



Pretreatment Tumor DNA Sequencing of *KIT* and *PDGFRA* in Endosonography-Guided Biopsies Optimizes the Preoperative Management of Gastrointestinal Stromal Tumors

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Published online: 2 March 2020
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Abstract

Background Neoadjuvant tyrosine kinase inhibitor (TKI) therapy increases the chance of organ-preserving, radical resection in selected patients with gastrointestinal stromal tumors (GISTs). We aimed to evaluate systematic, immediate DNA sequencing of *KIT* and *PDGFRA* in pretreatment GIST tissue to guide neoadjuvant TKI therapy and optimize preoperative tumor response.

Methods All patients who were candidates for neoadjuvant therapy of a suspected GIST [the study cohort (SC)] were prospectively included from January 2014 to March 2018. Patients were subjected to pretreatment endosonography-guided fine-needle biopsy (EUS-FNB) or transabdominal ultrasound-guided needle biopsy (TUS-NB), followed by immediate tumor DNA sequencing (<2 weeks). A historic (2006–2013) reference cohort (RC) underwent work-up without sequencing before neoadjuvant imatinib ($n=42$). The rate of optimal neoadjuvant therapy (Therapy_{OPTIMAL}) was calculated, and the induced tumor size reduction (Tumor Regression_{MAX}, %) was evaluated by computed tomography (CT) scan.

Results The success rate of pretreatment tumor DNA sequencing in the SC ($n=81$) was 77/81 (95%) [EUS-FNB 71/74 (96%); TUS-NB 6/7 (86%)], with mutations localized in *KIT* ($n=58$), *PDGFRA* ($n=18$), or neither gene, wild type ($n=5$). In patients with a final indication for neoadjuvant therapy, the Therapy_{OPTIMAL} was higher in the SC compared with the RC [61/63 (97%) versus 33/42 (79%), $p=0.006$], leading to a significantly higher Tumor Regression_{MAX} in patients treated with TKI (27% vs. 19%, $p=0.015$).

Conclusions Pretreatment endosonography-guided biopsy sampling followed by immediate tumor DNA sequencing of *KIT* and *PDGFRA* is highly accurate and valuable in guiding neoadjuvant TKI therapy in GIST. This approach minimizes mal-treatment with inappropriate regimens and leads to improved tumor size reduction before surgery.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s40291-020-00451-0>) contains supplementary material, which is available to authorized users.

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Key Points

To date, the pretreatment diagnosis and genetic profiling of tumors has been challenging and imperfect in patients with suspected gastrointestinal stromal tumors (GISTs).

In the current work, we show that endosonography-guided fine-needle biopsy sampling followed by next-generation sequencing of *KIT* and *PDGFRA* is highly accurate for the diagnosis and mutational analysis of GISTs already at an early, pretreatment stage.

The suggested work-up enables neoadjuvant, tyrosine kinase inhibitor therapy firmly based on tumor genomics data. Moreover, we demonstrate that the suggested approach leads to a significantly lower number of patients being maltreated with suboptimal neoadjuvant therapy regimens and results in a significantly greater tumor size reduction before surgery.

1 Introduction

In modern cancer care, it is often as important as it is challenging to initiate an effective treatment with limited toxicity. This statement is certainly valid regarding gastrointestinal stromal tumors (GISTs). The anti-tumoral effect of targeted therapy with tyrosine kinase inhibitors (TKIs) can be dramatic in GISTs [1, 2], but TKIs, such as imatinib and sunitinib, are also associated with substantial side effects [3–5].

Patient survival has improved significantly thanks to imatinib therapy, both in patients suffering from metastatic GISTs [3, 4, 6] and in resected patients (adjuvant therapy) with tumors larger than 3 cm [5]. Neoadjuvant imatinib for preoperative, tumor downsizing induces rapid tumor cell apoptosis [7], facilitates surgical, radical resection [8–10], and leads to a high disease-specific survival [11] without an increased post-operative complication rate [7, 12].

However, the individual tumor response to imatinib therapy is intimately related to the tumor genetic profile [13]. A majority of sporadic GISTs occur due to primary mutations in either the *KIT* or *PDGFRA* genes. Some genetic subgroups, such as the common *KIT* exon 11 mutants and the rare *PDGFRA* exon 12 mutants, respond well to standard dose imatinib (400 mg daily) [14–16] (Table 1). A rare exception is the *KIT* exon 11 p.(L576P) variant, which is far less sensitive [17]. An intermediate response is seen in *KIT* exon 9 mutants requiring high-dose imatinib (800 mg daily) [6, 15, 18]. A few other mutations, such as the *PDGFRA* exon 18 p.(D842V) mutant, are completely resistant to imatinib [16, 19] (Table 1). Sporadic tumors without any detected mutation in *KIT* or *PDGFRA* are called wild type (WT) tumors and are regarded as nonsensitive to imatinib therapy [20]. Instead, such patients are candidates for upfront surgery or clinical trials evaluating alternative therapies [21].

