersson J, et al. Population-based study of the diagnosis and treatment of gastrointestinal stromal tumours. Br J Surg. 2006 Jul;93(7):836-43.

Bümming P, Nilsson O, Ahlman H, et al. Gastrointestinal stromal tumors regularly express synaptic vesicle proteins: evidence of a neuroendocrine phenotype. Endocr Relat Cancer. 2007 Sep;14(3):853-63.

Camussi G, Deregibus MC, Tetta C. Tumor-derived microvesicles and the cancer microenvironment. Curr Mol Med. 2013 Jan;13(1):58-67.

Carney JA, Sheps SG, Go VL, et al. The triad of gastric leiomyosarcoma, functioning extra-adrenal paraganglioma and pulmonary chondroma. N Engl J Med. 1977 Jun 30;296(26):1517-8.

Carney JA, Stratakis CA. Familial paraganglioma and gastric stromal sarcoma: a new syndrome distinct from the Carney triad. Am J Med Genet. 2002 Mar 1;108(2):132-9.

Caterino S, Lorenzon L, Petrucciani N, et al. Gastrointestinal stromal tumors: correlation between symptoms at presentation, tumor location and prognostic factors in 47 consecutive patients. World J Surg Oncol. 2011 Feb 1;9:13.

Chan JK. Mesenchymal tumors of the gastrointestinal tract: a paradise for acronyms (STUMP, GIST, GANT, and now GIPACT), implication of c-kit in genesis, and yet another of the many emerging roles of the interstitial cell of Cajal in the pathogenesis of gastrointestinal diseases? Adv Anat Pathol. 1999 Jan;6(1):19-40.

Chan KH, Chan CW, Chow WH, et al. Gastrointestinal stromal tumors in a cohort of Chinese patients in Hong Kong. World J Gastroenterol. 2006 Apr 14;12(14):2223-8.

Chen S, Burgin S, McDaniel A, et al. Nf1-/- Schwann cell-conditioned medium modulates mast cell degranulation by c-Kit-mediated hyperactivation of phosphatidylinositol 3-kinase. Am J Pathol. 2010 Dec;177(6):3125-32.

Chan PM, Ilangumaran S, La Rose J, et al. Autoinhibition of the kit receptor tyrosine kinase by the cytosolic juxtamembrane region. Mol Cell Biol. 2003 May;23(9):3067-78.

Chen H, Ordög T, Chen J, et al. Differential gene expression in functional classes of interstitial cells of Cajal in murine small intestine. Physiol Genomics. 2007 Nov 14;31(3):492-509.

Chi P, Chen Y, Zhang L, et al. ETV1 is a lineage survival factor that cooperates with KIT in gastrointestinal stromal tumours. Nature. 2010 Oct 14;467(7317):849-53.

Cho S, Kitadai Y, Yoshida S, et al. Deletion of the KIT gene is associated with liver metastasis and poor prognosis in patients with gastrointestinal stromal tumor in the stomach. Int J Oncol. 2006 Jun;28(6):1361-7.

Chompret A, Kannengiesser C, Barrois M, et al. PDGFRA germline mutation in a family with multiple cases of gastrointestinal stromal tumor. Gastroenterology. 2004 Jan;126(1):318-21.

Chou A, Chen J, Clarkson A, et al. Succinate dehydrogenase-deficient GISTs are characterized by IGF1R overexpression. Mod Pathol. 2012 Sep;25(9):1307-13.

Chourmouzi D, Sinakos E, Papalavrentios L, et al. Gastrointestinal stromal tumors: a pictorial review. J Gastrointestin Liver Dis. 2009 Sep;18(3):379-83.

Corless CL. Gastrointestinal stromal tumors: what do we know now? Mod Pathol. 2014 Jan;27 Suppl 1:S1-16.

Corless CL, Barnett CM, Heinrich MC. Gastrointestinal stromal tumours: origin and molecular oncology. Nat Rev Cancer. 2011 Nov 17;11(12):865-78.

Corless CL, Schroeder A, Griffith D, et al. PDGFRA mutations in gastrointestinal stromal tumors: frequency, spectrum and in vitro sensitivity to imatinib. J Clin Oncol. 2005 Aug 10;23(23):5357-64.

Dagher R, Cohen M, Williams G, et al. Approval summary: imatinib mesylate in the treatment of metastatic and/or unresectable malignant gastrointestinal stromal tumors. Clin Cancer Res. 2002 Oct;8(10):3034-8.

Davids MS, Letai A, Brown JR. Overcoming stroma-mediated treatment resistance in chronic lymphocytic leukemia through BCL-2 inhibition. Leuk Lymphoma. 2013 Aug;54(8):1823-5.

Davies HE, Wathen CG, Gleeson FV. The risks of radiation exposure related to diagnostic imaging and how to minimise them. BMJ. 2011 Feb 25;342:d947.

Dematteo RP, Ballman KV, Antonescu CR, et al.; American College of Surgeons Oncology Group (ACOSOG) Intergroup Adjuvant GIST Study Team. Adjuvant imatinib mesylate after resection of localised, primary gastrointestinal stromal tumour: a randomised, double-blind, placebo-controlled trial. Lancet. 2009 Mar 28;373(9669):1097-104.

