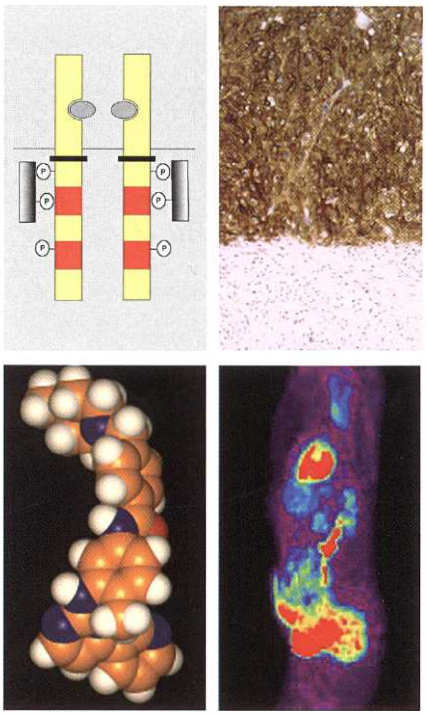
Gastrointestinal Stromal Tumours

- on diagnosis and treatment

Per Bümming



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V. Bümming P, Nilsson O, Ahlman H, Welbencer A, Nilsson B. Gastrointestinal stromal tumors (GIST) regularly express synaptic vesicle proteins – evidence of a neuroendocrine phenotype.
Submitted.

The Sahlgrenska Academy
AT GÖTEBORG UNIVERSITY

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Abstract

Gastrointestinal stromal tumours (GIST) are thought to originate from the interstitial cells of Cajal, which show many properties in common with neurons of the gastrointestinal tract. High-risk GIST has a very poor prognosis and tumour recurrence is common after intentionally curative surgery. With recent advances in our understanding of the molecular pathology of this disease and now that a specific KIT tyrosine kinase inhibitor, imatinib, is available, the prognosis for these patients has dramatically changed.

A population-based study from western Sweden with a total population of approximately 1.5 million was conducted, and 259 patients with clinically detected GIST were included. The annual incidence of GIST in the region was estimated to be 14.5 per million inhabitants. The majority of patients with high-risk GIST and all those with overtly malignant tumours experienced recurrence after complete (R0) resection. Tumour size, proliferative index (Ki67 max%), R0 resection, and *KIT* exon 11 deletion were independent prognostic factors. Prediction of prognosis for patients with GIST was simplified by a risk score based on tumour size and Ki67 max%.

Early on, we treated patients with high-risk or overtly malignant GIST with imatinib in three different clinical settings (neoadjuvant, adjuvant and palliative) and response to treatment was found to be correlated with *KIT* mutational status and tumour regression. The response to treatment was studied by functional imaging of tumour glucose uptake using ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG-PET). In one patient, neoadjuvant treatment facilitated later surgical treatment. Adjuvant imatinib seemed promising, but long-term effects on survival must be evaluated in randomised clinical trials. Palliative imatinib was safe and effective, particularly in patients with *KIT* exon 11 mutations.

A 2-tracer PET, using ^{18}F-FDG and ^{11}C-hydroxyephedrine, was used to simultaneously detect GIST and pheochromocytoma in patients with neuroendocrine (NE) tumour syndromes, e.g. Carney triad and neurofibromatosis type 1.

GISTs were examined for a possible NE phenotype by immunohistochemistry, western blot and quantitative gene expression studies. GIST showed an abundant expression of synaptic-like microvesicle (SLMV) proteins both at the transcriptional and the translational level. Subsets of GIST appear to express peptide hormone receptors, which may be used for receptor-based radionuclide therapy.

In summary, the incidence of GIST was shown to be higher than previously estimated. Radical surgery and *KIT* exon 11 mutation were important prognosticators. Adjuvant treatment with imatinib seems to be promising in patients with high-risk GIST. Pre-treatment with imatinib is an attractive option in patients with tumours that are non-resectable initially. The 2-tracer PET technique may be useful in patients with NE tumour syndromes. The expression of SLMV proteins in GIST indicates a certain degree of NE differentiation, which has possible potential therapeutic implications.

Key words: *adjuvant, epidemiology, GIST, imatinib, KIT, mutation, neoadjuvant, neuroendocrine phenotype, PET, prognosis, surgery, vesicle proteins.*

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Vetande utan förnuft är dubbel dårskap
// Baltasar Gracián y Morales

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ABBREVIATIONS

ATP	adenosine triphosphate
CD117	KIT antibody
Cg	chromogranin
CT	computed tomography
ddH_2O	double-distilled water
dHPLC	denaturating high-performance liquid chromatography
DNA	deoxyribonucleic acid
^{18}F-FDG	^{18}F-fluorodeoxyglucose
GI	gastrointestinal
GIST	gastrointestinal stromal tumour
HED	hydroxyephedrine
hpf	high-power field
HRP	horseradish peroxidase
ICC	interstitial cells of Cajal
i.v.	intravenous
LDCV	large dense-core vesicle
MAP	mitogen-activated protein
MBq	megabecquerel
mc	monoclonal
MRI	magnetic resonance imaging
NF1	neurofibromatosis type 1
pc	polyclonal
PCR	polymerase chain reaction
PDGFRA	platelet-derived growth factor receptor alpha
PET	positron emission tomography
PI3K	phosphatidylinositol 3-kinase
Q-PCR	quantitative reverse transcriptase polymerase chain reaction
R0	no residual tumour
R1	microscopic residual tumour
R2	macroscopic residual tumour
RECIST	response evaluation criteria in solid tumours
SCF	stem cell factor
SLMV	synaptic-like microvesicle
SMA	smooth-muscle actin
SNOMED	systematised nomenclature of medicine
SV2	synaptic vesicle protein 2
VMAT	vesicular monoamine transporter

INTRODUCTION

Historical Aspects

In the past, gastrointestinal stromal tumours (GISTs) were thought to be of smooth-muscle origin (Golden & Stout, 1941) and consequently classified as leiomyomas, leiomyosarcomas, or leiomyoblastomas (Appleman, 1986). Ultrastructural studies in the 1960s and 1970s did not give consistent evidence of smooth-muscle differentiation in GIST (Franquemont, 1995). With the introduction of immunohistochemistry, it was shown that the expression of smooth-muscle markers, i.e. actin and desmin, was highly variable in these tumours. Mazur & Clark (1983) introduced the term "stromal tumour" to describe this heterogeneous tumour entity. A subset of stromal tumours stained positively for neural crest markers (S-100, neuron-specific enolase) not found in other smooth-muscle neoplasms (Franquemont, 1995). Gastrointestinal autonomic nerve tumours (GANT) were described as a phenotypic variant of GIST with neuroendocrine (NE) differentiation (Miettinen *et al.*, 1999).

With the finding that most gastrointestinal (GI) stromal tumours are positive for CD34, a glycoprotein normally expressed on endothelial cells and hematopoietic stem cells, the term GIST became more widely used (Miettinen *et al.*, 1995). Today, GISTs are regarded as the most common mesenchymal (non-epithelial) neoplasms of the GI tract. True smooth-muscle tumours, i.e. leiomyoma and leiomyosarcoma, are rare in the GI tract (Miettinen & Lasota, 2001).

Definition of GIST

The lack of smooth-muscle differentiation combined with occasional neural characteristics in GIST led to speculation that these stromal tumours were related to the interstitial cells of Cajal (ICC), a network of spindle-shaped cells in the intestinal wall (Perez-Atayde *et al.*, 1993). ICC are intercalated between gut smooth-muscle cells and nerve terminals (Faussone-Pellegrini, 1990), where they function as regulators of intestinal peristalsis and mediators of neurotransmission (Sanders, 1996). The observation that the tyrosine kinase receptor KIT is expressed by ICC and is of vital importance for the development of these cells (Huizinga *et al.*, 1995) led to the discovery that GISTs also express KIT. Due to the striking similarities between GIST cells and ICC, it is currently believed that GISTs originate from precursor cells that may differentiate towards ICC (Kindblom *et al.*, 1998). The KIT protein, detected immunohistochemically by CD117 (antibody to KIT), is expressed in the vast majority of GISTs. CD117 is presently regarded as a sensitive and specific marker for these tumours (Hornick & Fletcher, 2002).

The histological appearance of GISTs is quite variable. About two-thirds of the tumours are composed of spindle-shaped cells or epithelioid cells, but mixed spindled-epithelioid tumours and tumours with NE features also occur. Typically, GISTs stain positive for KIT (CD117) in 95% of cases, CD34 in 70%, smooth-muscle actin (SMA) in 35%, S-100 in 5% of cases, but rarely for desmin (Miettinen & Lasota, 2001). GISTs are currently defined as intra-abdominal mesenchymal tumours that express the KIT protein, or have *KIT*- or *PDGFRA* mutations (see *Molecular Pathology* section).

Recent studies have demonstrated strong expression of a novel protein, DOG-1 (West *et al.*, 2004), and protein kinase C theta (PKCθ), a molecule associated with T-cell activation and neural differentiation (Blay *et al.*, 2004). Gene expression studies have revealed the secretory granule NE protein 1 and chromogranin C (CgC) in GIST, which may indicate a possible NE differentiation (Buragas *et al.*, 2005).

It is important to correctly diagnose GIST, since these patients can be offered several therapeutic options. The main differential diagnoses are other intra-abdominal soft-tissue tumours (**Table 1**).

Table 1. *Differential diagnosis of gastrointestinal stromal tumours (GIST).*

	KIT	***CD34***	***SMA***	***S-100***	***Desmin***
GIST	+	60-70%	30-40%	5%	Rare
Smooth-muscle tumour	-	10-15%	+	Rare	+
Schwannoma	-	+	-	+	-
Fibromatosis	Disputed	Rare	+	-	Rare cells

Modified from Fletcher *et al.* (2002).

Clinical Features

Due to difficulties in classification, the true incidence of GIST is unknown. Previous reports have estimated the annual incidence to be approximately 4 per million inhabitants (DeMatteo *et al.*, 2000; Jemal *et al.*, 2002). GIST has an equal sex distribution, and the median age at presentation is about 60 years. The majority of GISTs are sporadic, but individuals from the same family with GIST have been reported (Nishida *et al.*, 1998; Beghini *et al.*, 2001). GISTs have also been associated with certain syndromes including NE tumours, i.e. Carney triad and neurofibromatosis type 1 (NF1, von Recklinghausen's disease) (Zöller *et al.*, 1997; Carney, 1999). There have also been case reports on multiple GIST and synchronous ileal carcinoids (Buragas *et al.*, 2005).

GISTs are most commonly seen in the stomach and small intestine, but can also originate in the oesophagus, the colo-rectum, mesentery, omentum, and retroperitoneum. Since GISTs tend to grow non-invasively, the tumours can be large before they cause symptoms. The vast majority of GISTs are solitary and well-delineated at diagnosis.

The clinical presentation is often related to tumour size; smaller tumours are found incidentally during endoscopy, radiological imaging, or surgery for other reasons. Larger tumours are usually diagnosed during work-up for symptoms. In reports from tertiary cancer centres, it has been shown that up to 50% of GISTs are metastatic at presentation (DeMatteo *et al.*, 2000; Crosby *et al.*, 2001).

In general, GIST recurs within the abdominal cavity, and frequently involves the peritoneum (52%), the liver (63%), or both (15%) (Ng *et al.*, 1992b; DeMatteo *et al.*, 2000). Extra-abdominal metastases (pulmonary and bone) are rare but can occur late in disease. Lymph node metastases are very rare (Fong *et al.*, 1993).

Diagnosis

With growing awareness of this tumour type in recent years, there has been considerable interest in establishing early diagnosis. On CT/MRI, GIST should be suspected when there is a well-defined mass closely associated with the stomach or intestine. Intra-tumoural necrosis, or haemorrhage, strengthens the suspicion. CT/MRI are essential investigations to determine the extent of the primary tumour anatomically, multicentricity, and presence of metastases. Endoscopy may identify submucosal lesions, with or without ulceration. On endoscopic ultrasound, GISTs are characterised by a marginal halo and relatively high echogenicity, and can therefore often be distinguished from leiomyomas or leiomyosarcomas (Okai *et al.*, 2003).

The role of functional imaging by positron emission tomography (PET) is rapidly expanding. GISTs, like several other malignancies, have increased glucose metabolism and thus take up the tracer ^{18}F-fluorodeoxyglucose (FDG). This technique can be used for staging of the tumour disease and is of value for planning of the surgical approach (van den Abbeele & Badawi, 2002). It was recently reported that tumour uptake on ^{18}F-FDG-PET was very rapidly extinguished (within 24–48 hours) in response to treatment with imatinib (Stroobants *et al.*, 2003). When PET was combined with another imaging modality such as CT (PET-CT), the accuracy of diagnosing and monitoring treatment responses in GIST was further improved (Antoch *et al.*, 2004).

Prognostic Features

GISTs represent a spectrum of disease that ranges from benign tumours with excellent prognosis to highly malignant tumours with very poor prognosis. The clinical behaviour has not always been easy to predict, and several prognostic factors have been proposed.

The most frequently used prognostic parameters for GIST have been tumour size and mitotic rate. During an NIH/NCI-workshop, consensus guidelines were prepared that emphasised the importance of tumour size and mitotic index in estimating the metastatic risk of primary GIST (Fletcher *et al.*, 2002) (**Table 2**).