Therefore, the adequacy and effect of neoadjuvant imatinib therapy is dependent on correct information regarding the precise mutation in each individual tumor. Admittedly, *PDGFRA* mutants are most commonly found in the stomach and *KIT* exon 9 mutants in the small intestine, but the tumor origin alone cannot predict the genetic profile [13]. Likewise, the epithelioid cell type is common among *PDGFRA* mutants, but the morphology of the tumor cells per se does not sufficiently reveal the underlying mutation [22].

The pretreatment diagnosis and genetic profiling of suspected GISTs have been limited by imperfect available diagnostic techniques. Performing a transabdominal ultrasound-guided needle biopsy (TUS-NB) requires an accessible tumor of a reasonably large size. Routine endoscopy with forceps biopsy and endoscopic ultrasound with fine-needle aspiration for cytology (EUS-FNA) both have a low

diagnostic accuracy [23, 24]. However, the use of a new generation of EUS needles aimed for histology [endosonography-guided fine-needle biopsy (EUS-FNB)] can ameliorate the shortcomings of other diagnostic approaches and lead to early genetic profiling of GISTs [25].

In summary, the effective, neoadjuvant therapy in GISTs is dependent on accurate diagnostic information including tumor genomic data. The overall goal of this work was to evaluate EUS-FNB and TUS-NB followed by immediate mutational analysis of *KIT* and *PDGFRA* in pretreatment tumor tissue for the early detection of unfavorable mutations with respect to imatinib therapy. An additional aim was to analyze the clinical impact, especially the efficacy of treatment, of such a standardized work-up in patients considered for neoadjuvant therapy.

2 Methods

2.1 Study Setting and Study Subjects

The Sahlgrenska University Hospital (SUH) is a tertiary center for EUS and for the work-up and care of all patients with GISTs in the region of West Sweden (population approximately 1.9 million). All incident GIST cases are managed via a preoperative, multidisciplinary therapy conference (MDTC).

All patients > 18 years referred to the SUH for the preoperative diagnostic work-up of a suspected GIST during the period January 2014–March 2018, hereafter called the study cohort (SC), were eligible for inclusion in this prospective, single-center study. Patients with probable GIST but with a condition requiring urgent surgery, such as ileus or tumor bleeding, were not eligible for study inclusion. Eligible patients were excluded if the diagnostic work-up resulted in a final diagnosis other than GIST.

The patients with GIST advocated for and treated with neoadjuvant therapy in the same center during the period 2006–2013 constituted the reference population—hereafter called the reference cohort (RC).

This study was approved by the Regional Ethical Review Board of Gothenburg. Written informed consent was obtained from all the study participants. The study was registered at ClinicalTrials.gov (NCT02360839).

2.2 Tumor Sampling Procedures

2.2.1 Study Cohort

The work-up strategy of all referrals was determined by the study surgeon (BN). Primarily, the study participants were subjected to high-priority, pretreatment EUS-FNB. In tumors considered out of reach by oral access EUS,

Table 1 Anti-tumoral effect of standard dose imatinib in GISTs in relation to the primary mutation of the tumor

Tumor genomic profile	Anti-tumoral effect of standard dose IMA ^a		
	Adequate	Reduced	Poor
<i>Favorable mutations</i>			
<i>KIT</i> exon 11 [mutants except p.(L576P)] [14, 15]	X		
<i>KIT</i> exon 13 [14]	X		
<i>PDGFRA</i> exon 12 [14, 15]	X		
<i>PDGFRA</i> exon 14 [16]	X		
<i>PDGFRA</i> exon 18[mutants except p.(D842V)] [28]	X		
<i>Unfavorable mutations</i>			
<i>KIT</i> exon 9 [18]		X	
Wild type [15, 48]		X	
<i>KIT</i> exon 11 p.(L576P) [17]			X
<i>KIT</i> exon 17 [28, 29]			X
<i>PDGFRA</i> exon 18 p.(D842V) [16, 19, 28]			X

GIST gastrointestinal stromal tumor, IMA imatinib

^aStandard dose IMA = 400 mg daily

high-priority TUS-NB was performed (Fig. 1). Any EUS-FNB was performed by one of the study endosonographers (RS/PH) using a linear echoendoscope (EG3870UTK, Pentax, Tokyo, Japan) and a 22-gauge biopsy needle (Procore™, Wilson-Cook Medical, Limerick, Ireland or Sharkcore™, Medtronic, Minneapolis, USA). Any TUS-NB were performed at the radiology department using an 18-gauge biopsy needle (Bard Biopsy Systems, Tempe, USA). All samples were sent to the study pathologist (ON).