Dematteo RP, Lewis JJ, Leung D, et al. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. Ann Surg. 2000 Jan;231(1):51-8.

Demetri GD, Garrett CR, Schöffski P, et al. Complete longitudinal analyses of the randomized, placebo-controlled, phase III trial of sunitinib in patients with gastrointestinal stromal tumor following imatinib failure. Clin Cancer Res. 2012 Jun 1;18(11):3170-9.

Demetri GD, von Mehren M, Blanke CD, et al. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med. 2002 Aug 15;347(7):472-80.

Demetri GD, Reichardt P, Kang YK, et al. Efficacy and safety of regorafenib for advanced gastrointestinal stromal tumours after failure of imatinib and sunitinib (GRID): an international, multicentre, randomised, placebo-controlled, phase 3 trial. Lancet. 2013 Jan 26;381(9863):295-302.

Demetri GD, Wang Y, Wehrle E, Racine A, et al. Imatinib plasma levels are correlated with clinical benefit in patients with unresectable/metastatic gastrointestinal stromal tumors. J Clin Oncol. 2009 Jul 1;27(19):3141-7.

Domon B, Aebersold R. Mass spectrometry and protein analysis. Science. 2006 Apr 14;312(5771):212-7.

Dowling P, Clynes M. Conditioned media from cell lines: a complementary model to clinical specimens for the discovery of disease-specific biomarkers. Proteomics. 2011 Feb;11(4):794-804.

Druker BJ, Tamura S, Buchdunger E, et al. Effects of a selective inhibitor of the Abl tyrosine kinase on the growth of Bcr-Abl positive cells. Nat Med. 1996 May;2(5):561-6.

Ducimetière F, Lurkin A, Ranchère-Vince D, et al. Incidence of sarcoma histotypes and molecular subtypes in a prospective epidemiological study with central pathology review and molecular testing. PLoS One. 2011;6(8):e20294.

Duensing A, Joseph NE, Medeiros F, et al. Protein Kinase C theta (PKCtheta) expression and constitutive activation in gastrointestinal stromal tumors (GISTs). Cancer Res. 2004 Aug 1;64(15):5127-31.

Duensing A, Medeiros F, McConarty B, et al. Mechanisms of oncogenic KIT signal transduction in primary gastrointestinal stromal tumors (GISTs). Oncogene. 2004 May 13;23(22):3999-4006.

Duvvuri U, Shiwarski DJ, Xiao D, et al. TMEM16A induces MAPK and contributes directly to tumorigenesis and cancer progression. Cancer Res. 2012 Jul 1;72(13):3270-81.

Dwight T, Benn DE, Clarkson A, et al. Loss of SDHA expression identifies SDHA mutations in succinate dehydrogenase-deficient gastrointestinal stromal tumors. Am J Surg Pathol. 2013 Feb;37(2):226-33.

Eechoute K, Fransson MN, Reyners AK, et al. A long-term prospective population pharmacokinetic study on imatinib plasma concentrations in GIST patients. Clin Cancer Res. 2012 Oct 15;18(20):5780-7.

Eechoute K, Sparreboom A, Burger H, et al. Drug transporters and imatinib treatment: implications for clinical practice. Clin Cancer Res. 2011 Feb 1;17(3):406-15.

Eisenberg BL, Trent JC. Adjuvant and neoadjuvant imatinib therapy: current role in the management of gastrointestinal stromal tumors. Int J Cancer. 2011 Dec 1;129(11):2533-42.

Elble RC, Pauli BU. Tumor suppression by a proapoptotic calcium-activated chloride channel in mammary epithelium. J Biol Chem. 2001 Nov 2;276(44):40510-7.

Erlandson RA, Klimstra DS, Woodruff JM. Subclassification of gastrointestinal stromal tumors based on evaluation by electron microscopy and immunohistochemistry. Ultrastruct Pathol. 1996 Jul-Aug;20(4):373-93.

ESMO / European Sarcoma Network Working Group. Gastrointestinal stromal tumors: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2012 Oct;23 Suppl 7:vii49-55.

Espinosa I, Lee CH, Kim MK, et al. A novel monoclonal antibody against DOG1 is a sensitive and specific marker for gastrointestinal stromal tumors. Am J Surg Pathol. 2008 Feb;32(2):210-8.

Evans DJ, Lampert IA, Jacobs M. Intermediate filaments in smooth muscle tumours. J Clin Pathol. 1983 Jan;36(1):57-61.

Everett M, Gutman H. Surgical management of gastrointestinal stromal tumors: analysis of outcome with respect to surgical margins and technique. J Surg Oncol. 2008 Dec 15;98(8):588-93.

Ferrera L, Caputo A, Galietta LJ. TMEM16A protein: a new identity for Ca(2+)-dependent Cl^- channels. Physiology (Bethesda). 2010 Dec;25(6):357-63.

Fiore M, Palassini E, Fumagalli E, et al. Preoperative imatinib mesylate for unresectable or locally advanced primary gastrointestinal stromal tumors (GIST). Eur J Surg Oncol. 2009 Jul;35(7):739-45.