Table 2. *Risk assessment of primary GIST.*

Risk	Size (cm)	Mitotic Count (per 50 hpf)
Very low risk	< 2	< 5
Low risk	2–5	< 5
Intermediate risk	< 5	6–10
	5–10	< 5
High risk	> 5	> 5
	> 10	Any mitotic rate
	Any tumour	> 10

Modified from Fletcher *et al.* (2002).

Surgery

Complete (R0) surgical resection provides the best chance of cure in patients with GIST. Only a localised resection of the tumour, i.e. wedge resection of the stomach or a segmental intestinal resection, is necessary for well-delineated tumours. Negative microscopic margins must be attempted. If necessary, en-bloc resection of adjacent organs should be considered. Avoidance of tumour rupture is imperative (Ng *et al.*,

1992a). Some authors have recommended wide peritonectomy because of the high risk of peritoneal recurrence (Roberts & Eisenberg, 2002). The value of regional lymph-node resection is unproven, and extensive lymphadenectomy is not recommended (Fong *et al.*, 1993; DeMatteo *et al.*, 2002). In recent years, laparoscopic resection has been advocated for smaller gastric GISTs (Hindmarsh *et al.*, 2005). This technique seems appropriate, but the present series are limited in size and long-term follow-up data are lacking.

Chemotherapy and Radiation Therapy

Systemic chemotherapy has been largely unsuccessful in GIST patients. The frequency of partial responses after either single-agent therapy or combination therapy has varied between 0 and 15% (DeMatteo *et al.*, 2002; De Pas *et al.*, 2003). One explanation may be that GISTs have increased levels of multidrug resistance proteins in comparison with leiomyosarcomas (Plaat *et al.*, 2000). Intraperitoneal chemotherapy was introduced to improve the results after surgery for peritoneal recurrence. However, it did not alter survival for patients with liver metastases, but it probably contributed to extending the mean time to recurrence from 8 to 21 months (Eilber, 1999). The experience of radiation therapy in GIST is limited; in one report, radiation therapy temporarily controlled metastatic GIST in 6 out of 9 patients (Crosby *et al.*, 2001).

Molecular Pathology

The *KIT* gene, located on chromosome 4q11-q12 (Spritz *et al.*, 1994), has 21 exons and is the cellular homologue of the oncogene *v-kit* of the Hardy-Zuckerman feline sarcoma virus (Besmer *et al.*, 1986). It encodes the tyrosine kinase receptor KIT (CD117), which is of fundamental importance in the pathogenesis of GIST. KIT belongs to the subclass III family of tyrosine kinase receptors, which is closely related to the receptors for platelet-derived growth factor (PDGF), macrophage colony-stimulating factor, and FLT3 ligand (Rousset *et al.*, 1995). KIT is a transmembrane receptor in which the extracellular region has a binding site for the ligand: stem cell factor (SCF). The intracellular region consists of an ATP-binding site and a split tyrosine kinase domain (**Fig. 1 A**). KIT is expressed by haematopoietic cells, melanocytes, mast cells, germ cells, and ICC (Heinrich *et al.*, 2002), and is important for differentiation, cell growth and survival. The unbound KIT protein is enzymatically inactive. It is activated by the binding of bivalent ligand dimers, i.e. two adjacent receptors are brought together in a process called homodimerisation, which results in activation of kinases and cross-phosphorylation of intracellular tyrosine residues (Broudy, 1997; Nishida & Hirota, 2000) (**Fig. 1 A**). The phosphorylation of KIT and PDGFRA activates similar downstream molecules: mitogen-activated protein (MAP) kinase, AKT, mTOR, p70/85S6K, STAT 1 and STAT 3 (Heinrich *et al.*, 2003b; Duensing *et al.*, 2004). The phosphorylation is mainly mediated via the PI3 kinase/AKT/mTOR pathways (**Fig. 1 A and B**), and inhibition of the PI3 kinase/mTOR pathway reduces proliferation and increases apoptosis (Duensing *et al.*, 2004).

KIT Mutations. The vast majority of sporadic GISTs have gain-of-function mutations in *KIT* (Hirota *et al.*, 1998; Rubin *et al.*, 2001; Corless *et al.*, 2002; Heinrich *et al.*, 2003a) that result in ligand-independent dimerisation, autophosphorylation and

activation of downstream signalling pathways. The mutations are confined to only 4 exons; exon 9 encoding the extracellular transmembrane domain, exon 11 encoding the intracellular juxtamembrane domain, exon 13 encoding the the first portion of the kinase domain, and exon 17 encoding the second portion of the kinase domain (**Fig. 1 B**).

Sixty to seventy per cent of the mutations involve exon 11, a domain that normally functions by inhibiting receptor dimerisation in the absence of SCF, thus leading to ligand-independent receptor activation. Most exon 11 mutations are deletions and/or point mutations located at the 5' end (Heinrich *et al.*, 2003a).

Exon 9 mutations are found in approximately 10% of cases. This type of mutation is interesting, since all but one of such mutations were found by Antonescu *et al.* (2003) to be identical (an insertion of 6 nucleotides resulting in duplication of alanine 501 and tyrosine 502) and almost all mutations were found in small intestinal GISTs.

Mutations in exon 13 (kinase 1 domain) and exon 17 (kinase 2 domain or activation loop) have been seen in about 1% and < 1% of cases, respectively (Lasota *et al.*, 2000; Lux *et al.*, 2000; Rubin *et al.*, 2001).

Even in the absence of a mutation, nearly all GISTs express a phosphorylated KIT protein, indicating that they are constitutively activated (Rubin *et al.*, 2001). Explanations for this could be an alteration in related proteins, gene amplification, or different methylation patterns (DeMatteo *et al.*, 2003). Mutations of *KIT* do not occur in leiomyomas or leiomyosarcomas (Lasota *et al.*, 1999). The *KIT* mutation is almost always somatic, but several family members with heritable mutations of exon 11 have been identified. The familial cases often have multiple and small tumours; they also display a diffuse hyperplasia of the Auerbach's plexus. In addition, many patients with familial GIST have systemic cutaneous hyperpigmentation. Abnormalities of mast cells have been reported, e.g. urticaria pigmentosa or systemic mast cell disease (Longley *et al.*, 1996). Clinical features and mutation analysis in families with inherited exon 11 mutations indicate an association between ICC hyperplasia/neoplasia, multiple GISTs, melanocytic dysfunction, and cutaneous mast cell proliferation (Nishida *et al.*, 1998; Hirota *et al.*, 2000; Beghini *et al.*, 2001; Maeyama *et al.*, 2001).

PDGFRA Mutations. A small subset of GIST patients with no mutation in *KIT* can have mutations in the *PDGFRA* gene (Heinrich *et al.*, 2003b; Hirota *et al.*, 2003). The mutations involve exons 12, 14, and 18, which are homologous to *KIT* exons 11, 13, and 17. The signal transduction profiles for these *PDGFRA*-mutant tumours were found to be the same as for *KIT*-mutant tumours, suggesting that PDGFRA can have a role similar to that of KIT in GIST oncogenesis (Heinrich *et al.*, 2003b). The majority of *PDGFRA*-mutant tumours express KIT only weakly, or not at all. These tumours are often gastric in origin and associated with a fairly benign clinical course (Lasota *et al.*, 2004; Miettinen *et al.*, 2005). About 5–10% of GISTs lack both *KIT* and *PDGFRA* mutations.

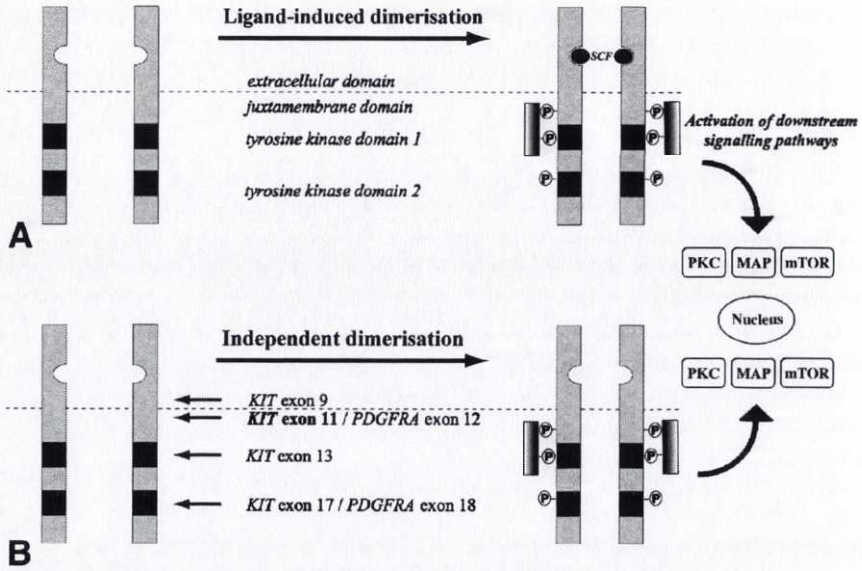

Fig. 1. A. Ligand-induced dimerisation *of KIT or PDGFRA results in autophosphorylation and activation of downstream signalling involved in cell differentiation, cell proliferation, and cell survival.* ***B. Independent dimerisation****, due to gain-of-function mutations in the KIT or PDGFRA genes, results in unrestrained cell proliferation.*

Imatinib

Imatinib mesylate (Glivec®, Novartis Pharmaceuticals, Basel, Switzerland)–initially developed to inhibit ABL as treatment for chronic myelogenous leukaemia–has caused a paradigm shift in cancer therapy. Imatinib is an ATP analogue that binds to the intracellular portion of KIT. In this way, the kinase is prevented from transferring phosphate groups from ATP to tyrosine residues of substrates. This inhibits downstream signalling from the kinase and switches the balance between cell proliferation and apoptosis towards apoptosis (**Fig. 2**).

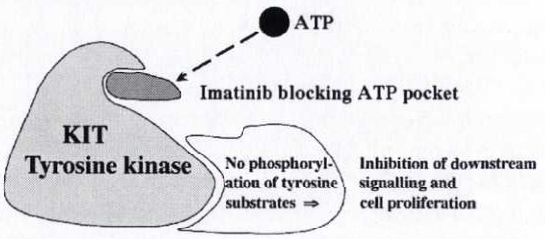

Fig. 2. *Mechanism of action of imatinib.*

Imatinib selectively inhibits several tyrosine kinases, including ABL, BCR-ABL fusion protein, KIT and PDGFRA (Carroll *et al.*, 1997). Imatinib is well absorbed after oral administration. The half-life is about 20 hours (Druker *et al.*, 2001; Demetri *et al.*, 2002), which is compatible with once-daily administration. Preclinical studies have shown that imatinib serum concentrations of greater than 1μmol/L are necessary for optimum therapeutic effects, which can be achieved by daily doses of ≥ 300 mg.

AIMS OF THE STUDY

The epidemiology, prognostic factors and outcome of the surgical treatment of GIST have not been addressed in population-based studies. With the introduction of the tyrosine kinase inhibitor imatinib, effective treatment of GIST became possible. Functional imaging with ^{18}F-FDG-PET has turned out to be a valuable tool for evaluation of tumour responses to treatment with imatinib. GISTs are thought to originate from ICC, which show properties in common with neurons of the GI tract.

The aims of the study were:

- To analyse the incidence and prevalence of GIST and to validate both the consensus risk stratification scheme and a new risk score based on tumour size and proliferative index (I).
- To evaluate radical surgery (R0 resection) and other prognostic factors in a population-based patient series with long follow-up (II).
- To explore the usefulness of imatinib in different clinical settings (neoadjuvant, adjuvant and palliative) and evaluate response to given treatment with respect to *KIT* exon 11 mutational status (III).
- To demonstrate the diagnostic value of a novel 2-tracer PET technique in patients with GIST as part of NE tumour syndromes (IV).
- To evaluate NE differentiation in GIST by analysing the expression of synaptic vesicle proteins (V).

MATERIALS & METHODS

Population-Based Studies (papers I & II)

Retrieval of Patients

Records on all potential patients with GIST, diagnosed between January 1983 and December 2000 within the Swedish region of Västra Götaland (population 1.3–1.6 million), were retrieved. Histopathological examination of all patients was performed in four hospital-based pathology laboratories, each covering a defined geographic area. Computerised files of histopathological diagnoses according to the topographic and morphological (T and M) coding system of SNOMED were used to retrieve all patients. T codes included all sites in the GI tract; intra-abdominal, retroperitoneal, mesenteric, omental and pelvic areas, and the liver. M codes included all benign and malignant mesenchymal lesions, tumours of the autonomic and peripheral nervous systems, and benign and malignant tumours not further classified. In all 1,460 patients who matched the T and M codes, clinical information and pathology reports were reviewed. Five hundred patients were excluded immediately, since they obviously did not have GIST. In the remaining 960 patients, slides were reviewed histologically, after which 310 other patients had to be excluded. The remaining 650 patients were studied in detail including immunohistochemical analyses.

Inclusion Criteria

Requirements for the diagnosis of GIST and inclusion in these studies were: (1) site in or adjacent to the GI tract, mesentery, omentum, or retroperitoneum; (2) spindle-shaped and/or epithelioid morphology compatible with GIST; and (3) positive immunoreactivity for CD117. Three hundred and ninety-eight of the 650 patients fulfilled these criteria. Ninety-five patients were excluded because they were referrals from other regions, and 15 patients were excluded because they had a primary GIST diagnosed before 1983. Two hundred and eighty-eight patients with primary GIST from our region remained and were included in paper I. For the analyses in paper II, 29 patients with autopsy-based diagnosis were excluded; 259 patients with clinically detected primary GIST were thus studied.