2.2.2 Reference Cohort

During the RC time frame (2006–2013), EUS constituted one diagnostic option, but the method was not systematically performed before MDTC. If EUS was conducted, sampling was mainly performed by the contemporary standard technique, i.e. EUS-FNA for cytology (22/25-gauge Echotip™, Wilson-Cook Medical). If TUS-NB was conducted, sampling was performed as above.

2.3 Tumor DNA Sequencing

In the SC, the samples of EUS-FNB and TUS-NB were subjected to tumor DNA sequencing immediately after the histopathology assessment (< 1 week). During 2014–2015, and for research purposes, the corresponding surgical specimens of all of the resected cases were also subjected to tumor DNA sequencing to confirm the detected mutations [25]. During 2016–2018, this procedure was performed only in cases with a WT profile identified in the

pretreatment sample (to confirm the absence of a mutation) and in cases with failed pretreatment sequencing.

In the RC, mostly due to the lack of appropriate pretreatment tumor samples, no sequencing of *KIT* and *PDGFRA* was performed within a reasonable time to enable genotype-guided management at the MDTC. Sequencing of the surgical specimens or of available preoperative work-up samples was instead performed at a later stage.

Before sequencing (SC and RC), the approximate number of tumor cells in each sample was estimated. Then, the tumor area was manually microdissected from the formalin-fixed paraffin-embedded (FFPE) tissue. Five micrometer thick sections from each sample were cut and put into a 1.5-ml tube. DNA was isolated using the QIAamp DNA FFPE tissue *KIT* (Qiagen GmbH, Hilden, Germany), following the manufacturer’s instructions. The DNA concentration was determined using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA).

2.3.1 Sanger Sequencing

Between January 2014 and December 2015, Sanger sequencing was the method used in all cases. For the purpose of sequencing, 200 ng of DNA was used to detect mutations in *KIT* exons 9, 11, and 13 and *PDGFRA* exons 12 and 18 with primers designed in-house (details available on request) and the Multiplex PCR *KIT* (Qiagen) according to the manufacturer’s instructions. Sanger sequencing of the amplicons was performed with both the forward and reverse primers using the BigDye™ Terminator v1.1 Cycle Sequencing *KIT*