Fletcher CD, Berman JJ, Corless C, et al. Diagnosis of gastrointestinal stromal tumors: A consensus approach. Hum Pathol. 2002 May;33(5):459-65.

Fletcher CDM, Bridge JA, Hogendoorn P, Mertens F. WHO Classification of Tumours of Soft Tissue and Bone. Fourth Edition. IARC press, Lyon, 2013.

Fletcher CDM, Unni KK, Mertens F. WHO Classification of Tumours: pathology and genetics of tumours of soft tissue and bone. IARC press, Lyon. 2002.

Frings S, Reuter D, Kleene SJ. Neuronal Ca^{2+} -activated Cl^- channels - homing in on an elusive channel species. Prog Neurobiol. 2000 Feb;60(3):247-89.

Furuzono S, Ohya S, Inoue S, et al. Inherent pacemaker function of duodenal GIST. Eur J Cancer. 2006 Jan;42(2):243-8.

Gastrointestinal Stromal Tumor Meta-Analysis Group (MetaGIST). Comparison of two doses of imatinib for the treatment of unresectable or metastatic gastrointestinal stromal tumors: a meta-analysis of 1,640 patients. J Clin Oncol. 2010 Mar 1;28(7):1247-53.

Geiger T, Wehner A, Schaab C, et al. Comparative proteomic analysis of eleven common cell lines reveals ubiquitous but varying expression of most proteins. Mol Cell Proteomics. 2012 Mar;11(3):M111.014050.

Ghanem N, Altehoefer C, Furtwängler A, et al. Computed tomography in gastrointestinal stromal tumors. Eur Radiol. 2003 Jul;13(7):1669-78.

Gibcus JH, Menkema L, Mastik MF, et al. Amplicon mapping and expression profiling identify the Fas-associated death domain gene as a new driver in the 11q13.3 amplicon in laryngeal/pharyngeal cancer. Clin Cancer Res. 2007 Nov 1;13(21):6257-66.

Goettsch WG, Bos SD, Breekveldt-Postma N, et al. Incidence of gastrointestinal stromal tumours is underestimated: results of a nation-wide study. Eur J Cancer. 2005 Dec;41(18):2868-72.

Gold JS, Gönen M, Gutiérrez A, et al. Development and validation of a prognostic nomogram for recurrence-free survival after complete surgical resection of localised primary gastrointestinal stromal tumour: a retrospective analysis. Lancet Oncol. 2009 Nov;10(11):1045-52.

Gomez-Pinilla PJ, Gibbons SJ, Bardsley MR, et al. Ano1 is a selective marker of interstitial cells of Cajal in the human and mouse gastrointestinal tract. Am J Physiol Gastrointest Liver Physiol. 2009 Jun;296(6):G1370-81.

Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002 Jan;2(1):48-58.

Gronchi A, Fiore M, Miselli F, et al. Surgery of residual disease following molecular-targeted therapy with imatinib mesylate in advanced/metastatic GIST. Ann Surg. 2007 Mar;245(3):341-6.

Gschwind HP, Pfaar U, Waldmeier F, et al. Metabolism and disposition of imatinib mesylate in healthy volunteers. Drug Metab Dispos. 2005 Oct;33(10):1503-12.

Gstaiger M, Aebersold R. Applying mass spectrometry-based proteomics to genetics, genomics and network biology. Nat Rev Genet. 2009 Sep;10(9):617-27.

Habela CW, Ernest NJ, Swindall AF, et al. Chloride accumulation drives volume dynamics underlying cell proliferation and migration. J Neurophysiol. 2009 Feb;101(2):750-7.

Hamada A, Miyano H, Watanabe H. Interaction of imatinib mesilate with human P-glycoprotein. J Pharmacol Exp Ther. 2003 Nov;307(2):824-8.

Hamill OP, Marty A, Neher E, et al. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 1981 Aug;391(2):85-100.

Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646-74.

Harris TJ, McCormick F. The molecular pathology of cancer. Nat Rev Clin Oncol. 2010 May;7(5):251-65.

Hartzell C, Putzier I, Arreola J. Calcium-activated chloride channels. Annu Rev Physiol. 2005;67:719-58.

Heinrich MC, Corless CL, Blanke CD, et al. Molecular correlates of imatinib resistance in gastrointestinal stromal tumors. J Clin Oncol. 2006 Oct 10;24(29):4764-74.

Heinrich MC, Corless CL, Demetri GD, et al. Kinase mutations and imatinib response in patients with metastatic gastrointestinal stromal tumor. J Clin Oncol. 2003 Dec 1;21(23):4342-9.

Heinrich MC, Corless CL, Duensing A, et al. PDGFRA activating mutations in gastrointestinal stromal tumors. Science. 2003 Jan 31;299(5607):708-10.

Heinrich MC, Owzar K, Corless CL, et al. Correlation of kinase genotype and clinical outcome in the North American Intergroup Phase III Trial of imatinib mesylate for treatment of advanced gastrointestinal stromal tumor: CALGB 150105 Study by Cancer and Leukemia Group B and Southwest Oncology Group. J Clin Oncol. 2008 Nov 20;26(33):5360-7.