Morphological Analysis

The following data were recorded for each patient: histological type, degree of tumour cell pleomorphism, mitotic rate per 50 high-power fields (hpf, with 1 hpf = 0.16 mm^2), average and maximum proliferative index (Ki67), and patterns of immunoreactivity for CD117 and CD34. The following antibodies were used: CD117 (pc), CD34 (mc), and Ki-67 (MIB1; mc) (paper I).

Clinical Information and Follow-up

Information regarding presenting symptoms and dates of diagnosis, tumour status at diagnosis, treatment, and diagnosis of local recurrences or metastases were recorded. For all patients, information on survival and cause of death was obtained from clinical records, autopsy reports and Official Population Registries. Surgical margins were assessed from clinical records and evaluation of the histological findings in the specimens. The margins were classified as R0 (no residual tumour), R1 (microscopic residual tumour), and R2 (macroscopic residual tumour) according to UICC (TNM classification of malignant tumours, 2002). All tumours were classified according to a consensus risk group stratification system based on tumour size and mitotic rate per 50 hpf (Fletcher *et al.*, 2002; **Table 2**). In these studies, we added a fifth group called overtly malignant, which included all patients with proven metastatic disease at diagnosis. The median follow-up time of surviving patients was 8 years (range 1.8–19.5 years) (paper II).

Statistical Analysis

A Poisson regression model (Breslow & Day, 1987) was used for the analysis of overall survival instead of the Kaplan-Meier model, because the latter cannot treat continuous risk variables efficiently

and does not allow all types of comparisons with the normal population. This model was used to calculate the survival function for the different risk groups according to Fletcher *et al.* (2002), with addition of the overtly malignant group. In the analysis, the follow-up was divided in intervals of 0.5 years, and the death rate within each interval was estimated by calculating the quotient between the number of events and patient years within the interval. From this, a stepwise constant hazard function was obtained and, by applying the mathematic relation between survival and hazard functions, the survival function was determined. The expected proportion of survivors was calculated, taking age, sex, and calendar time into account using the mathematical relations applied to hazard functions derived from official statistics of Sweden. Continuous variables were compared using the non-parametric Mann-Whitney *U* test and categorical variables using Fisher's exact test. In the analysis of all prognostic factors in paper I, the Poisson regression (Breslow & Day, 1987) was used to estimate a hazard function of the form $\exp(\beta_0 + \beta_1 \times x_1 + \ldots)$, where the β values were coefficients and x_i, $i = 1, 2, \ldots$, were variables. The function was continuous as a function of all continuous variables, including time since diagnosis. The analysis was performed by a stepwise procedure, which, at the end, only included variables that were significant in the multivariate context. The Poisson regression analysis resulted in a risk score, which was a linear combination defined by the variables and the β coefficients. A special analysis was performed to elucidate how well the risk score captured the large variation in the risk of dying. The probability of dying within 5 years after diagnosis was calculated depending on the risk score. The calculation of the percentile points was based on a piecewise linear transformation of the score yielding a normally distributed variable. Regression on age was applied to the normally distributed variable. The incidence of GIST was calculated from the number of clinically detected primary GIST cases divided by the accumulated number of person years (17,862,068) during the period 1983–2000. The prevalence was calculated from the age specific incidence figures and the estimated death hazard function, taking into account risk groups and calendar time.

In paper II, disease-free survival time was calculated as the interval from the initial diagnosis to the diagnosis of recurrence, persistent disease, or tumour-related death. Recurrence-free survival time was calculated as the interval from the initial diagnosis to the diagnosis of the first recurrence. Patients with no evidence of recurrence at last follow-up and those who died from non-GIST causes were censored in the analysis of disease-free survival. Patients without evidence of recurrence or metastasis at last follow-up were censored and those with persistent disease were excluded from the analysis of recurrence-free survival. Survival estimates in paper II were calculated by the Kaplan-Meier method and differences between groups were compared by the log rank test. As in paper I, stepwise Poisson regression procedures were used to estimate the hazard functions for recurrence and tumour-related death, and to identify independent risk factors. All statistical tests were two-sided. $P < 0.05$ was considered statistically significant.

New Therapeutic Options Combining Surgical and Medical Treatment (paper III)

Patients

Seventeen consecutive patients with high-risk, or overtly malignant, GIST were admitted to Sahlgrenska University Hospital from May 2001 to September 2002, and included in this study. There were two females and 15 males, with a median age at primary surgery of 62 years (range 10–74 years).

One patient with initially non-resectable GIST was treated neoadjuvantly with imatinib so that surgical treatment could be performed later. Three out of 5 patients were treated with adjuvant imatinib after R1 resection for rectal GIST: two had primary tumours and the other a local recurrence. All three patients had no clinical or radiological signs of residual tumour after surgery; however, all had microscopic intra-lesional margins. A fourth patient with a small intestinal GIST also had intra-lesional margins (observed 4 months). The fifth patient, a young female, had a gastric GIST (observed 144 months). She was first operated upon at age 10, and had then, during a 12-year period prior to imatinib, undergone 5 operations due to recurrence. Eleven patients received palliative imatinib, 10 due to unresectable primary tumour, peritoneal and/or liver metastases, and one due to pulmonary metastases.

Morphological Analysis

All resected or biopsied tumours were reviewed histologically. Histologically, three (18%) of the tumours were predominantely epithelioid, 8 (47%) were predominantely spindle-shaped, and 6 (35%) mixed spindle-shaped and epithelioid. The mitotic rate per 50 consecutive hpfs was recorded as < 2, 2–5, 6–10, and > 10 per 50 hpfs. Proliferative activity was assessed visually, estimating the percentage of Ki67-immunopositive tumour cells using MIB1. Immunostains for CD117, CD34, α-SMA, desmin, and S100 protein were performed in all 17 cases and found to be positive in 17 (100%), 14 (82%), two (12%), zero, and zero cases, respectively.

Clinical Information and Follow-up

Treatment with imatinib, 400 mg p.o. once-daily, was given neoadjuvantly (n = 1) three months prior to surgery, adjuvantly (n = 5) over 7–13 months, or palliatively (n = 11) over 6–18 months. Side effects were monitored in agreement with the US National Cancer Institute Common Toxicity Criteria (version 2.0). Tumour response was evaluated by spiral CT (5-mm contiguous reconstruction algorithm) 3, 6, and 12 months after induction of imatinib, and categorised according to the RECIST (Therasse *et al.*, 2000). MRI (contiguous cuts of 10 mm or less) was performed in the patient who received neoadjuvant therapy. ^{18}F-FDG-PET was performed before and during treatment with imatinib in 10 patients.

New Imaging Options (paper IV)

Patients

In this study we reported two patients with NE tumour syndromes, one with complete Carney triad and one with NF 1, in whom accurate diagnosis of both pheochromocytoma and GIST in the same patient was made by a sensitive 2-tracer PET technique.

PET

Attenuation-corrected whole-body PET with a dedicated scanner (CTI/Siemens) was performed 10 min after i.v. injection (800 MBq) of ^{11}C-hydroxyephedrine (HED) to visualise regional catecholamine accumulation and 1 hour after i.v. injection (400 MBq) of ^{18}F-FDG to visualise regional glucose uptake. Both examinations were performed on the same day. All images were corrected for attenuation and iteratively reconstructed with a 6-mm Hann filter. All post-processing was done with standard software supplied by the manufacturer.

Immunohistochemistry

The following monoclonal and polyclonal antibodies directed against the following proteins were used: CD117 (pc), CgA (pc), tyrosine hydroxylase (pc), vimentin (mc), SMA (mc), S-100 (pc), and Ki67 (MIB1; mc). All slides were subjected to antigen retrieval by microwave treatment. Bound antibodies were visualised by indirect immunoperoxidase techniques (Dako EnVision+).

Neuroendocrine Differentiation in GIST (paper V)

Tumours

Forty-one tumours obtained from the population-based material (papers I & II) were studied by immunohistochemistry. Tumours were separated into low-risk profile GIST (very low-risk, low-risk and intermediate-risk; n = 29) and high-risk profile GIST (high-risk and overtly malignant; n = 12). In low-risk profile tumours, 27 primaries, one local recurrence and one metastasis were used for analysis. In high-risk profile tumours, 7 primaries, one local recurrence and 4 metastases were used.

Biopsies from 10 patients with GIST were analysed by western blot: four patients with low-risk profile tumours and 6 patients with high-risk profile tumours. In low-risk profile tumours, only primaries were used for analysis. In high-risk profile tumours, one primary, one local recurrence and 4 metastases were used.

Biopsies from 10 patients with GIST were analysed by quantitative reverse transcriptase polymerase chain reaction (Q-PCR). There was equal distribution between low-risk and high-risk

profile tumours. In low-risk profile tumours, 4 primaries and one local recurrence were used for analysis. In high-risk profile tumours, one primary, one local recurrence and 3 metastases were used.

Immunohistochemistry

Tissue sections from paraffin-embedded specimens were placed on positively-charged slides, deparaffinised, rehydrated, and subjected to antigen retrieval by microwave treatment. Incubation with primary antibodies (**Table 3**) was followed by detection with DakoCytomation EnVision+ System-HRP labelled polymer or DakoCytomation LSAB®+ System-HRP. Diaminobenzidine was used as a chromogen. After counterstaining, sections were dehydrated and mounted. Immunolabelling was graded as: 0 = < 1% positive tumour cells; 1+ = 1–24% positive tumour cells; 2+ = 25–75% positive tumour cells; 3+ = > 75% positive tumour cells.

Western Blot

Frozen tumour tissues (200 mg/sample) were homogenised in 10 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA, 9.8 mM 3-(3-cholamidopropyl) dimethylammonium 1-propane sulphate and protease inhibitors. Homogenates were sonicated, followed by centrifugation and assay for protein content. Aliquots of proteins (40 µg) were electrophoresed on polyacrylamide gels (10% NuPAGE Bis-Tris gels; Invitrogen). Proteins were transferred to polyvinyl difluoride membranes using a NOVEX blotting system. Membranes were incubated with primary antibodies (**Table 3**) at 4°C overnight, followed by alkaline phosphatase-conjugated secondary antibodies and CDP-star (Tropix) as substrate. Membranes were then exposed to ECL film at room temperature. Molecular weight markers (See-Blue Plus 2 and MagicMark; Invitrogen) were used to calculate the apparent sizes of immunoreactive proteins.

Table 3. *Antibodies used for immunohistochemistry and western blot.*

Antibody	*Species*	*Clone*	*Code No.*	*Source*
Amphiphysin (mc)	Mouse	3	VAM-SV030	Nordic Biosite AB, Täby, Sweden
B-actin (mc)	Mouse	MAbcam 8226	AB8226	Abcam Ltd., Cambridge, UK
CD 117 (pc)	Rabbit	-	A4502	DakoCytomation Denmark A/S, Glostrup, Denmark
CgA (mc)	Mouse	LK2H10	MAB319	Chemicon International, Inc., Temecula, CA, USA
GAP 43 (mc)	Mouse	IG7	NCL-GAP43	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
SNAP 25 (mc)	Mouse	SP12	NB09	Oncogene™ Research Products, Darmstadt, Germany
SV2 (mc)	Mouse	SP2/0	SV2	Developmental Studies Hybridoma Bank, Iowa city, IA, USA
Synapsin 1 (mc)	Mouse	A10C	NB08	Oncogene™ Research Products, Darmstadt, Germany
Synapsin 2A (mc)	Mouse	1	S56820	Transduction Laboratories, Lexington, KY, USA
Synaptobrevin (mc)	Mouse	SP10	MMS-616R	Nordic Biosite AB, Täby, Sweden
Synaptophysin (mc)	Mouse	SY38	M0776	DakoCytomation Denmark A/S, Glostrup, Denmark
Syntaxin (mc)	Mouse	SP8	MAB336	Chemicon International, Inc., Temecula, CA, USA
VMAT 1 (pc)	Goat	-	Sc-7718	Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA
VMAT 2 (pc)	Rabbit	-	AB1767	Chemicon International, Inc., Temecula, CA, USA

mc, monoclonal; pc, polyclonal.

Q-PCR

RNA Extraction. Total RNA was prepared by homogenising tumour biopsies in Trizol Reagent (Invitrogen) using a Tissuelyser (Qiagen) followed by RNA purification using the FastRNA kit (QBIO

gene). RNA yields were measured by reading absorbance at 260 nm (Nano Drop ND-1000). The quality of extracted RNA was examined by gel electrophoresis (Agilent 2100 Bioanalyzer). Before cDNA-synthesis, 4 µg of total RNA per sample was subjected to DNase treatment using DNA-Free (Ambion).

cDNA Synthesis. cDNA was synthesised from extracted RNA using TaqMan reverse transcription reagents with random hexamers (Applied Biosystems). From each tumour sample, 0,4 µg RNA was used for the cDNA synthesis, and a control reaction without reverse transcriptase was run.

Primers and Probes. Primers and probes against synaptobrevin 1 & 2 (VAMP 1 & 2), amphiphysin (AMPH), synapsin 1 (SYN 1), synaptic vesicle protein 2A (SV2A), KIT (KIT), chromogranin A (CHGA), synaptophysin (SYP), VMAT 1 & 2 (VMAT 1 & 2) and β-actin were purchased from Applied Biosystems.