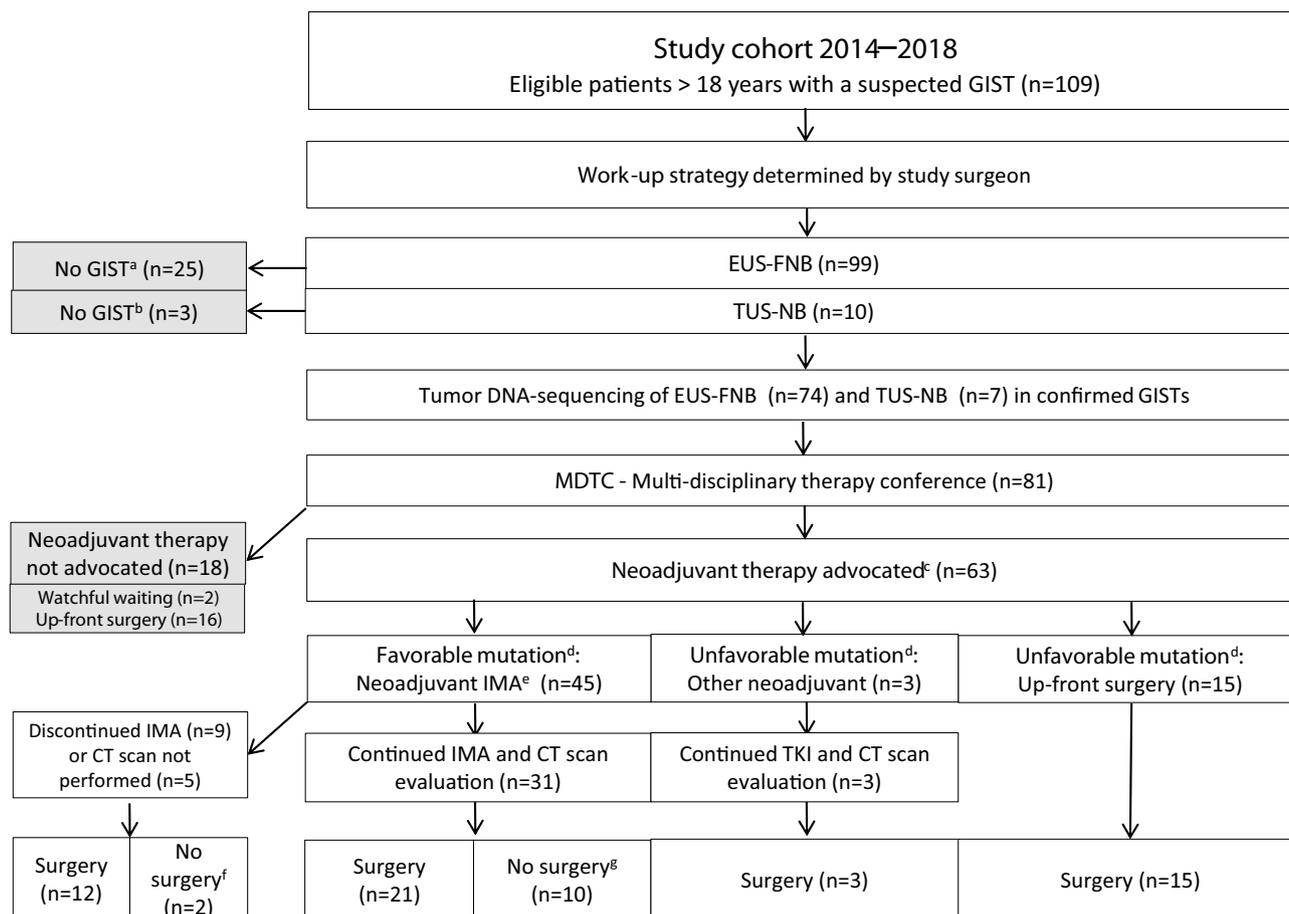


Fig. 1 Flow chart of patients with a suspected GIST eligible for study enrollment January 2014–March 2018 in Sahlgrenska University Hospital. ^aDiagnostic entities in non-GIST lesions subjected to EUS-FNB: leiomyoma ($n=10$); schwannoma ($n=5$); adenocarcinoma ($n=3$); leiomyosarcoma ($n=2$); epithelioid sarcoma ($n=1$); benign fibrinoid tumor ($n=1$); ganglioneurinoma ($n=1$); lipoma ($n=1$); malignant peripheral nerve sheath tumor ($n=1$). ^bDiagnostic entities in non-GIST lesions subjected to TUS-NB: desmoid tumor ($n=1$); adenocarcinoma ($n=1$); leiomyosarcoma ($n=1$). ^cCriteria for the initiation of down-sizing therapy according to the main body

of the manuscript. ^dAccording to Table 1. ^eNeoadjuvant therapy with imatinib mesylate in standard dose (400 mg daily). ^fThe two patients were considered unfit for the extensive surgery needed. Instead, the patients were provided the best palliative treatment. ^gThe reason for surgery not being performed at the end of the study period among the ten patients was: (a) patients awaiting surgery, $n=1$; (b) patients with concomitant malignancy being unfit for surgery, $n=1$; (c) patients with a remaining large tumor burden not yet considered eligible for radical resection, $n=4$; (d) patients with advanced comorbidity being unfit for surgery, $n=3$

with the ABI PRISM™ 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3.2 Next-Generation Sequencing

From January 2016 onwards, next-generation sequencing (NGS) was the method used in all cases. For the purpose of sequencing, 10 ng of DNA was used to prepare barcoded libraries with the Ion AmpliSeq™ Library *KIT* 2.0 (Life Technologies). The Cancer Hotspot Panel v2 (Life Technologies) covering 207 targeted regions in 50 cancer-related genes (Supplementary Table 1 in the Electronic Supplementary Material) was used to identify mutations in *KIT* (exons 9–11, 13–15, and 17–18), *PDGFRA* (exons 12, 14, 15, and

18), and *BRAF* (exon 15). Template preparation and enrichment were performed with the IonChef (Life Technologies). Eight barcoded samples were pooled per Ion 318™ chip and sequenced on the Ion PGM™ System (Life Technologies). All steps were performed according to the manufacturer's instructions.

Alignment to the hg19 human reference genome and variant calling were performed by the Torrent Suite Software v5.2.2 (Life Technologies). To identify hotspot variants, the Variant Caller v5.2.0.34 (Life Technologies) was used. Alignment was visually inspected with the Integrative Genomics Viewer (IGV) modified by IonTorrent v5.01 (0) (Broad Institute, Cambridge, MA, USA).

2.4 The MDTC and Neoadjuvant Therapy

The results of pathology and pretreatment sequencing with mutational analysis were presented to the Sahlgrenska MDTC for decisions on further preoperative management. The SUH multidisciplinary GIST team advocates neoadjuvant TKI therapy in all patients with GISTs (without advanced comorbidity) if the tumor diameter > 3 cm and/or if the tumor is borderline resectable, e.g., situated in the gastroesophageal junction or in the duodenum. The SUH experience (nonpublished data) is that the use of neoadjuvant therapy in relatively small tumors (3–5 cm) facilitates surgery and minimizes the perioperative complication rate. The cases not advocated for neoadjuvant therapy were managed by upfront resection or watchful waiting (Fig. 1), and these cases were not further analyzed within the study.