Higgins CF. Multiple molecular mechanisms for multidrug resistance transporters. Nature. 2007 Apr 12;446(7137):749-57.

Hirota S, Isozaki K, Moriyama Y, et al. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science. 1998 Jan 23;279(5350):577-80.

Hirota S, Ohashi A, Nishida T, et al. Gain-of-function mutations of platelet-derived growth factor receptor alpha gene in gastrointestinal stromal tumors. Gastroenterology. 2003 Sep;125(3):660-7.

Hislop J, Mowatt G, Sharma P, et al. Systematic review of escalated imatinib doses compared with sunitinib or best supportive care, for the treatment of people with unresectable/metastatic gastrointestinal stromal tumours whose disease has progressed on the standard imatinib dose. J Gastrointest Cancer. 2012 Jun;43(2):168-76.

Hwang SJ, Blair PJ, Britton FC, et al. Expression of anoctamin 1/TMEM16A by interstitial cells of Cajal is fundamental for slow wave activity in gastrointestinal muscles. J Physiol. 2009 Oct 15;587(Pt 20):4887-904.

Jahn R, Fasshauer D. Molecular machines governing exocytosis of synaptic vesicles. Nature. 2012 Oct 11;490(7419):201-7.

Jakob J, Mussi C, Ronellenfitsch U, et al. Gastrointestinal stromal tumor of the rectum: results of surgical and multimodality therapy in the era of imatinib. Ann Surg Oncol. 2013 Feb;20(2):586-92.

Janeway KA, Kim SY, Lodish M, et al. Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. Proc Natl Acad Sci U S A. 2011 Jan 4;108(1):314-8.

Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin. 2011 Mar-Apr;61(2):69-90.

Jiang Q, Wong AO. Signal transduction mechanisms for autocrine/paracrine regulation of somatolactin-α secretion and synthesis in carp pituitary cells by somatolactin-α and -β. Am J Physiol Endocrinol Metab. 2013 Jan 15;304(2):E176-86.

Jo VY, Fletcher CD. WHO classification of soft tissue tumours: an update based on the 2013 (4th) edition. Pathology. 2014 Feb;46(2):95-104.

Joensuu H. Risk stratification of patients diagnosed with gastrointestinal stromal tumor. Hum Pathol. 2008 Oct;39(10):1411-9.

Joensuu H, DeMatteo RP. The management of gastrointestinal stromal tumors: a model for targeted and multidisciplinary therapy of malignancy. Annu Rev Med. 2012;63:247-58.

Joensuu H, Eriksson M, Sundby Hall K, et al. One vs three years of adjuvant imatinib for operable gastrointestinal stromal tumor: a randomized trial. JAMA. 2012 Mar 28;307(12):1265-72.

Joensuu H, Fletcher C, Dimitrijevic S, et al. Management of malignant gastrointestinal stromal tumours. Lancet Oncol. 2002 Nov;3(11):655-64.

Joensuu H, Hohenberger P, Corless CL. Gastrointestinal stromal tumour. Lancet. 2013 Sep 14;382(9896):973-83.

Joensuu H, Trent JC, Reichardt P. Practical management of tyrosine kinase inhibitor-associated side effects in GIST. Cancer Treat Rev. 2011 Feb;37(1):75-88.

Joensuu H, Vehtari A, Riihimäki J, et al. Risk of recurrence of gastrointestinal stromal tumour after surgery: an analysis of pooled population-based cohorts. Lancet Oncol. 2012 Mar;13(3):265-74.

Judson I. Therapeutic drug monitoring of imatinib - new data strengthen the case. Clin Cancer Res. 2012 Oct 15;18(20):5517-9.

Kang HJ, Nam SW, Kim H, et al. Correlation of KIT and platelet-derived growth factor receptor alpha mutations with gene activation and expression profiles in gastrointestinal stromal tumors. Oncogene. 2005 Feb 3;24(6):1066-74.

Katoh M, Katoh M. FLJ10261 gene, located within the CCND1-EMS1 locus on human chromosome 11q13, encodes the eight-transmembrane protein homologous to C12orf3, C11orf25 and FLJ34272 gene products. Int J Oncol. 2003 Jun;22(6):1375-81.

Kawanowa K, Sakuma Y, Sakurai S, et al. High incidence of microscopic gastrointestinal stromal tumors in the stomach. Hum Pathol. 2006 Dec;37(12):1527-35.

Khan J, Wei JS, Ringnér M, et al. Classification and diagnostic prediction of cancers using gene expression profiling and artificial neural networks. Nat Med. 2001 Jun;7(6):673-9.

Kim CW, Lee HM, Lee TH, et al. Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. Cancer Res. 2002 Nov 1;62(21):6312-7.

Kindblom LG, Remotti HE, Aldenborg F, et al. Gastrointestinal pacemaker cell tumor (GIPACT): gastrointestinal stromal tumors show phenotypic characteristics of the interstitial cells of Cajal. Am J Pathol. 1998 May;152(5):1259-69.

Kinoshita K, Hirota S, Isozaki K, et al. Absence of c-kit gene mutations in gastrointestinal stromal tumours from neurofibromatosis type 1 patients. J Pathol. 2004 Jan;202(1):80-5.