Real-Time PCR Assay. The PCR assays were performed in 96-well optical plates, using an ABI Prism 700. The reaction consisted of 40 cycles of 90°C for 15 sec and 60°C for 1 min. Samples were analysed in triplicate. The cycle threshhold (C_t) for the target gene and β-actin were determined for each sample. Values were expressed as - ΔC_t (target gene - β-actin) or number of target copies per 1,000 copies of β-actin.

Mutation Analysis (papers II–V)

DNA/RNA Isolation and Amplification

Genomic DNA was extracted from formalin-fixed, paraffin-embedded archival tumour tissue and 100 ng was amplified in 50 µl PCR reactions using BD Advantage 2 or BD Sprint (Clonetech, BD Biosciences) according to the manufacturer's recommendations with primers designed to amplify *KIT* exons 9, 11, 13, and 17 as well as *PDGFRA* exons 12 and 18, respectively. Total RNA was extracted from fresh-frozen tumour tissue and used as template in the cDNA synthesis. cDNA was amplified in 100 µl PCR reactions with primers PCRKIT21s and PCRKIT22as designed to amplify exon 11 of the *KIT* gene.

Denaturing High-Performance Liquid Chromatography and Nucleotide Sequence Analysis

Denaturing High-Performance Liquid Chromatography (dHPLC) is a rapid and sensitive method for screening for mutations within DNA samples, and was used in paper II. This technique is based on liquid chromatography in a column where a stationary phase is used to retain samples and a mobile phase is used to release samples from the column. In WT DNA (no mutation), the alleles in the sample form two homoduplexes. In contrast, in samples with mutation, there is a mismatch and both homo- and heteroduplexes are formed. Heteroduplexes have a weaker affinity for the stationary phase than homoduplexes and are therefore eluted from the column at lower concentrations of the mobile phase. Heteroduplex formation was carried out by denaturing the PCR products at 95°C, and then allowing the samples to re-anneal by gradually decreasing the temperature to 45°C. 5-10 µl of the PCR product was injected on a Helix DNA HPLC Column 50 x 3.0 mm (Varian) and eluted at a flow rate of 0.45 ml/min. Samples showing an aberrant elution profile were re-extracted, re-amplified and the mutated sequence was then determined by bidirectional direct sequencing using the same primers as in the PCR reactions.

RESULTS AND DISCUSSION

Population-Based Studies (papers I & II)

Incidence and Prevalence

The true incidence and prevalence of GIST is still controversial. The reason for this has been the absence of well-defined pathological criteria, variable nomenclature over time, and the fact that the majority of such tumours have been diagnosed as benign or as tumours of uncertain malignant potential. Under such circumstances, some of these tumours may not have been reported to the National Cancer Registries. Our approach–to review all mesenchymal and nervous system GI tumours histopathologically from a well-defined population over a long period of time–gives a unique opportunity to estimate incidence/prevalence of authentic GIST. The annual incidence of clinically detected GIST in our region was estimated to be 14.5 per million inhabitants (95% CI: 12.8–16.4 per million), and it did not vary over time. The prevalence of GIST was calculated from the incidence figures and the estimated death hazard function, and was found to be 129 per million; for patients with high-risk GIST, the prevalence was estimated to be 22.2 per million (paper I). The median age at presentation was 68 years, and there was an equal gender distribution (paper II). GIST was occasionally found in young adults (only 4 out of 259 patients were younger than 30 years at presentation) in accordance with the results of Miettinen *et al.* (2005). Using information on the incidence of GIST and the age- and sex-specific distribution of the general population in our region, the age- and sex-specific incidence of GIST could be calculated (**Fig. 3**) (paper II).

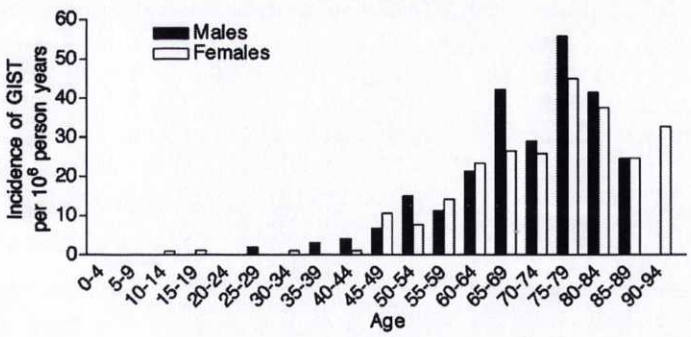

Fig. 3. *Age- and sex-specific incidence of GIST in a western Swedish population (n = 259).*

In a recent US population-based study, a low annual incidence rate of GIST was reported: 7 cases per million inhabitants (Tran *et al.*, 2005). Blanke *et al.* (2005) commented that one major limitation to this study was that only tumours reported to be malignant were included, which would lead to an underestimation of the true incidence. In line with our results, Tryggvason *et al.* (2005) reported an annual incidence of 11 cases per million inhabitants in an Icelandic population-based series (1990-2003) of similar design to that of ours.

Associated Malignancies

Other malignancies (diagnosed before, after, or concomitant with GIST) were seen in almost one-third (27.8%) of the patients. Adenocarcinomas of the GI and urinary tract predominated. Interestingly, as many as 8 patients had NF1 or NE tumours (small cell lung carcinoma, carcinoid, pheochromocytoma) (**Fig. 4**).

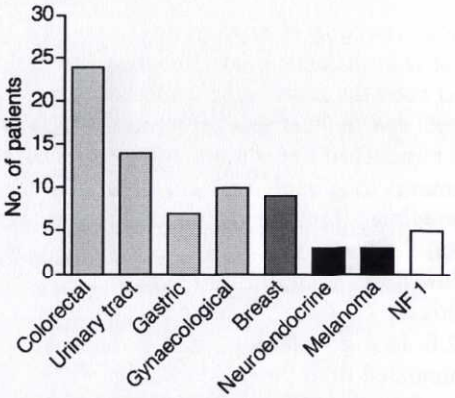

Fig. 4. *Summary of 72/259 patients with other malignancies diagnosed before, after, or concomitant with GIST.*

A recent German single-centre study (Agaimy & Wuensch, 2005) demonstrated other malignancies associated with GIST–most commonly GI carcinomas–in 18 out of 97 GIST patients (18.6%). These results and ours contrast with the findings of DeMatteo *et al.* (2000) and Crosby *et al.* (2001), who reported associated malignancies (before or concomitant with GIST) in only 6% and 5% of their respective study populations. This obvious discrepancy may relate to shorter follow-up and biased data from tertiary cancer centres with follow-up at other hospitals.

The finding of 5 patients with NF1-associated GIST in our study is interesting, since there have been several smaller series indicating an association between NF 1 and GIST (Cheng *et al.*, 2004; Kinoshita *et al.*, 2004; Yantiss *et al.*, 2005). In an autopsy series of NF1 patients, GISTs were detected in up to one-third of the patients (Zöller *et al.*, 1997). A recent study of 15 NF1 patients with GIST showed that these tumours were preferentially multicentric, of small intestinal origin, and devoid of *KIT* and *PDGFRA* mutations. NF1-GISTs were associated with a rather favourable prognosis; NE tumours (duodenal carcinoid or pheochromocytoma) were diagnosed in 6 of the 15 patients (Andersson *et al.*, 2005).

Symptoms, Preoperative Investigations, Tumour Site, and Surgical Procedures

In general, previous studies analysing surgical treatment, clinical course, and prognostic factors in GIST have suffered from methodological limitations. The early series were confounded by inclusion of non-GIST sarcomas. In several retrospective series, the tumour specimens had not been histopathologically reviewed and some studies only addressed subsets of tumours, e.g. "gastric or intestinal sarcomas" (Shiu *et al.*, 1982; Akwari *et al.*, 1978). Historically, the grading system has differed between studies, and the series from large referral centres were biased by having numerous patients with large and aggressive tumours (DeMatteo *et al.*, 2000; Pierie *et al.*, 2001; Singer *et al.*, 2002). Papers I and II were the first to evaluate a large population-based

series of GIST patients with *long-term* follow-up during a time period when surgery was the only available treatment (**Fig. 5**):

GIST-related **symptoms** were seen in 77% of the patients (paper II). GI bleeding (51%) and abdominal pain (32%) were the most common symptoms. It should be noted that it was more common to diagnose GIST incidentally than as a palpable mass. Almost half of the symptomatic GISTs were classified as high-risk, or overtly malignant.

Preoperative investigation was performed in almost all symptomatic patients. Due to symptoms such as bleeding and abdominal pain, endoscopy was frequently used and often found to be diagnostic. However, diagnostic tumour tissue was only obtained in one-third of the endoscopic biopsies, which may reflect deep submucosal growth with indirect impression by the tumour.

The most common **tumour site** was the stomach (55%), followed by the small intestine (37%). A few GISTs of retroperitoneal origin were seen, but no oesophageal GIST was diagnosed in our series. The tumour distribution was in accordance with the results of previous studies (DeMatteo *et al.*, 2000; Miettinen & Lasota, 2001).

The **surgical procedures** were predominantly gastric or intestinal resections. Only a minority of the patients underwent major surgical procedures, i.e. total gastrectomy or Whipple resection.

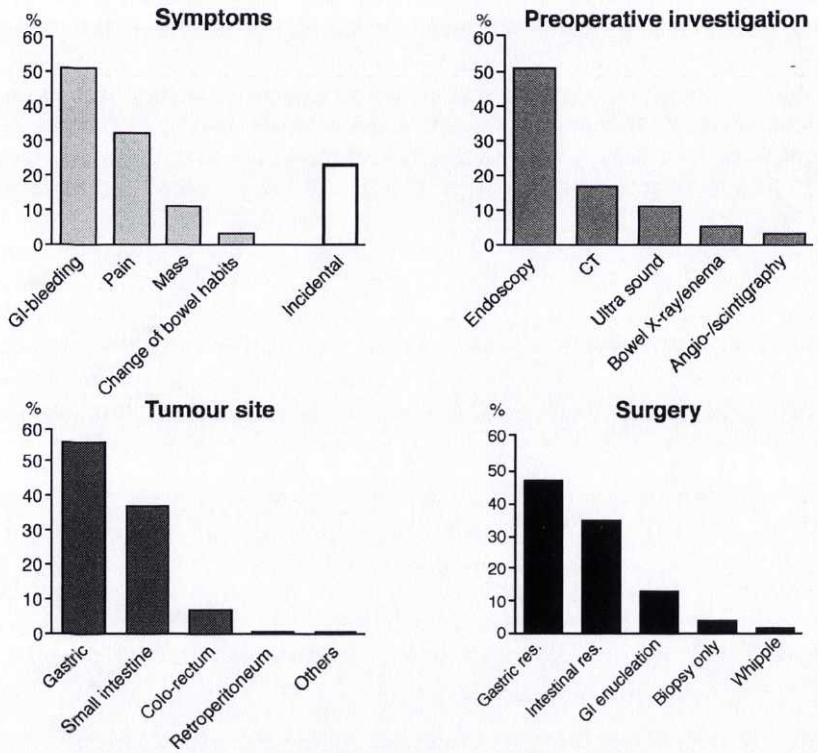

Fig. 5. *Clinical features, diagnostic tools and treatment of GIST (n = 259).*

The median tumour size in patients with clinically detected GIST was 8.9 cm (range 1.0–35.0 cm). A solitary tumour was seen in 86% of the patients, and only a minority of these tumours invaded adjacent organs. This finding is in contrast to other non-population-based studies that have reported approximately 30% invasive tumours at presentation (Nishida & Hirota, 2000). Very large tumours, measuring > 20 cm in diameter, may occur (Joensuu *et al.*, 2002; DeMatteo *et al.*, 2003). In our series, 12% of the patients had such large tumours. The type of clinical presentation was related to tumour size; small tumours (< 2 cm) were found incidentally during endoscopy, radiological imaging, or surgery for unrelated conditions, whereas larger tumours were diagnosed during work-up for abdominal pain, or palpable mass. Eight patients, including three with NF1, presented with multiple tumours. Twenty-nine patients (11%) presented with distant metastases and comprised the overtly malignant group.

Although patients were treated at several different hospitals, complete (R0) resection was achieved in 85% of the patients (paper II); R0 resection was performed in 80% of the patients with high-risk tumours, but only in 17% of the patients with overtly malignant tumours. The radical resection rate in this study confirmed previous results from single institutions (DeMatteo *et al.*, 2000; Langer *et al.*, 2003; Bucher *et al.*, 2006).

Recurrent Tumour Disease

The recurrence rate was low in the entire series of patients, but the risk of recurrence differed significantly between risk groups. The rate of recurrent disease after R0 resection was only 2% in the very low-, low- and intermediate-risk groups; these recurrent tumours were associated with negative prognostic factors: two patients had *KIT* exon 11 deletion, and one patient had high Ki67 max% (10%). In contrast, recurrent disease occurred in 62% and 100% of the R0-resected high-risk, or overtly malignant tumours, respectively; repeat surgery did not appear to influence survival (**Table 4**).

The median tumour size and median Ki67 max% for the patients with recurrence was 12 cm (range 1.5–33 cm) and 25% (range 0.5–40%), respectively. The median time to first recurrence in patients with high-risk, or overtly malignant, GIST was 19 months (range 5–72 months) and 7 months (range 4–19 months), respectively. Recurrent tumours were thus larger, showed higher proliferation (Ki67 max%), and there was a higher proportion of *KIT* exon 11 deletions (53%) than non-recurrent tumours (22%) ($P < 0.001$).