In the SC patients, standard dose imatinib (400 mg daily) was primarily initiated by the MDTC, but only if the pretreatment mutation detected was favorable and indicated full sensitivity to imatinib (Table 1). If the mutation detected was unfavorable and indicated imatinib resistance or reduced sensitivity to imatinib, an alternative downsizing strategy was chosen or upfront surgery was performed (Table 1 and Fig. 1). Inexorably, neoadjuvant therapy will result in a low mitotic index in surgical specimens of GISTs with favorable mutations. Therefore, adjuvant therapy after surgery was more liberally advocated by the MDTC in patients having tumors with an indeterminate risk.

In the RC patients, standard dose imatinib (400 mg daily) was initiated in all cases advocated for neoadjuvant therapy, as no data on the tumor mutation profiles were or could be provided to the MDTC before decisions were made on further management and therapy.

2.5 Follow-Up and Surgery

After the MDTC, the study subjects were monitored by regular visits to the outpatient unit. Neoadjuvant therapy was continued for a minimum of 3 months and a maximum of 15 months. Therapy was stopped and surgery was attempted either when the estimated maximal tumor size reduction was obtained or when the tumor size reduction was high enough to enable organ-saving radical resection.

Any side effects of neoadjuvant therapy were evaluated and recorded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) system [26] ranging from mild to life threatening (grade 1: mild; grade 2: moderate; grade 3: severe; grade 4: life-threatening or disabling; grade 5: death related to side effects). Mild side effects were usually treated by symptomatic therapy. In patients experiencing continuous, moderate-severe side effects (and with

the intent to avoid complete therapy discontinuation), the dosage of imatinib was reduced to 100–200 mg daily. If imatinib dose reduction did not lead to reduced side effects, neoadjuvant therapy was discontinued.

As in other studies analyzing the value of neoadjuvant imatinib therapy [11, 12], repeated computed tomography (CT) scans and the application of the RECIST criteria [27] were used for tumor response evaluation. The tumor size reduction, Tumor Regression_{MAX} (%), was calculated by comparing the maximum cross-sectional tumor size (mm) of the pretreatment CT scan (Pre-CT_{max}) with the follow-up CT scan (Post-CT_{max}) performed 12–16 weeks after the initiation of neoadjuvant TKI therapy:

$$\text{Tumor Regression}_{\text{MAX}}(\%) = 100 \times (\text{Pre-CT}_{\text{max}} - \text{Post-CT}_{\text{max}}) / \text{Pre-CT}_{\text{max}}$$

Patients in any cohort were excluded from the Tumor Regression_{MAX} analysis if they discontinued therapy before 12 weeks of treatment or an evaluation CT scan for some reason was not performed at the appropriate time. Based on the tumor characteristics and the tumor response to therapy, the appropriate time for resection was determined by the study surgeon (BN).

Finally, each case was categorized either as subjected to optimal neoadjuvant therapy (OT) or non-optimal neoadjuvant therapy (NOT) based on the detected genomic status, the therapy initiated, and according to Table 1 [14–20, 28, 29]. The rate of optimal neoadjuvant therapy, Therapy_{OPTIMAL} (%), in each cohort (SC and RC) was calculated:

$$\text{Therapy}_{\text{OPTIMAL}} = 100 \times n_{\text{OT}} / (n_{\text{OT}} + n_{\text{NOT}})$$

2.6 Outcomes

The primary outcome was the success rate (%) of pretreatment sequencing using the EUS-FNB/TUS-NB samples in an intention-to-diagnose analysis. Success was defined as a conclusive mutational analysis as assessed by the study geneticist (FE) and accomplished in time for the MDTC (< 2 weeks after sampling). In tumors classified as WT in pretreatment samples, confirmation of the WT profile was also required by sequencing of the corresponding resection specimens.

The secondary outcomes were (1) the Therapy_{OPTIMAL} (%), (2) the preoperative tumor size reduction, Tumor Regression_{MAX} (%), (3) the R0-resection rate, and (4) short-term parameters related to surgical resection.

2.7 Statistics

Descriptive, pretreatment baseline data of the study subjects and the tumors were expressed as the median and the interquartile range (IQR). The NIH classification was not, and could not, be used to calculate the prognostic risk in surgical specimens as neoadjuvant therapy affects both the tumor size and the mitotic rate. Instead, imaging data and pretreatment tumor samples were used to calculate the tumor size and the tumor proliferation rate (Ki-67 index).