Kitamura Y, Hirotab S. Kit as a human oncogenic tyrosine kinase. Cell Mol Life Sci. 2004 Dec;61(23):2924-31.

Lasota J, Dansonka-Mieszkowska A, Sobin LH, et al. A great majority of GISTs with PDGFRA mutations represent gastric tumors of low or no malignant potential. Lab Invest. 2004 Jul;84(7):874-83.

Leitner JW, Sussman KE, Vatter AE, et al. Adenine nucleotides in the secretory granule fraction of rat islets. Endocrinology. 1975 Mar;96(3):662-77.

Liegl-Atzwanger B, Fletcher JA, et al. Gastrointestinal stromal tumors. Virchows Arch. 2010 Feb;456(2):111-27.

Liegl B, Kepten I, Le C, et al. Heterogeneity of kinase inhibitor resistance mechanisms in GIST. J Pathol. 2008 Sep;216(1):64-74.

Liu HN, Ohya S, Nishizawa Y, et al. Serotonin augments gut pacemaker activity via 5-HT3 receptors. PLoS One. 2011;6(9):e24928.

Lux ML, Rubin BP, Biase TL, et al. KIT extracellular and kinase domain mutations in gastrointestinal stromal tumors. Am J Pathol. 2000 Mar;156(3):791-5.

Maier J, Lange T, Kerle I, et al. Detection of mutant free circulating tumor DNA in the plasma of patients with gastrointestinal stromal tumor harboring activating mutations of CKIT or PDGFRA. Clin Cancer Res. 2013 Sep 1;19(17):4854-67.

Malle P, Sorschag M, Gallowitsch HJ. FDG PET and FDG PET/CT in patients with gastrointestinal stromal tumours. Wien Med Wochenschr. 2012 Oct;162(19-20):423-9.

Marrari A, Trent JC, George S. Personalized cancer therapy for gastrointestinal stromal tumor: synergizing tumor genotyping with imatinib plasma levels. Curr Opin Oncol. 2010 Jul;22(4):336-41.

Mastrangelo G, Coindre JM, Ducimetière F, et al. Incidence of soft tissue sarcoma and beyond: a population-based prospective study in 3 European regions. Cancer. 2012 Nov 1;118(21):5339-48.

Mazur MT, Clark HB. Gastric stromal tumors. Reappraisal of histogenesis. Am J Surg Pathol. 1983 Sep;7(6):507-19.

McAuliffe JC, Hunt KK, Lazar AJ, et al. A randomized, phase II study of preoperative plus postoperative imatinib in GIST: evidence of rapid radiographic response and temporal induction of tumor cell apoptosis. Ann Surg Oncol. 2009 Apr;16(4):910-9.

Miettinen M. Gastrointestinal stromal tumors. An immunohistochemical study of cellular differentiation. Am J Clin Pathol. 1988 May;89(5):601-10.

Miettinen M, Lasota J. Gastrointestinal stromal tumors: pathology and prognosis at different sites. Semin Diagn Pathol. 2006 May;23(2):70-83.

Miettinen M, Sobin LH, Lasota J. Gastrointestinal stromal tumors of the stomach: a clinicopathologic, immunohistochemical, and molecular genetic study of 1765 cases with long-term follow-up. Am J Surg Pathol. 2005 Jan;29(1):52-68.

Miettinen M, Virolainen M, Maarit-Sarlomo-Rikala. Gastrointestinal stromal tumors--value of CD34 antigen in their identification and separation from true leiomyomas and schwannomas. Am J Surg Pathol. 1995 Feb;19(2):207-16.

Miettinen M, Wang ZF, Lasota J. DOG1 antibody in the differential diagnosis of gastrointestinal stromal tumors: a study of 1840 cases. Am J Surg Pathol. 2009 Sep;33(9):1401-8.

Miller IV, Raposo G, Welsch U, et al. First identification of Ewing's sarcoma-derived extracellular vesicles and exploration of their biological and potential diagnostic implications. Biol Cell. 2013 Jul;105(7):289-303.

Min KW, Leabu M. Interstitial cells of Cajal (ICC) and gastrointestinal stromal tumor (GIST): facts, speculations, and myths. J Cell Mol Med. 2006 Oct-Dec;10(4):995-1013.

Miranda C, Nucifora M, Molinari F, et al. KRAS and BRAF mutations predict primary resistance to imatinib in gastrointestinal stromal tumors. Clin Cancer Res. 2012 Mar 15;18(6):1769-76.

Mucciarini C, Rossi G, Bertolini F, et al. Incidence and clinicopathologic features of gastrointestinal stromal tumors. A population-based study. BMC Cancer. 2007 Dec 20;7:230.

Mullady DK, Tan BR. A multidisciplinary approach to the diagnosis and treatment of gastrointestinal stromal tumor. J Clin Gastroenterol. 2013 Aug;47(7):578-85.

Namkung W, Phuan PW, Verkman AS. TMEM16A inhibitors reveal TMEM16A as a minor component of calcium-activated chloride channel conductance in airway and intestinal epithelial cells. J Biol Chem. 2011 Jan 21;286(3):2365-74.