Survival and Prognostic Factors

Estimation of overall survival for the different risk groups was performed using Poisson regression, which enables comparison with an age- and sex-matched normal population (paper I). Median overall survival for the patients with high-risk GIST and overtly malignant GIST was 40 months and 16 months, respectively, as compared to 214 months for the age- and sex-matched normal population ($P < 0.001$).

Table 4. *Clinical and histopathological data in 38 patients with low-, intermediate-, high-risk, and overtly malignant GIST with recurrent tumour disease after complete (R0) resection.*

Size (cm)	*Ki67 max%*	*RG*	*Kinase receptor mutation*	*Site of primary recurr.*	*TTFR (mos)*	*No. of add. surg.*	*Disease status*	*FU (mos)*
4	0.5	Low	M552_Y553del (ex 11)	Liv	55	0	TRD	57
1.5	1	Low	W557C K558_V559del (ex 11)	Loc	24	1	NED	153
4	10	Im	WT	Diss	26	0	TRD	28
11	40	High	WT	Diss	5	0	TRD	6
7.5	40	High	L576P (ex 11)	Diss	5	1	TRD	6
8	10	High	WT	Liv	6	0	TRD	8
10	40	High	WT	Diss	7	0	TRD	10
9	25	High	W557_K558del (ex 11)	Liv	8	0	TRD	12
10	30	High	K550_K558del (ex 11)	Diss	13	0	TRD	14
30	25	High	V559D (ex 11)	Liv	8	0	TRD	14
6	25	High	WT	Liv	12	0	TRD	14
4	30	High	V561D (ex 12)	Liv	14	0	TRD	16
3.5	25	High	WT	Diss	19	0	TRD	19
13	5	High	M552_V555del Q556K (ex 11)	Liv	19	0	TRD	20
17	1	High	WT	Liv	12	0	TRD	23
16	0.5	High	D572_D579dupl (ex 11)	Loc	9	1	TRD	24
20	25	High	Y553_E554del (ex 11)	Diss	5	2	TRD	27
19	1	High	Q556H W557_V560del (ex 11)	Diss	13	0	TRD	28
12	10	High	WT	Diss	18	0	TRD	28
11	10	High	K558_V560del (ex 11)	Diss	13	2	TRD	39
19	25	High	W557_K558del (ex 11)	Diss	12	4	TRD	42
11	1	High	K550I P551_E554del (ex 11)	Loc	31	0	TRD	42
26	10	High	WT	Diss	28	1	TRD	42
16	1	High	D579del (ex 11)	Diss	17	0	TRD	45
20	5	High	V555_V559del (ex 11)	Liv	15	1	TRD	46
33	5	High	W557_Q575del (ex 11)	Liv	6	2	TRD	60
15	10	High	P551_E554del (ex 11)	Liv	28	0	TRD	73
15	0.5	High	V560_N566del (ex 11)	Diss	72	0	TRD	76
4.5	15	High	WT	Diss	72	1	TRD	105
5	25	High	WT	Diss	10	0	AWD	21
12	10	High	E554_W557del (ex 11)	Liv	65	0	AWD	68
6	10	High	K558_V559del V560I (ex 11)	Loc	16	5	AWD	100
4	25	High	WT	Loc	12	1	NED	48
20	10	OM	D579_F591dupl (ex 11)	Diss	4	1	TRD	6
18	20	OM	E554_V555del (ex 11)	Diss	7	0	TRD	8
30	10	OM	W557_E561del (ex 11)	Diss	5	1	TRD	8
17	10	OM	W557C K558_V559del (ex 11)	Diss	14	3	TRD	24
30	10	OM	WT	Diss	19	2	TRD	38

add, additional; AWD, alive with disease; Diss, disseminated disease (liver / peritoneal / omental / mesenteric / pulmonary, and/or bone metastases); FU, follow-up; Im, intermediate; Liv, liver metastases only; Loc, local recurrence; mos, months; NED, no evidence of disease; OM, overtly malignant; recurr, recurrence; RG, risk group; surg, surgery; TRD, tumour-related death; TTFR, time to first recurrence; WT, wild-type.

Univariate Analysis. In paper II, **large tumour size**, **high mitotic rate**, the presence of ***KIT* exon 11 deletion**, **severe pleomorphism**, **high Ki67 max%**, **high age**, **short observation time after surgery**, and **non-R0 resection** ($P < 0.001$ in all variables) were all associated with recurrent tumour disease or tumour-related death (**Table 5**).

Multivariate Analysis. In paper II, only **large tumour size**, **high Ki67 max%**, and the presence of ***KIT* exon 11 deletion** were independent predictors of recurrence. Independent predictors of tumour-related death were **large tumour size**, **high Ki67 max%**, **high age**, **short observation time after surgery**, and **non-R0 resection**. It should be noted that sex or tumour site (stomach *vs*. small intestine or colo-rectum) did not correlate with overall survival, although patients with gastric GIST had a 10%

lower risk (lower hazard function) of dying than patients with small intestinal tumours (**Table 5**).

Table 5. *Prognostic factors for* ***recurrent tumour disease*** *in R0-resected GIST or* ***tumour-related death*** *in the entire series.*

	Recurrent tumour disease (R0, n = 221)						***Tumour-related death (n = 259)***					
	Univariate analysis			***Multivariate analysis***			***Univariate analysis***			***Multivariate analysis***		
Variable	***β***	***P***	***HR***	***β***	***P***	***HR***	***β***	***P***	***HR***	***β***	***P***	***HR***
MR/50 hpf	1.16	< 0.001	3.18		0.390		0.48	< 0.001	1.62		0.796	
Size (cm)	0.15	< 0.001	1.16	0.12	< 0.001	1.13	0.07	< 0.001	1.07	0.04	0.003	1.04
Ki67max%	0.12	< 0.001	1.13	0.12	< 0.001	1.12	0.07	< 0.001	1.07	0.05	< 0.001	1.05
***KIT* ex 11 deletion**	1.42	< 0.001	4.15	0.98	0.003	2.66						
R0 resection							-1.93	< 0.001	0.14	-0.85	0.008	0.43

A hazard ratio (HR) of 2.66 for patients with *KIT* exon 11 deletion = 2.66 times greater risk for recurrence compared to patients without this mutation. A hazard ratio of 0.43 for patients with R0 resection = 57 (1-0.43 x 100) % lower risk for tumour-related death compared to patients with R1/R2 resection. MR, mitotic rate.

Previous studies have indicated several prognostic factors. In the NIH/NCI-workshop, tumour size and mitotic rate were considered to be the strongest predictors (Fletcher *et al.*, 2002). In our study, we could confirm the importance of tumour size, but not mitotic rate. On the other hand, Ki67 max% (based on identification of "hot spots") had stronger predictive value than mitotic rate. DeMatteo *et al.* (2000) had earlier found advanced stage and large tumour size to be associated with poor survival. In contrast to our study, others have demonstrated significantly better survival for patients with gastric GIST (Emory *et al.*, 1999; Miettinen & Lasota, 2001).

The prognostic significance of *KIT* exon 11 mutations has been unclear. Some reports (Ernst *et al.*, 1998; Taniguchi *et al.*, 1999; Kim *et al.*, 2004) have shown that GISTs with *KIT* exon 11 mutations were associated with more aggressive behaviour than wild-type (WT) tumours, while other investigators (Sakurai *et al.*, 1999; Andersson *et al.*, 2002; Wardelmann *et al.*, 2002) could not find such correlation. Interestingly, our study showed that patients with tumours with *KIT* exon 11 deletions had shorter recurrence-free survival than patients with other *KIT* mutations or WT tumours. Singer *et al.* (2002) had earlier demonstrated that a group of patients with *KIT* exon 11 deletions (single or combined with insertions) had a worse prognosis than patients with *KIT* exon 11 substitutions.

There have been several studies reporting significantly improved survival rates after R0 resection *vs.* R1/R2 resection (Ng *et al.*, 1992a; DeMatteo *et al.*, 2000; Crosby *et al.*, 2001). R2 resection was demonstrated to be a strong independent risk factor for tumour-related death in our study (paper II). The dismal prognosis for patients with high-risk, or overtly malignant, GIST in this study (paper II) corroborates the findings of previous large series (Ng *et al.*, 1992b; Mudan *et al.*, 2000; DeMatteo *et al.*, 2000).

GIST Risk Score. The estimated influence of Ki67 max% on survival for fixed tumour size and the estimated influence of tumour size on survival for fixed Ki67 max% is shown in **Fig. 6 A and B**. The Poisson regression, based on analysis of tumour size, Ki67 max%, and overall survival, resulted in a risk score of 0.0486 ≤ (tumour size in cm) + 0.0491 ≤ (Ki67 max%), which was a linear combination defined by the variables size and Ki67 max%, and the beta-coefficients (0.0486 and 0.0491). Since both coefficients were almost identical (approximately 5%), a risk score could be calculated (i.e. the probability of dying within 5 years) depending on the percentile point of the risk score (size + Ki67 max%) and age at diagnosis (**Fig. 6 C**).

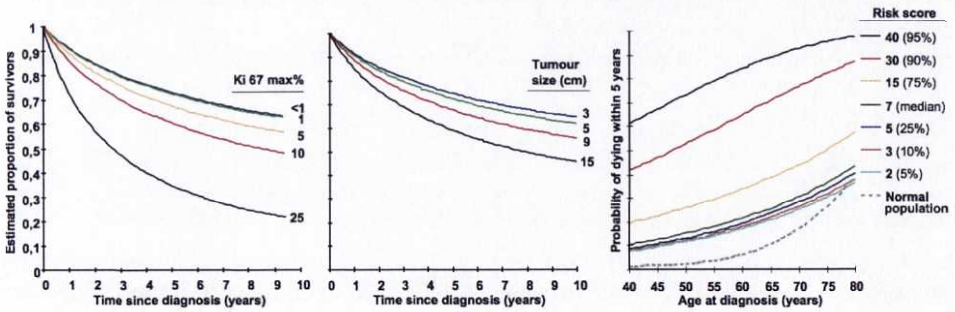

Fig. 6. *Estimated proportion of survivors according to Ki67 max% (**A**) and tumour size (**B**) and estimated probability of dying within 5 years according to various risk scores (tumour size in cm + Ki67 max%) and percentiles (**C**) in patients with clinically detected GIST (n = 251). Each % point increase in Ki67 max%, when tumour size fixed at 5 cm, was associated with a 5% increased risk of dying (**A**). Each 1-cm increase in tumour size, when Ki67 max% was fixed at 1%, was associated with a 5% increased risk of dying (**B**).*

Up to the median percentile (risk score = 7), the estimated survival was no different from that of the normal population. In contrast, scores > 7 were associated with an increased risk of dying from the tumour disease within 5 years. The risk rapidly increased with increasing scores (**Fig. 6 C**). The risk score effectively stratified GIST patients into one group with excellent prognosis (≤ 7) and one group with poor prognosis (> 7). **From a practical standpoint, any GIST larger than 6 cm, regardless of Ki67 max%, and any GIST with Ki67 max% ≥ 5%, regardless of size, should be considered malignant.**

Recurrence-Free Survival. The Kaplan-Meier estimates for recurrence-free survival in GIST patients stratified according to risk group are shown in **Fig. 7**. The risk of recurrence was very high (median time to recurrence less than 20 months) for the high-risk or overtly malignant (median time less than 10 months) groups and extremely low for the very low-, low-, and intermediate-risk groups. The vast majority of primary recurrences in patients with high-risk GIST were diagnosed within three years of primary surgery, but 3 of 14 patients at risk developed recurrence 3–6 years after the first intervention. No recurrences were observed after 6 years (**Fig. 7, Table 4**) (see *Future Perspectives* section).

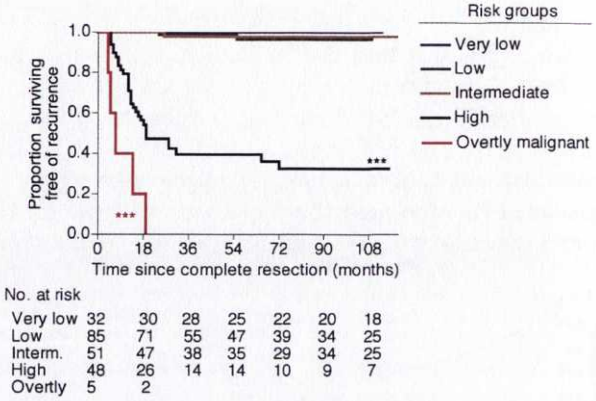

Fig. 7. *Recurrence-free survival in GIST after complete (R0) resection stratified according to risk group (n = 221). P < 0.001, high-risk and overtly malignant vs. very low-, low- and intermediate-risk GIST (log rank test).*

Indication for Adjuvant Treatment with Imatinib

From our results, it follows that adjuvant imatinib is not indicated in patients with very low-risk and low-risk GIST–and most probably not in patients with intermediate-risk tumours. Using our proposed GIST risk score, this corresponds to scores of ≤ 7. In line with our results, disease-free patients with high-risk GIST with a follow-up of less than 6 years after surgery are at risk, high during the first 36 months (**Fig. 7**). All 5 patients with overtly malignant GIST and R0 resection died rapidly from the disease (**Fig. 7, Table 4**). The results further support the inclusion criteria of ongoing prospective studies that preferably patients with high-risk GIST are candidates for adjuvant imatinib after R0 resection. In our large series, we identified 60 patients with high-risk GIST (16 out of 20 alive had no evidence of disease at the latest follow-up) and 29 patients with overtly malignant GIST (persistent disease in the 4 patients still alive) (**Fig. 8**). Currently, all 8 patients (4 high-risk and 4 overtly malignant) with non-resectable disease are on palliative treatment with imatinib. The 16 disease-free patients will be investigated with PET-CT. If resectable recurrence is found, repeat surgery will be performed. If R0 resection is achieved, a period of adjuvant imatinib will be offered to these patients (**Fig. 8, Table 6**).