Fisher's exact test and Student's *t* test (Tumor Regression_{MAX}) were used to test for significant differences in the secondary outcome variables comparing the SC and the RC. The 95% confidence interval (CI) was calculated when appropriate. All tests were two-tailed and conducted at a statistical significance level of $p < 0.05$. The software SPSS Statistics version 22.0 (IBM Corp., Chicago, IL, USA) was used for all calculations.

3 Results

Of 109 eligible patients, 81 study participants with a final diagnosis of GIST were included in the SC, of which 63 participants were advocated neoadjuvant therapy (Fig. 1, Table 2, and Supplementary Table 2).

All cases ($n = 81$) subjected to EUS-FNB ($n = 74$) and TUS-NB ($n = 7$) received a preoperative diagnosis of GIST including conclusive immunohistochemistry. In one case only, a repeated EUS-FNB procedure was required due to a nonconclusive index EUS. One adverse event was recorded [adverse event rate: TUS-NB 0/7 (0%); EUS-FNB 1/74 (1.4%)]. The event was local bleeding post-EUS from a GIST situated in the stomach. The bleeding was stopped via gastroscopy by the injection of epinephrine in the tumor. No case was lost from follow-up.

The RC consisted of 42 patients (Table 2).

3.1 The Primary Outcome

In the SC, the success rate of pretreatment sequencing was 77/81 (95%) of all cases counted (Table 3) and 61/63 (95%) in the cases advocated for neoadjuvant therapy. There was no significant difference in success rate comparing Sanger sequencing with NGS (Table 3). The distribution of the detected mutations was according to Table 2 and Supplementary Table 1, with no primary mutations detected in less frequently mutated genes such as the *BRAF* gene.

3.1.1 Sequencing of the EUS-FNB Samples

The success rate of pretreatment sequencing of the EUS-FNB samples was 71/74 (96%) [Sanger 30/31 (97%); NGS 41/43 (95%), $p = 1.0$, Table 3]. In the first unsuccessful case (a 2-cm gastric GIST situated near the gastroesophageal junction), the amount of EUS-FNB tissue was insufficient for the analysis. Sequencing of the corresponding surgical specimen showed a *KIT* exon 11 mutation. In the second (a 3-cm duodenal GIST) and the third (a 22-cm gastric GIST) unsuccessful cases, the pretreatment sequencing showed WT profiles, while the post-operative specimen sequencing detected a *KIT* exon 11 mutation in both cases. In both the first and the second case, the indication for neoadjuvant therapy was not the tumor size, but rather the tumor position and the possibility of avoiding extensive surgery after tumor downsizing. In 21 cases, sequencing of the corresponding surgical specimen confirmed the primary mutation detected in the pretreatment EUS-FNB sample.

3.1.2 Sequencing of TUS-NB Samples

The success rate of pretreatment sequencing of the TUS-NB samples was 6/7 (86%) [Sanger 3/4 (75%); NGS 3/3 (100%), $p = 1.0$, Table 3]. In three cases, sequencing of the corresponding surgical specimen confirmed the primary mutation detected in the pretreatment TUS-NB sample. In one of these three cases, a patient treated with neoadjuvant imatinib, there was a secondary mutation in the *BRAF* gene detected by the NGS in the surgical specimen. The primary mutation of this case was *KIT* exon 11 p.Q556_N564delinsH.

3.2 The Secondary Outcomes

3.2.1 Rate of Optimal Neoadjuvant Therapy

A favorable mutation was detected in 45/63 (71%) of the SC tumors advocated for neoadjuvant therapy (Fig. 1 and Table 4). In these patients, a standard dosage of imatinib 400 mg daily was initiated.

The rate of optimal neoadjuvant therapy (Therapy_{OPTIMAL}) was significantly higher in the SC compared with the RC [61/63 (97%) vs. 33/42 (79%), $p = 0.006$, Table 4].

3.2.2 Tumor Size Reduction

In total, 90 patients were initiated on neoadjuvant TKI therapy [SC $n = 48$ (imatinib $n = 45$, sunitinib $n = 3$) (Fig. 1); RC $n = 42$ (imatinib $n = 42$)].