Namkung W, Yao Z, Finkbeiner WE, et al. Small-molecule activators of TMEM16A, a calcium-activated chloride channel, stimulate epithelial chloride secretion and intestinal contraction. FASEB J. 2011 Nov;25(11):4048-62.

Neher E, Sakmann B. Single-channel currents recorded from membrane of denervated frog muscle fibres. Nature. 1976 Apr 29;260(5554):799-802.

Nielsen TO, West RB, Linn SC, et al. Molecular characterisation of soft tissue tumours: a gene expression study. Lancet. 2002 Apr 13;359(9314):1301-7.

Nilsson B, Bümming P, Meis-Kindblom JM, et al. Gastrointestinal stromal tumors: the incidence, prevalence, clinical course, and prognostication in the pre-imatinib mesylate era - a population-based study in western Sweden. Cancer. 2005 Feb 15;103(4):821-9.

Nilsson T, Mann M, Aebersold R, et al. Mass spectrometry in high-throughput proteomics: ready for the big time. Nat Methods. 2010 Sep;7(9):681-5.

Nishida T, Hirota S, Taniguchi M, et al. Familial gastrointestinal stromal tumours with germline mutation of the KIT gene. Nat Genet. 1998 Aug;19(4):323-4.

Norman A. Flow cytometry. Med Phys. 1980 Nov-Dec;7(6):609-15.

Novitsky YW, Kercher KW, Sing RF, et al. Long-term outcomes of laparoscopic resection of gastric gastrointestinal stromal tumors. Ann Surg. 2006 Jun;243(6):738-45; discussion 745-7.

Old WM, Meyer-Arendt K, Aveline-Wolf L, et al. Comparison of label-free methods for quantifying human proteins by shotgun proteomics. Mol Cell Proteomics. 2005 Oct;4(10):1487-502.

Ozvegy-Laczka C, Cserepes J, Elkind NB, Sarkadi B. Tyrosine kinase inhibitor resistance in cancer: role of ABC multidrug transporters. Drug Resist Updat. 2005 Feb-Apr;8(1-2):15-26.

Pan CY, Kao LS. Catecholamine secretion from bovine adrenal chromaffin cells: the role of the Na^{+}/Ca^{2+} exchanger and the intracellular Ca^{2+} pool. J Neurochem. 1997 Sep;69(3):1085-92.

Pappo AS, Janeway KA. Pediatric gastrointestinal stromal tumors. Hematol Oncol Clin North Am. 2009 Feb;23(1):15-34.

Patrikidou A, Chabaud S, Ray-Coquard I, et al. Influence of imatinib interruption and rechallenge on the residual disease in patients with advanced GIST: results of the

BFR14 prospective French Sarcoma Group randomised, phase III trial. Ann Oncol. 2013 Apr;24(4):1087-93.

Pellegatti P, Raffaghello L, Bianchi G, et al. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. PLoS One. 2008 Jul 9;3(7):e2599.

Peng B, Dutreix C, Mehring G, et al. Absolute bioavailability of imatinib (Glivec) orally versus intravenous infusion. J Clin Pharmacol. 2004 Feb;44(2):158-62.

Peng B, Lloyd P, et al. Clinical pharmacokinetics of imatinib. Clin Pharmacokinet. 2005;44(9):879-94.

Pérez-Gutiérrez S, González-Cámpora R, Amérigo-Navarro J, et al. Expression of P-glycoprotein and metallothionein in gastrointestinal stromal tumor and leiomyosarcomas. Clinical implications. Pathol Oncol Res. 2007;13(3):203-8.

Plaat BE, Hollema H, Molenaar WM, et al. Soft tissue leiomyosarcomas and malignant gastrointestinal stromal tumors: differences in clinical outcome and expression of multidrug resistance proteins. J Clin Oncol. 2000 Sep 15;18(18):3211-20.

Poste G. Bring on the biomarkers. Nature. 2011 Jan 13;469(7329):156-7.

Prenen H, Cools J, Mentens N, et al. Efficacy of the kinase inhibitor SU11248 against gastrointestinal stromal tumor mutants refractory to imatinib mesylate. Clin Cancer Res. 2006 Apr 15;12(8):2622-7.

Raut CP, Posner M, Desai J, et al. Surgical management of advanced gastrointestinal stromal tumors after treatment with targeted systemic therapy using kinase inhibitors. J Clin Oncol. 2006 May 20;24(15):2325-31.

Reichardt P, Reichardt A, Pink D. Molecular targeted therapy of gastrointestinal stromal tumors. Curr Cancer Drug Targets. 2011 Jul;11(6):688-97.

Reichardt P, Blay JY, Boukovinas I, et al. Adjuvant therapy in primary GIST: state-of-the-art. Ann Oncol. 2012 Nov;23(11):2776-81.

Reid T. Reintroduction of imatinib in GIST. J Gastrointest Cancer. 2013 Dec;44(4):385-92.

Ricci C, Scappini B, Divoky V, et al. Mutation in the ATP-binding pocket of the ABL kinase domain in an STI571-resistant BCR/ABL-positive cell line. Cancer Res. 2002 Nov 1;62(21):5995-8.