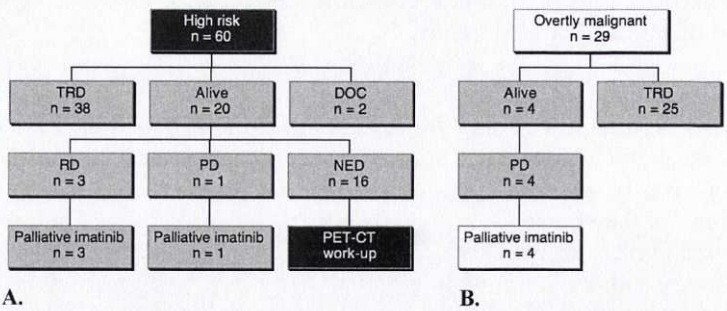

Fig. 8. *Management algorithm for identified survivors with high-risk (**A**) or overtly malignant GIST (**B**) from the entire series. TRD, tumour-related death; DOC, death of other causes; RD, recurrent disease; PD, persistent disease; NED, no evidence of disease.*

Table 6. *Clinical data and tumour characteristics in 16 R0-resected patients with high-risk GIST, alive and disease-free at the latest follow-up.*

Age (years)	Size (cm)	Ki67 max%	Kinase receptor mutation	FU (mos)
51	12	5	WT	21*
75	6	10	P577_F591dupl (ex 11)	21*
70	15	0.5	WT	22*
27	3.5	10	WT	24*
69	5	5	K550_E554del, V555I (ex 11)	28*
81	6	5	V559A (ex 11)	29*
73	4.0	25	WT	48*
63	14	1	I571_D579dupl (ex 11)	60*
46	18	1	M552V (ex 11)	62*
81	6	10	WT	87
25	25	5	WT	91
35	6	5	K558T, V559D (ex 11)	116
69	11	5	Q556H, W557_L576del (ex 11)	126
71	4.5	20	W557_K558del, V559F (ex 11)	137
64	12	1	P551del, M552L (ex 11)	144
72	12	5	V559A (ex 11)	151
Median: 69	8.5	5		61

del, deletion; dupl, duplication; FU, follow-up; WT, wild-type.
*observation period shorter than 6 years.

New Therapeutic Options Combining Surgical and Medical Treatment (paper III)

Neoadjuvant Imatinib

This early centre-based study which included 17 patients with high-risk, or overtly malignant, GIST was designed to study the efficacy of imatinib in different clinical settings. One patient with a very large non-resectable tumour was the first to receive neoadjuvant treatment with imatinib (400 mg once-daily over 3 months), which led to rapid tumour regression and facilitated standard surgical resection (**Fig. 9**). A pre-treatment core-needle biopsy revealed a mixed spindled-epithelioid GIST, immunoreactive for KIT, and with a Ki67 index of 10%. The tumour had a *KIT* exon 11 substitution (W557R).

In the surgical specimen, extensive tumour necrosis was evident with few viable tumour cells; Ki67 index was zero. Fifty-five months after surgery, the patient is still on imatinib due to liver metastases, and is fully active. The liver metastases have been calcified. Repeat ^{18}F-FDG-PET examinations show no radiotracer uptake. The neoadjuvant setting is very attractive, especially from the patient's point of view, since responding tumours may be removed with minimal sacrifice of normal tissues. To date, we have treated 7 patients in this manner; all primary tumours were removed with minimal sacrifice of adjacent organs and in one patient with recurrent tumour, abdominoperineal rectum amputation was avoided. One elderly patient with oesophageal GIST has shown marked tumour regression and will probably avoid a thoraco-abdominal procedure. The clinical value of down-staging therapy, rendering primaries and metastases resectable, has later been shown by others (Scaife *et al.*, 2003; Hohenberger *et al.*, 2003).

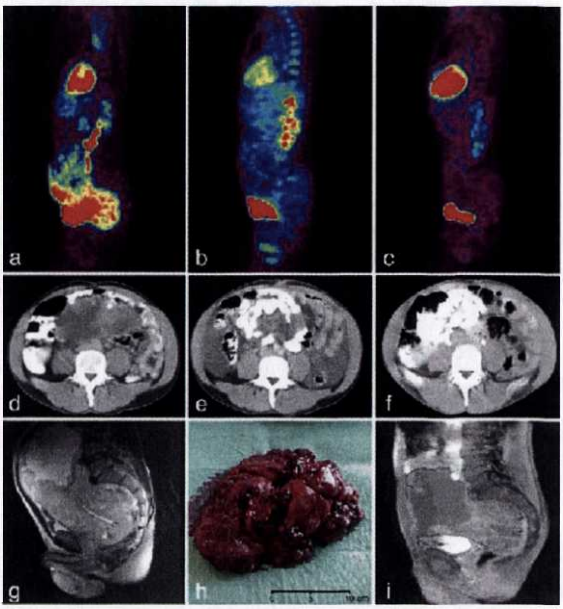

Fig. 9. *^{18}F-FDG PET examinations before (**a**), after 3 weeks (**b**), and after 3 months (c) of imatinib treatment. The high uptake of ^{18}F-FDG seen in the tumour prior to imatinib treatment could not be detected after 3 weeks and 3 months of treatment. CT scans before (**d**), after 3 weeks (**e**), and after 3 months (f) of imatinib treatment. Over 3 months of imatinib treament, the tumour decreased in size from 35 to 18 cm. After 3 months of treatment not detectable at the L2 level (f). Corresponding T_2- and T_1-weighted MRI before (**g**) and after 3 months (i) of treatment, showing tumour reduction. After 3 months of treatment, a GIST of small intestinal origin was completely resected, leaving adjacent structures intact (**h**).*

Adjuvant Imatinib

Five patients received adjuvant treatment with imatinib after R0/R1 resection. Four of 5 patients had *KIT* exon 11 mutations (deletions only: n = 3; substitution only: n = 1), and one patient was WT for exon 11. No patient given adjuvant imatinib developed recurrent tumour disease over 7–13 months of follow-up. There were no serious adverse effects from the drug; one patient developed transient minor oedema. In our consecutive series, we have now treated 21 patients with high-risk GIST with imatinib (400 mg once-daily over 12 months) after R0/R1 resection. The mean observation time is 35 months (SD 15 months). The recurrence-free survival for patients treated with adjuvant imatinib seems much improved relative to historic controls from the pre-imatinib era (papers I & II) (**Fig. 10**).

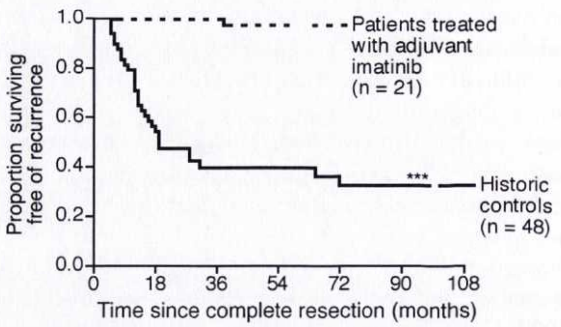

Fig. 10. *Recurrence-free survival in patients with high-risk GIST treated with adjuvant imatinib after R0/R1 resection (mean follow-up: 35 months, SD 15 months) relative to historic controls treated solely with surgery.*
**** $P < 0.0002$ (log rank test).*

Only one GIST treated with adjuvant imatinib has recurred (*case 4* in paper III) (**Fig. 10**); this patient underwent resections due to local recurrence/peritoneal metastases on several occasions. After the fifth tumour resection (right-sided hemihepatectomy), she

was treated with adjuvant imatinib for 12 months. Initial mutation analysis revealed a tumour lacking a *KIT* exon 11 mutation. Thirty-eight months after liver surgery, a solitary pulmonary metastasis was diagnosed and treated. She will not be further treated with imatinib since the tumour was verified WT for both *KIT* and *PDGFRA*. The role of adjuvant imatinib following **repeat** surgery for recurrent GIST has been reported by Lai *et al.* (2005). Of two patients, one remained tumour-free for 12 months, while the other recurred 3 months after discontinuation of imatinib. No mutation analysis was performed and ^{18}F-FDG-PET was not used during follow-up.

Palliative Imatinib

Eleven patients, all with metastatic non-resectable disease, received palliative treatment with imatinib. Seven of the 11 patients had *KIT* exon 11 mutations; deletions only (n = 4), or deletions combined with substitutions (n = 3). Of the 11 patients receiving imatinib in a palliative setting, 8 patients had partial tumour responses during a follow-up of 6–18 months. Two patients in this group died of myocardial infarction. Shortly before death, both showed progressive tumour disease on CT. There were no serious adverse effects by the drug in this group; three patients had minor oedema.

The first patient treated with imatinib was a Finnish woman (*Patient Zero*) with widely metastatic GIST that was resistant to other therapies. The tumour had a *KIT* exon 11 mutation. Within a few weeks, the ^{18}FDG-PET uptake was extinguished, and serial tumour biopsies during treatment showed myxoid degeneration and fibrosis (Joensuu *et al.*, 2001). This remarkable therapeutic success led to rapid phase I (van Oosterom *et al.*, 2001), II (Demetri *et al.*, 2002), and III (Benjamin *et al.*, 2003; Verweij *et al.*, 2004) trials, which all confirmed the efficacy of imatinib treatment; partial responses were seen in 40–70% of the patients and stable disease in 15–30%. The randomised studies have not shown any difference in response induction between 400 mg imatinib given once-daily as opposed to twice a day; however, the EORTC phase III trial demonstrated a significantly longer progression-free survival at the higher dose (**Table 7**).

Table 7. *Interim results in trials of imatinib as palliative treatment for metastatic GIST.*

					Response (%)				
Trial	***Phase***	***No. pts.***	***Dose (mg/day)***	***FU***	***CR***	***PR***	***SD***	***PD***	***PFS***
EORTC	I	36	400–1000	> 10 mos	-	51	31	8	82
US	II*	73	400	52 mos		68	14	15	84
multicentre		74	600		2	65	18	8	
EORTC	III*	470	400	25 mos	5	45	32	13	88
		472	800		6	48	32	9	108
US	III*	361	400	14 mos	-	43	32	-	80
intergroup		360	800			41	32		82

CR, complete response; EORTC, European Organisation for the Research and Treatment of Cancer; PD, progressive disease; PFS, progression-free survival (in weeks); PR, partial response; SD, stable disease. *randomised clinical trials. Modified from van der Zwan & DeMatteo (2005).

The median time to response, as judged by CT, was about three months. Metabolic responses, as judged by ^{18}F-FDG-PET, were demonstrated within hours to days after

initiation of treatment (Joensuu & Dimitrijevic, 2001). Since the introduction of imatinib as treatment for metastatic GIST, the early clinical outcome has been dramatically improved; the 2-year overall survival for patients with metastatic disease is now approximately 70% (Verweij *et al.*, 2004).

Several studies have shown that the response to imatinib depends on the mutational status of *KIT*. Recent 4-year follow-up analysis of the US multicentre randomised phase II trial demonstrated that patients with metastatic GIST with *KIT* exon 11 mutations had a partial tumour response in 87% as opposed to 48% in tumours harbouring *KIT* exon 9 mutations and 0% in WT tumours, respectively. Accordingly, patients with tumours harbouring *KIT* exon 11 mutations had longer median event-free survival (> 100 weeks) than those with exon 9 mutations (< 28 weeks) or WT tumours (< 14 weeks) (Blanke *et al.*, 2006). These results corroborate our early series, in which all 9 responsive patients had a *KIT* mutation (8 exon 11 and one exon 9).

New Imaging Options (paper IV)

GIST can be a component of rare syndromes, i.e. Carney triad and NF1. In 1977, Carney *et al.* reported an association of multiple epithelioid gastric GIST, extra-adrenal pheochromocytoma, and pulmonary chondroma in a small series of mainly young women. NF1 can be associated with several different malignancies, most commonly duodenal NE tumours and pheochromocytomas. GIST has been described as the most common GI manifestation in patients with NF1 (Fuller & Williams, 1991). However, the genetic basis for these associations is largely unknown (**Table 8**). Mutation analysis has revealed that all Carney-GISTs (Diment *et al.*, 2005) and most NF1-GISTs (Takazawa *et al*, 2005) do not harbour *KIT*, or *PDGFRA* mutations.