Among the 48 SC patients, at least one moderate side effect related to neoadjuvant TKI therapy was recorded in 14 patients (29%) (all treated with imatinib). The discontinuation of imatinib therapy due to side effects was recorded in

Table 2 The baseline characteristics of the study cohort and the reference cohort

	Study cohort			Reference cohort
	All cases	Neoadjuvant advocated	Neoadjuvant not advocated	
<i>Patient characteristics</i>				
Number of patients, <i>n</i>	81	63	18	42
Patient age, median (IQR)	69 (62–74)	69 (64–75)	65 (57–73)	66 (60–74)
Patient gender, M/F	39/42	32/31	7/11	25/17
<i>Tumor characteristics (pretreatment)</i>				
Tumor origin, <i>n</i>				
Esophagus	1	1	0	0
Stomach (fundus/body/antrum)	71 (24/40/7)	55 (18/33/4)	16 (6/7/3)	31 (16/14/1)
Small bowel (duod/jejun/ileum)	9 (7/0/2)	7 (5/0/2)	2 (2/0/0)	11 (4/3/4)
Tumor size ^a , mm, median (IQR)	45 (29–75)	52 (40–90)	24 (20–28)	90 (49–163)
Tumor proliferation rate ^b (Ki-67 index), median (IQR)	4.0 (2.0–6.0)	4.0 (2.0–6.0)	3.3 (2.2–4.0)	7.0 (2.6–11.8)
Tumor genomic profile (gene/exon)				
<i>KIT</i> exon 9	2	2	0	1
<i>KIT</i> exon 11 ^c	52	39	13	34
<i>KIT</i> exon 13 ^d	3	3	0	1
<i>KIT</i> exon 17	1	1	0	0
<i>PDGFRA</i> exon 12	3	2	1	0
<i>PDGFRA</i> exon 14	1	0	1	0
<i>PDGFRA</i> exon 18 p. (D842V)	12	10	2	5
<i>PDGFRA</i> exon 18 non-p. (D842V)	2	2	0	0
Wild type	5	4	1	1

F female, IQR interquartile range, M male

^aPretreatment tumor size

^bThe Ki-67 index was assessed and calculated in the available and adequate pretreatment samples with no recorded statistical difference in the Ki-67 index comparing tumors in various locations (esophagus vs. stomach vs. duodenum) and tumors of various genomic status (*KIT* mutation vs. *PDGFRA* mutation vs. WT profile)

^cDetails on the *KIT* exon 11 mutations are provided in Supplementary Table 1 (see the electronic supplementary material)

^dAll mutants were p. (K642E)

Table 3 The primary outcome: the success rate of pretreatment tumor DNA-sequencing

Sample type	Sequencing modality		P value	Total
	Sanger	NGS		
EUS-FNB (<i>n</i> = 74)	30/31 (97%)	41/43 (95%)	1.0	71/74 (96%)
TUS-NB (<i>n</i> = 7)	3/4 (75%)	3/3 (100%)	1.0	6/7 (86%)
All samples (<i>n</i> = 81)	33/35 (94%)	44/46 (96%)	1.0	77/81 (95%)

EUS-FNB endosonography-guided fine-needle biopsy, NGS next-generation sequencing, TUS-NB transabdominal ultrasound-guided needle biopsy

nine out of 45 patients (20%) (edema *n* = 4; dermatitis *n* = 2; nausea *n* = 2; diarrhea *n* = 1, Fig. 1).

Among the 42 RC patients, at least one moderate side effect related to neoadjuvant TKI therapy was recorded in ten patients (24%). The discontinuation of imatinib therapy due to side effects was recorded in six out of 42 patients (14%) (edema *n* = 3; dermatitis *n* = 2; nausea *n* = 1).

Finally, 66 out of 90 patients (73%) [SC *n* = 34/48 (imatinib *n* = 31; sunitinib *n* = 3); RC *n* = 32/42 (imatinib *n* = 32)] completed neoadjuvant TKI and had at least one follow-up CT scan performed (Fig. 1). The mean tumor size reduction (Tumor Regression_{MAX}) was significantly higher in the SC compared with the RC [27% (95% CI 22–31)

vs. 19% (95% CI 14–24), $p=0.015$, Fig. 2a]. The Tumor Regression_{MAX} results in relation to the tumor genomic profile of individual cases are shown in Figs. 2b and c. The mean Tumor Regression_{MAX} in cases with exclusively favorable mutations was comparable in the SC and the RC [27% (95% CI 23–31) vs. 24% (95% CI 21–27), $p=0.27$].

3.2.3 R0-Resection Rate and Other Parameters Related to Surgical Resection

Among the cases completing neoadjuvant TK therapy and finally proceeding to surgical resection (SC $n=29$, RC $n=28$, Fig. 1), the R0-resection rate was not significantly higher comparing the SC (24/29, 83%) and the RC (24/28,

86%, $p=1.0$). The mean size of the resection specimens in the SC and the RC was 64 mm (95% CI 46–80) and 78 mm (95% CI 58–98), respectively. Other short-term outcome parameters related to surgery are presented in Table 5. The mean duration of neoadjuvant therapy before surgery did not differ between the two cohorts [SC 8.2 months (95% CI 6.2–10.2) vs. RC 8.3 months (95% CI 6.0–10.6), $p=0.94$].