Rossi F, Ehlers I, Agosti V, et al. Oncogenic Kit signaling and therapeutic intervention in a mouse model of gastrointestinal stromal tumor. Proc Natl Acad Sci U S A. 2006 Aug 22;103(34):12843-8.

Rossi S, Gasparotto D, Toffolatti L, et al. Molecular and clinicopathologic characterization of gastrointestinal stromal tumors (GISTs) of small size. Am J Surg Pathol. 2010 Oct;34(10):1480-91.

Rubin BP, Heinrich MC, Corless CL. Gastrointestinal stromal tumour. Lancet. 2007 May 19;369(9574):1731-41.

Rubin BP, Singer S, Tsao C, et al. KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. Cancer Res. 2001 Nov 15;61(22):8118-21.

Ruiz C, Martins JR, Rudin F, et al. Enhanced expression of ANO1 in head and neck squamous cell carcinoma causes cell migration and correlates with poor prognosis. PLoS One. 2012;7(8):e43265.

Rutkowski P, Gronchi A, Hohenberger P, et al. Neoadjuvant imatinib in locally advanced gastrointestinal stromal tumors (GIST): the EORTC STBSG experience. Ann Surg Oncol. 2013 Sep;20(9):2937-43.

Sadygov RG, Cociorva D, Yates JR 3rd. Large-scale database searching using tandem mass spectra: looking up the answer in the back of the book. Nat Methods. 2004 Dec;1(3):195-202.

Sakurai S, Oguni S, Hironaka M, et al. Mutations in c-kit gene exons 9 and 13 in gastrointestinal stromal tumors among Japanese. Jpn J Cancer Res. 2001 May;92(5):494-8.

Sanders KM, Zhu MH, Britton F, et al. Anoctamins and gastrointestinal smooth muscle excitability. Exp Physiol. 2012 Feb;97(2):200-6.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5463-7.

Saunders NA, Simpson F, Thompson EW, et al. Role of intratumoural heterogeneity in cancer drug resistance: molecular and clinical perspectives. EMBO Mol Med. 2012 Aug;4(8):675-84.

Sepe PS, Moparty B, Pitman MB, et al. EUS-guided FNA for the diagnosis of GI stromal cell tumors: sensitivity and cytologic yield. Gastrointest Endosc. 2009 Aug;70(2):254-61.

Shahi PK, Choi S, Zuo DC, Yeum CH, et al. 5-hydroxytryptamine generates tonic inward currents on pacemaker activity of interstitial cells of cajal from mouse small intestine. Korean J Physiol Pharmacol. 2011 Jun;15(3):129-35.

Sihto H, Sarlomo-Rikala M, Tynninen O, et al. KIT and platelet-derived growth factor receptor alpha tyrosine kinase gene mutations and KIT amplifications in human solid tumors. J Clin Oncol. 2005 Jan 1;23(1):49-57.

Simon S, Grabellus F, Ferrera L, et al. DOG1 regulates growth and IGFBP5 in gastrointestinal stromal tumors. Cancer Res. 2013 Jun 15;73(12):3661-70.

Smith CB, Betz WJ. Simultaneous independent measurement of endocytosis and exocytosis. Nature. 1996 Apr 11;380(6574):531-4.

Sommer G, Agosti V, Ehlers I, et al. Gastrointestinal stromal tumors in a mouse model by targeted mutation of the Kit receptor tyrosine kinase. Proc Natl Acad Sci U S A. 2003 May 27;100(11):6706-11.

Sontheimer H. An unexpected role for ion channels in brain tumor metastasis. Exp Biol Med (Maywood). 2008 Jul;233(7):779-91.

Spitzner M, Martins JR, Soria RB, et al. Eag1 and Bestrophin 1 are up-regulated in fast-growing colonic cancer cells. J Biol Chem. 2008 Mar 21;283(12):7421-8.

Stiller CA, Trama A, Serraino D, et al. RARECARE Working Group. Descriptive epidemiology of sarcomas in Europe: report from the RARECARE project. Eur J Cancer. 2013 Feb;49(3):684-95.

Subramanian S, West RB, Corless CL, et al. Gastrointestinal stromal tumors (GISTs) with KIT and PDGFRA mutations have distinct gene expression profiles. Oncogene. 2004 Oct 14;23(47):7780-90.

Suehara Y, Kondo T, Seki K, et al. Pfetin as a prognostic biomarker of gastrointestinal stromal tumors revealed by proteomics. Clin Cancer Res. 2008 Mar 15;14(6):1707-17.

Sugahara K, Michikawa Y, Ishikawa K, et al. Combination effects of distinct cores in 11q13 amplification region on cervical lymph node metastasis of oral squamous cell carcinoma. Int J Oncol. 2011 Oct;39(4):761-9.

Takazawa Y, Sakurai S, Sakuma Y, et al. Gastrointestinal stromal tumors of neurofibromatosis type I (von Recklinghausen's disease). Am J Surg Pathol. 2005 Jun;29(6):755-63.