Table 8. *Syndromes including GIST.*

Syndrome	*Locus name*	*Gene locus*	*Inheritance*	*Gene*
Familial GIST	Unknown	Unknown	AD	*KIT* (JM) or *PDGFRA*
GIST, hyperpigmentation, urticaria pigmentosa	Unknown	Unknown	AD	*KIT* (JM)
GIST and dysphagia	Unknown	Unknown	AD	*KIT* (TK-2)
Paraganglioma-GIST	Unknown	Unknown	AD	Unknown
Carney triad	Unknown	Unknown	Sporadic	Unknown
Neurofibromatosis type 1	NF1	17q11.2	AD	*NF1*

AD, autosomal dominant; JM, juxtamembrane domain; TK-2, tyrosine kinase 2 domain.
Modified from Perry *et al.* (2006).

In this study we reported two patients, one patient with complete Carney triad and one patient with NF1, in whom accurate diagnosis of both pheochromocytoma and GIST in the same patient was made by a 2-tracer PET technique using ^{18}F-FDG for GIST and ^{11}C-HED for pheochromocytoma.

Carney Triad

During follow-up of a 54-year-old female patient who presented with gastric GIST 39 years ago, functional imaging using ^{18}F-FDG for recurrent GIST and ^{11}C-HED for

pheochromocytoma demonstrated a liver tumour and two components of a left-sided juxta-adrenal tumour. High FDG uptake was seen in the liver tumour and the upper component of the juxta-adrenal lesion, indicating GIST. The lower juxta-adrenal portion had HED uptake only, thus indicating pheochromocytoma (**Fig. 11**).

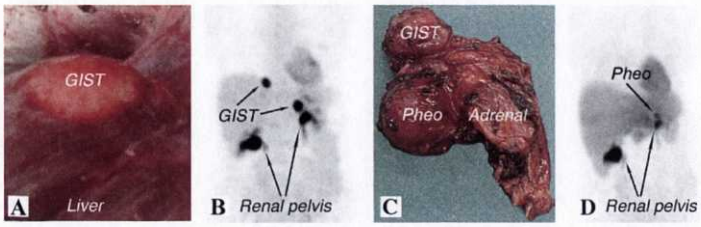

Fig. 11. *A small GIST liver metastasis (**A**). This tumour and a synchronous GIST were visualised by ^{18}F-FDG in the upper portion (**B**) of a time-glass shaped tumour adjacent to the left adrenal (**C**). The lower tumour portion was an extra-adrenal pheochromocytoma, imaged by ^{11}C-HED (**D**).*

Neurofibromatosis Type 1

A 64-year-old male patient with NF1 had severe attacks of hypertension and syncope due to pheochromocytoma. Urinary catecholamines and plasma CgA were markedly elevated. CT showed a left-sided adrenal tumour. At adrenalectomy, a large number of small intestinal tumours were seen, later proven to be GIST with no *KIT* mutation. With recent experience of the Carney patient, a 2-tracer PET was performed postoperatively, which demonstrated multiple FDG uptake in the abdomen and a small HED uptake in the residual right adrenal. After a 5-month period on imatinib, 2-tracer PET was repeated; the previously observed FDG uptakes were extinguished, but the adrenal HED uptake was unchanged. At second-look laparotomy, all residual GIST lesions were excised and a very small pheochromocytoma was enucleated with preservation of the adrenal cortex.

Signalling through the MAP kinase pathway in WT-KIT was first demonstrated by Chian *et al.* (2001). This pathway may be operational in NF1-GIST, since *NF1* mutations result in constitutive *RAS* activation and increased signalling via the MAP kinase. This may be one explanation for the imatinib response seen in our patient with NF1-GIST with no *KIT*/*PDGFRA* mutation.

To the best of our knowledge, this is the first study on a novel 2-tracer PET-technique, which–with high precision–detected GIST **and** pheochromocytoma in patients with NE tumour syndromes. The small liver GIST in the Carney patient and the very small pheochromocytoma in the NF1 patient were not detected by CT/MRI. In a recent study by Trampal *et al.* (2004), it was concluded that ^{11}C-HED-PET is an imaging technique with high sensitivity and specificity in detecting pheochromocytomas. ^{11}C-HED-PET is one of the new functional imaging techniques, which awaits comparison with the most recent 6-[^{18}F]-fluorodopamine-PET to detect small or multiple pheochromocytomas (Ilias *et al.*, 2003).

Neuroendocrine Differentiation in GIST (paper V)

The origin and differentiation of GIST has been under debate for decades. Today, GIST is considered to originate from ICC, or their precursor cells (**Fig. 12**). The KIT protein is expressed both by ICC and GISTs. Ultrastructural studies have demonstrated similarities between ICC and GISTs, e.g. the presence of cytoplasmic vesicles (Erlandson *et al.*, 1996; Kindblom *et al.*, 1998), also found in neurons and NE cells (Komuro, 1999).

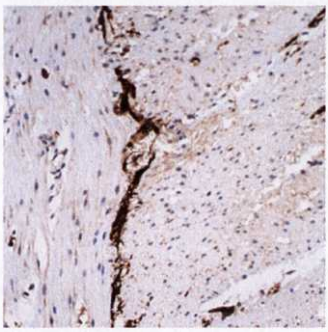

Fig. 12. *Immunohistochemical demonstration of CD117 (*KIT*) in ICC, intercalated between intramural neurons and smooth muscle cells of the intestinal wall.*

NE function is defined by the presence of a regulated secretory pathway, which requires specific storage organelles. Two types of regulated secretory pathways have been characterised. One pathway makes use of large dense-core vesicles (LDCV) containing e.g. chromogranins and vesicular monamine transporters (VMAT 1 & 2). The other pathway uses synaptic-like microvesicles (SLMV), e.g. synapsin 1, synaptophysin, synaptobrevin, and SV2 (Rindi *et al.*, 2004). Expression of CgA and synaptophysin have been hallmarks of NE differentiation, but other synaptic vesicle proteins are also expressed in abundance in several NE tumour types, e.g. SV2, VMAT 1 & 2, and amphiphysin (Jakobsen *et al.*, 2001 & 2002; Zanner *et al.*, 2004).

SLMV Proteins

Our immunohistochemical study demonstrated positive staining for synapsin 1, SV2, amphiphysin, and synaptobrevin in the majority of low-risk profile GISTs and all high-risk profile GISTs (**Table 9**).

Table 9. *Immunohistochemical demonstration of SLMV proteins in GIST (n = 41).*

Marker	***Protein***	***Low-risk profile (VL, L, Im), n = 29***	***High-risk profile (H, OM), n = 12***
SLMV	Synapsin 1	25/26	8/8
	SV 2	27/29	12/12
	Amphiphysin	23/26	8/8
	Synaptobrevin	24/29	12/12

In positive tumours, the staining pattern was neurite- and bouton-like for synapsin 1, cytoplasmic and granular for SV2 and synaptobrevin, and cytoplasmic (perinuclear) for amphiphysin (**Fig. 13**).

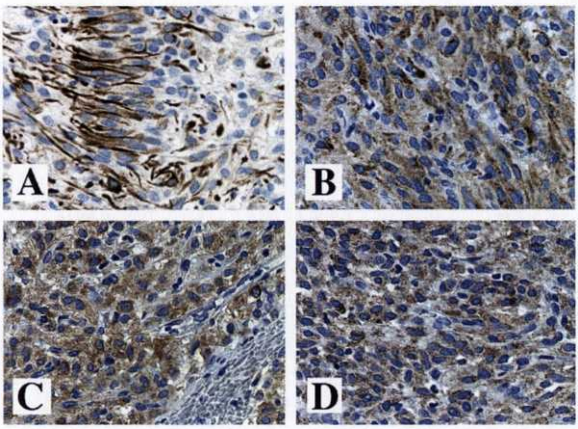

Fig. 13. *Immunohistochemical demonstration of synaptic vesicle proteins in GIST.*
A. ***synapsin 1****: strong staining of tumour cell neurites and boutons.*
B. SV2: *moderate granular staining of cytoplasm.*

C. amphiphysin: *moderate granular staining of cytoplasm (perinuclear).*
D. synaptobrevin: *moderate granular staining of cytoplasm.*

Characterisations by western blot demonstrated SV2, amphiphysin, and synaptobrevin, with major bands of 75–100 kD, 85 kD, and 15 kD, respectively. Q-PCR demonstrated expression of *SV2A*, *synapsin 1*, *amphiphysin*, and *synaptobrevin 1* & *2* in all GISTs (**Fig. 14**).

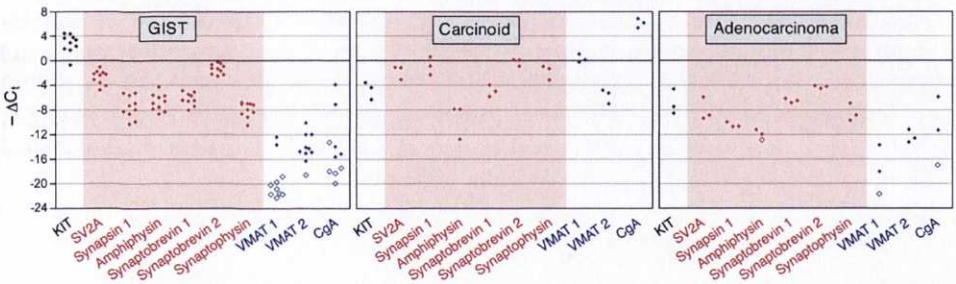

Fig. 14. *Scatter plots of KIT-, and synaptic vesicle protein expression in GIST. Expression of SLMV markers (red) was higher than the expression of LDCV markers (blue). Ileal carcinoid and colorectal adenocarcinoma served as control tumours. Log-transformed gene expression ratios obtained from Q-PCR analysis are plotted. Each dot represents data derived from one sample. Unfilled dots represent expression values below the detection limit.*

The median expression values for the *SV2A* and *synaptobrevin 1* & *2* genes in GIST were similar to those observed in ileal carcinoids. The expression values for the *synapsin 1* gene were two orders of magnitude lower than those observed in carcinoids.

LDCV Proteins

Expression of LDCV proteins was demonstrated in a minority of GISTs. CgA and VMAT 1 & 2 were examined by immunohistochemistry, but only VMAT 1 could be demonstrated: a small number of tumour cells were stained in one-third of the tumours. Characterisation of LDCV proteins by western blot was negative for both VMAT 1 & 2. Although Q-PCR analysis showed expression of *CgA* and *VMAT 1 & 2* in 5, 2, and 9 tumours out of 10 tumours, respectively, the expression values were 5, 4, and 3 orders of magnitude lower than those observed in carcinoids.

Lack of Correlation between SLMV Expression and Clinicopathological Features

There were no significant differences in the staining pattern, molecular size or expression levels of SLMV proteins when low-risk and high-risk profile GISTs were compared. Similarly, there were no significant differences in SLMV protein expression between GISTs with WT or mutated *KIT/PDGFRA*.

NE differentiation has been documented in some non-endocrine tumours, e.g. prostatic carcinoma, breast carcinoma, and GI carcinoma (Hirano *et al.*, 2005; van Krimpen *et al.*, 2004; Grabowski *et al.*, 2002). In contrast to GIST, the non-endocrine tumours show NE differentiation in only a subset of tumours. NE differentiation in prostatic carcinoma has been associated with poor prognosis (Di Sant'Agnese, 2001). However, in our GIST series, we were unable to detect any correlation between risk profile, mutational status of *KIT/PDGFRA*, and NE differentiation.

This study shows that GISTs regularly express SLMV proteins, indicating that these tumours are related to the NE tumour family. High expression of G protein-coupled receptors, e.g. somatostatin receptors, is a common finding in NE tumours and occasionally found in GIST (Montella *et al.*, 2005). Recently, Reubi *et al.* (2004) demonstrated extremely high densities of several brain-gut peptide receptors in GIST, which may open other opportunities to treat patients with radiolabelled peptides/analogues in cases that are resistant to therapy.

CONCLUSIONS

- The incidence of GIST (14.5 per million inhabitants per year) was found to be higher than previously estimated. The consensus risk stratification scheme has proven to be most useful. Prediction of prognosis for patients with GIST was simplified by a risk score based on the independent risk factors tumour size and proliferative index (Ki67 max%).

- Complete (R0) resection and *KIT* exon 11 deletion were found to be independent prognostic factors. Imatinib in adjuvant setting is not indicated for patients with very low-, low-, or intermediate-risk GIST due to the good results obtained by surgery alone.

- Treatment with imatinib in high-risk, or overtly malignant, GIST was found to be safe and effective, particularly in patients with *KIT* exon 11 mutations. Adjuvant treatment with imatinib seems promising, but long-term effects on survival must be evaluated in randomised clinical trials. In selected patients, neoadjuvant treatment can facilitate later surgical treatment.

- A 2-tracer PET, using ^{18}F-FDG and ^{11}C-HED, has the unique capacity to simultaneously detect GIST and pheochromocytoma in patients with NE tumour syndromes, e.g. Carney triad and NF1.

- Both low- and high-risk GISTs regularly express synaptic vesicle proteins of SLMV type. One-third of the tumours also expressed the LDCV marker VMAT 1. These features demonstrate a NE differentiation in a majority of GIST.

FUTURE PERSPECTIVES

Clinical Trials

Today, it is evident that patients with metastatic, disseminated, or unresectable GIST benefit from treatment with imatinib (paper III). Whether selected patients should be treated with imatinib prior to surgery, or with imatinib after complete (R0) resection of the primary tumour, is presently being evaluated in prospective American and European trials. For inclusion in these studies, information about *KIT-/PDGFRA* mutational status or objective response to imatinib, e.g. extinction of FDG uptake on PET, is not required. The results from our retrospective studies (papers I & II) demonstrate that adjuvant imatinib is not indicated for patients with very low-, low-, or intermediate-risk GIST due to the good results obtained by surgery alone. Our consecutive series (paper III) of patients with high-risk GIST treated with imatinib after R0/R1 resection (with mean follow-up of 35 months) indicate that these patients benefit from adjuvant treatment; the only recurrence observed was in a patient WT for both *KIT* and *PDGFRA*.

In the international trials on adjuvant treatment, patients with low-risk GIST were excluded, but in the EORTC and ACOSOG Z9001 studies patients with intermediate-risk tumours were included (**Table 10**).

Table 10. *Currently running trials combining surgery with neoadjuvant or adjuvant imatinib for GIST.*

Group/Trial	*Disease*	*Setting*	*Inclusion*	*Dose*
RTOG S0132	Any	Neoadjuvant	Potentially resectable primary tumour ≥ 5 cm or potentially resectable recurrent tumour ≥ 2 cm	600 mg/day x 8-10 weeks preop. and 600 mg/day x 24 months postop.
ACOSOG Z9000	Primary	Adjuvant	R0 resection and high-risk	400 mg/day x 12 months
ACOSOG Z9001	Primary	Adjuvant	R0 resection and tumour ≥ 3 cm	400 mg/day *vs.* placebo x 12 months
SSG XVIII	Primary with or without metastases	Adjuvant	R0 resection and high-risk	400 mg/day x 12 months *vs.* 36 months
EORTC 62024	Primary	Adjuvant	R0 resection and intermediate-/high-risk	400 mg/day *vs.* no treatment x 24 months

ACOSOG, American College of Surgeons Oncology Group; EORTC, European Organisation for Research and Treatment of Cancer; RTOG, Radiation Therapy Oncology Group; SSG, Scandinavian Sarcoma Group. Modified from van der Zwan & DeMatteo (2005).

New Targeted Therapies

Novel Medical Approaches

Many patients with advanced GIST and long-term treatment with imatinib develop resistance. The median time to tumour progression is about 24 months (Antonescu *et al.*, 2005). Four mechanisms for drug failure have been proposed: (1) acquisition of a secondary mutation in *KIT* or *PDGFRA*; (2) genomic amplification of *KIT* or *PDGFRA* with consequent kinase over-expression; (3) activation of an alternate

tyrosine kinase receptor with loss of KIT or PDGFRA oncoprotein expression and (4) functional resistance *in vivo* of tumours that are sensitive to imatinib *in vitro* (e.g. *KIT* exon 9 and WT *KIT*). Primary resistance is defined as rapid progression within the first 6 months of treatment, usually at multiple sites. These tumours often exhibit WT *KIT*, *KIT* exon 9 mutations, or a D842V mutation of *PDGFRA*. Drug failure beyond 6 months of treatment is defined as secondary resistance; only a few tumours continue to grow, while the majority of tumours are controlled by medication (Blay *et al.*, 2005). In the case of resistance, the therapeutic options may include repeat surgery or other interventions (e.g. radiofrequency or cryo-laser ablation, or hepatic arterial embolisation), tyrosine kinase inhibitors with wider profile than imatinib, or targeting of other signalling pathway intermediaries (van der Zwan & DeMatteo, 2005). Some of the new drugs in the current clinical trials are the multikinase inhibitor AMG706, the mTOR inhibitor RAD001 and the protein kinase C inhibitor PKC412 (**Fig. 15**).

We have treated 4 patients with imatinib resistance (three with a *KIT* exon 11 mutation and one with WT) with the mTOR inhibitor rapamycin, which is usually used for immunosuppression in transplant patients. In the three patients with *KIT* mutations, the time to tumour progression was 6, 13, and 15 months, respectively. The patient with a WT tumour did not show any objective response.

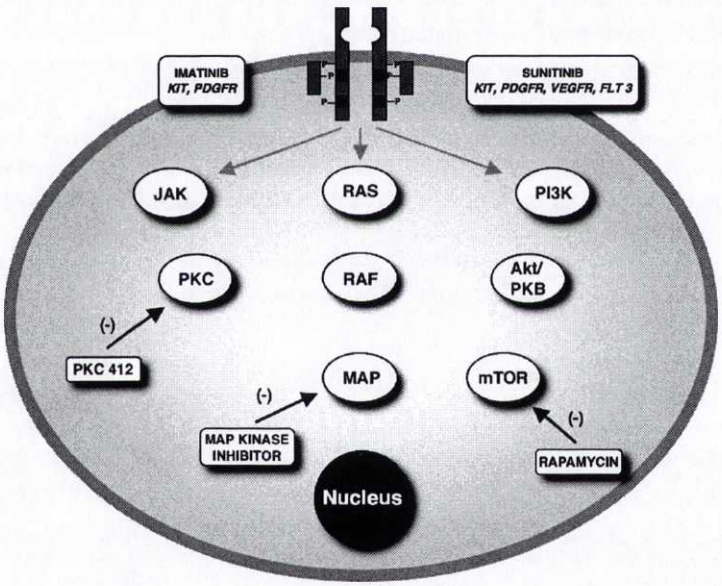

Fig. 15. *The downstream signalling pathways in GIST with drug targets.*

A novel tyrosine kinase inhibitor, currently in phase III clinical trials, is sunitinib malate (Sutent®; Pfizer Inc., New York, NY) (**Fig. 15**). This oral agent, which has activity against KIT, PDGFR, VEGFR, RET and FLT3, has yielded objective responses, or stable disease, in 26 of 48 evaluable patients with imatinib-resistance. It seems as if patients with *KIT* exon 9 mutations respond particularly well (79%)

(Demetri *et al.*, 2004), which can make this drug a valuable complement to imatinib. We are currently evaluating sunitinib as treatment for metastatic, imatinib- and rapamycin-resistant GIST in a clinical trial (Protocol no. A6181036). All 4 patients, first treated with rapamycin upon tumour progression, now receive sunitinib.

Cost and Quality

The new generation of expensive targeted cancer drugs in combination with limited healthcare resources highlights the need for improved methods to estimate the benefits of different treatment strategies. One method of estimating the benefit of a certain therapy is to calculate its expected gain in quality-adjusted life years (QALYs). One QALY is defined as one year of perfect health. Comparisons can be made between different therapies, and priorities based on cost and quality can be made. A key question is whether the expected gain in QALYs outweighs the costs associated with the actual cost of the specific treatment. By using special Poisson regression models, which allow hazard functions to be estimated as continuous functions, we can derive and calculate these probabilities, expected length of life and expected gain in QALYs in detail and with precision from our large series of GIST (papers I &II).

GIST–a Neuroendocrine Tumour?

GISTs express multiple synaptic vesicle proteins (paper V). These findings suggest that GISTs secrete hormones that may be used as markers in the diagnosis and follow-up. Peptide receptors are important in the regulation of hormone secretion and have been demonstrated in both NE tumours and GISTs. We have observed individual patients with GISTs that were distinctly imaged by somatostatin receptor scintigraphy (**Fig. 16**). GISTs also express very high densities of other peptide receptors, e.g. bombesin subtype 2, vasoactive intestinal peptide subtype 2, and cholecystokinin subtype 2 (Reubi *et al.*, 2004).

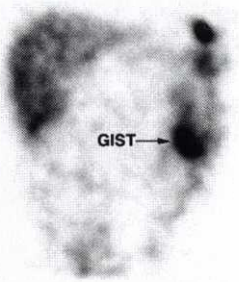

Fig. 16. *Somatostatin receptor scintigraphy visualising a GIST of small intestinal origin.*

High expression of somatostatin receptors in NE tumours has led to successful receptor-mediated radiotherapy using somatostatin analogues (Kwekkeboom *et al.*, 2003; Reubi, 2003). With access to suitable peptide analogues, receptor-mediated radiotherapy targeted to bombesin-, vasoactive intestinal peptide-, and cholecystokinin receptors may become a future alternative in patients with therapy-resistant GISTs.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Gastrointestinal stromacellstumör (GIST) hör till gruppen mjukdelstumörer, dit även tumörer från ben, brosk, fett, muskulatur och blodkärl räknas. GIST kan utgå från hela magtarmkanalen men ses oftast i magsäck och tunntarm. Forskarna tror idag att GIST härstammar från förstadier till Cajal's celler, som ligger insprängda mellan muskel- och nervceller i tarmväggen och ansvarar för magtarmkanalens motorik. Eftersom GIST inte går att behandla med cellgifter har kirurgi varit enda möjligheten att bota drabbade. Många patienter får dock återfall och dör av sin sjukdom trots upprepade operationer. GIST har varit en nyckfull tumör med svårbedömd prognos. I ett försök att bättre kunna beskriva prognosen för en enskild tumör har ett system att skatta risken föreslagits, som baserar sig på tumörstorlek och celldelningsförmåga (analyserat som mitosfrekvens = antal tumörceller i delningsfas). Beroende på dessa egenskaper har tumörerna delats in i följande grupper: mycket låg, låg, intermediär och hög risk-tumörer.

GIST har en receptor (mottagare för stimulering) kallad KIT på cellytan, som genom signalering till cellkärnan reglerar viktiga funktioner som celltillväxt och celldöd. Den gen (*KIT*), som kodar för denna mottagarmolekyl, har i majoriteten av GIST visat sig vara muterad, det vill säga genen förmedlar felaktig information som leder till överaktivering av KIT-receptorn med ökad celltillväxt/minskad celldöd till följd. Läkemedlet imatinib binder sig till KIT-receptorn och hämmar dess signalering, vilket visat sig vara effektivt för behandling av spridd eller icke-operabel GIST. Speciellt väl fungerar medicinen hos patienter med mutation i en specifik del (exon 11) av *KIT*-genen. Nyttan av tilläggsbehandling med imatinib efter genomförd radikal operation av "risk-tumörer" samt imatinib-behandling för att krympa en stor tumör inför operation analyseras för närvarande i flera studier. Tumörutbredning påvisas vanligtvis med datortomografi eller magnetröntgen, men det har visat sig att positron-emissions-tomografi (PET) med en radioaktivt märkt sockermolekyl, ^{18}fluorodeoxyglukos (FDG), är en känslig funktionell metod för att tidigt utvärdera effekten av behandling.

GIST-studier har hittills utgått från stora cancercentra vilket innebär att resultaten i stor utsträckning baserar sig på patientmaterial med större och mer aggressiva tumörer än vad man kan förvänta sig i en normalbefolkning. Syftet med denna avhandling har varit att (1) analysera hur vanligt GIST är i Västra Götalandsregionen samt att utvärdera den föreslagna riskskattningen; (2) utvärdera betydelsen av radikal operation och andra prognosfaktorer i ett stort populationsbaserat patientmaterial med lång uppföljningstid; (3) undersöka olika typer av behandling med imatinib, hur dessa kan kombineras med kirurgi samt utvärdera behandlingseffekten vid mutation i exon 11 i *KIT*-genen; (4) visa på det diagnostiska värdet av en ny typ av PET-undersökning hos patienter med GIST som del i syndrom innefattande olika tumörtyper; samt (5) utvärdera om GIST kan ha drag gemensamt med nervliknande hormonproducerande tumörer, så kallad neuroendokrin differentiering, eftersom GIST just utgår från Cajal's celler i tarmen.

Våra resultat visar att:
(1) GIST är vanligare än tidigare beräknat; förekomsten av kliniskt upptäckt, GIST i Västra Götalands regionen är 14,5 patienter per en miljon invånare och år. Den gällande riskskattningen, baserad på tumörstorlek och mitosfrekvens, är väl användbar. Vi föreslår dock en modifierad riskskattning, som baseras på tumörstorlek och högsta observerade celldelning (Ki67 max%), som på ett enklare sätt skiljer ut GIST med god prognos.
(2) Radikal operation och bortfallsmutation i *KIT* exon 11 är viktiga prognosfaktorer vid sidan av tumörstorlek och Ki67 max%. På grund av att patienter med mycket låg-, låg- och intermediär risk-GIST uppvisar synnerligen god prognos efter genomförd radikal operation, föreligger ingen indikation för behandling med imatinib för dessa patienter.
(3) Imatinib-behandling av patienter med hög risk-GIST, eller uppenbart elakartad GIST, är säker och effektiv, särskilt om *KIT* exon 11-mutation föreligger. Behandling med imatinib efter kirurgi vid hög risk-GIST verkar lovande, men långtidseffekter måste studeras närmare i större studier. För patienter med mycket stor tumör kan förbehandling med imatinib leda till bättre resultat av en efterföljande operation.
(4) En ny PET-undersökning användande 2 olika radioaktivt märkta spårmolekyler, deoxyglukos och hydroxyefedrin, kan samtidigt upptäcka såväl GIST som den neuroendokrina tumören feokromocytom hos patienter med neuroendokrina tumörsyndrom, det vill säga patienter som kan utveckla flera olika tumörtyper.
(5) GIST uttrycker vissa synaptiska vesikelprotein som normalt förekommer i nerver och neuroendokrina tumörer. Detta fynd visar att GIST till viss grad har neuroendokrin differentiering. Vissa GIST kan vidare uttrycka peptider och peptidreceptorer, vilket kan ge möjlighet till strålbehandling, peptidanaloger (konstgjorda peptider) bundna till radioaktiva läkemedel binds då till dessa receptorer på tumörytan och ger en stråleffekt på tumören.

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