Teng JF, Mabasa VH, Ensom MH. The role of therapeutic drug monitoring of imatinib in patients with chronic myeloid leukemia and metastatic or unresectable gastrointestinal stromal tumors. Ther Drug Monit. 2012 Feb;34(1):85-97.

Théou N, Gil S, Devocelle A, Julié C, et al. Multidrug resistance proteins in gastrointestinal stromal tumors: site-dependent expression and initial response to imatinib. Clin Cancer Res. 2005 Nov 1;11(21):7593-8.

Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. Nat Rev Immunol. 2002 Aug;2(8):569-79.

Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood. 2004 Dec 1;104(12):3739-45.

Torihashi S, Nishi K, Tokutomi Y, et al. Blockade of kit signaling induces transdifferentiation of interstitial cells of cajal to a smooth muscle phenotype. Gastroenterology. 1999 Jul;117(1):140-8.

Tran T, Davila JA, El-Serag HB. The epidemiology of malignant gastrointestinal stromal tumors: an analysis of 1,458 cases from 1992 to 2000. Am J Gastroenterol. 2005 Jan;100(1):162-8.

Tryggvason G, Gíslason HG, Magnússon MK, et al. Gastrointestinal stromal tumors in Iceland, 1990-2003: the icelandic GIST study, a population-based incidence and pathologic risk stratification study. Int J Cancer. 2005 Nov 1;117(2):289-93.

Tsvilovskyy VV, Zholos AV, Aberle T, et al. Deletion of TRPC4 and TRPC6 in mice impairs smooth muscle contraction and intestinal motility in vivo. Gastroenterology. 2009 Oct;137(4):1415-24.

Verkman AS, Galietta LJ. Chloride channels as drug targets. Nat Rev Drug Discov. 2009 Feb;8(2):153-71.

Verweij J, Casali PG, Zalcberg J, et al. Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. Lancet. 2004 Sep 25-Oct 1;364(9440):1127-34.

Wang D, Zhang Q, Blanke CD, et al. Phase II trial of neoadjuvant/adjuvant imatinib mesylate for advanced primary and metastatic/recurrent operable gastrointestinal stromal tumors: long-term follow-up results of Radiation Therapy Oncology Group 0132. Ann Surg Oncol. 2012 Apr;19(4):1074-80.

Wardelmann E, Merkelbach-Bruse S, Pauls K, et al. Polyclonal evolution of multiple secondary KIT mutations in gastrointestinal stromal tumors under treatment with imatinib mesylate. Clin Cancer Res. 2006 Mar 15;12(6):1743-9.

Welsh RA, Meyer AT. Ultrastructure of gastric leiomyoma. Arch Pathol. 1969 Jan;87(1):71-81.

West RB, Corless CL, Chen X, et al. The novel marker, DOG1, is expressed ubiquitously in gastrointestinal stromal tumors irrespective of KIT or PDGFRA mutation status. Am J Pathol. 2004 Jul;165(1):107-13.

White DL, Saunders VA, Dang P, et al. OCT-1-mediated influx is a key determinant of the intracellular uptake of imatinib but not nilotinib (AMN107): reduced OCT-1 activity is the cause of low in vitro sensitivity to imatinib. Blood. 2006 Jul 15;108(2):697-704.

Widmer N, Colombo S, Buclin T, et al. Functional consequence of MDR1 expression on imatinib intracellular concentrations. Blood. 2003 Aug 1;102(3):1142.

Wiener MC, Sachs JR, Deyanova EG, et al. Differential mass spectrometry: a label-free LC-MS method for finding significant differences in complex peptide and protein mixtures. Anal Chem. 2004 Oct 15;76(20):6085-96.

Wilkins MR, Sanchez JC, Gooley AA, et al. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Eng Rev. 1996;13:19-50.

Yamaguchi U, Nakayama R, Honda K, et al. Distinct gene expression-defined classes of gastrointestinal stromal tumor. J Clin Oncol. 2008 Sep 1;26(25):4100-8.

Yang YD, Cho H, Koo JY, et al. TMEM16A confers receptor-activated calcium-dependent chloride conductance. Nature. 2008 Oct 30;455(7217):1210-5.

Yano H, Nakanishi S, Kimura K, et al. Inhibition of histamine secretion by wortmannin through the blockade of phosphatidylinositol 3-kinase in RBL-2H3 cells. J Biol Chem. 1993 Dec 5;268(34):25846-56.

Yantiss RK, Rosenberg AE, Sarran L, et al. Multiple gastrointestinal stromal tumors in type I neurofibromatosis: a pathologic and molecular study. Mod Pathol. 2005 Apr;18(4):475-84.

Yates JR, Ruse CI, Nakorchevsky A. Proteomics by mass spectrometry: approaches, advances, and applications. Annu Rev Biomed Eng. 2009;11:49-79.

Zahm SH, Fraumeni JF Jr. The epidemiology of soft tissue sarcoma. Semin Oncol. 1997 Oct;24(5):504-14.

Zhong L, Roybal J, Chaerkady R, et al. Identification of secreted proteins that mediate cell-cell interactions in an in vitro model of the lung cancer microenvironment. Cancer Res. 2008 Sep 1;68(17):7237-45.