4 Discussion

In this prospective study on consecutive patients, we showed that the rapid and accurate pretreatment genomic profiling of GISTs can be achieved in a vast majority of cases. The early

Table 4 The secondary outcome: the rate of optimal neoadjuvant therapy (Therapy_{OPTIMAL})

	Study cohort	Reference cohort	<i>P</i> value
Therapy _{OPTIMAL}	61/63 (97%)	33/42 (79%)	0.006
	IMA standard ^a	Alternative TKI ^b	Other therapy ^d
<i>Study cohort</i>			
Favorable mutations			
<i>KIT</i> exon 11 ^e	37		1^h
<i>KIT</i> exon 13	3		
<i>PDGFRA</i> exon 12	2		
<i>PDGFRA</i> exon 18 ^f	2		
Unfavorable mutations			
<i>KIT</i> exon 9	1^g	1	
Wild type		2	2
<i>KIT</i> exon 11 p. (L576P)			1
<i>KIT</i> exon 17 p. (Y823D)			1
<i>PDGFRA</i> exon 18 p. (D842V)			10
<i>Reference cohort</i>			
Favorable mutations			
<i>KIT</i> exon 11 ^e	32		
<i>KIT</i> exon 13	1		
Unfavorable mutations			
<i>KIT</i> exon 9	1		
Wild type	1		
<i>KIT</i> exon 11 p. (L576P)	2		
<i>PDGFRA</i> exon 18 p. (D842V)	5		

The number of cases subjected to the different types of neoadjuvant therapy and with respect to the tumor's genetic profile. Numbers in bold signify the cases subjected to non-optimal neoadjuvant therapy according to Table

IMA imatinib, TKI tyrosine kinase inhibitor, WT wild type

^aIMA standard dose (400 mg daily)

^bIMA high dose (800 mg daily) or other TKI therapy besides IMA, i.e., sunitinib

^dNon-TKI based therapy, such as regular chemotherapy or upfront surgery

^eIncludes all variants of mutations in *KIT* exon 11 except p. (L576P)

^fAll variants of mutations in *PDGFRA* exon 18 except p. (D842V)

^gPretreatment mutational analysis failed, resulting in the incorrect regimen of standard dose IMA in a *KIT* exon 9 mutant

^hPretreatment mutational analysis falsely showed a WT profile, resulting in upfront surgery of a *KIT* exon 11 mutant

mutational analysis of the tumors had an important impact on the preoperative management of patients, demonstrated by only a few cases subjected to inadequate neoadjuvant therapy. Consequently, first-line, standard dose imatinib was initiated only in patients with a favorable tumor mutation, leading to a maximized chance of effective, preoperative tumor size reduction. Primarily, the results were enabled via the combination of high-priority EUS-guided biopsy sampling followed by immediate sequencing of *KIT* and *PDGFRA* in the acquired tumor tissue.

Without tumor genomic information, the prediction of the efficacy of neoadjuvant TKI therapy in GISTs will be more or less a qualified guess. Obviously, some tumors will respond to standard dose imatinib by chance [10, 12, 30]. However, there is a substantial risk of initiating a suboptimal regimen and delaying alternative strategies or surgery, as in patients with WT tumors or *PDGFRA* exon 18 p.(D842V) tumors. In the presented work, we could define the subset of patients eligible for standard dose imatinib in all but one patient. Importantly, 18 patients advocated for neoadjuvant therapy had a tumor mutation associated with nil or reduced imatinib sensitivity. In 17 of these patients, the mutation was detected before therapy was initiated, which led to a modified treatment plan.

The toxicity profile of imatinib is another argument for pretreatment mutational analysis. The frequency of therapy discontinuation reached as high as 35% in a study using 600 mg imatinib daily for neoadjuvant treatment [12]. High-dose 800 mg imatinib is associated with even more toxicity [3, 4] and should be initiated only when genetically motivated, such as in *KIT* exon 9 mutants. In a relatively recent publication by Kurokawa and coworkers, daily 400 mg imatinib was used for neoadjuvant therapy as in our study [10]. The authors recorded a need for imatinib dose reduction in 14 out of 53 patients (26%) and a final need for imatinib discontinuation in seven out of 53 patients (13%). Even though we recorded a somewhat higher need for imatinib discontinuation in the SC, but not in the RC, these numbers are comparable to the data reported by Kurokawa et al. [10] and lower than the rate of discontinuation reported by Wang et al. [12].

By comparing the SC with a historic cohort of comparable GIST patients, we could demonstrate that the mean tumor size reduction induced by neoadjuvant therapy was significantly higher if pretreatment mutational analysis of GIST was performed to guide therapy. That finding is important as the radiographic tumor response may affect the chance of radical resection (R0) and subsequently the risk of tumor recurrence, at least in patients with advanced GISTs [9, 11]. In the presented work, we did not record any increased frequency of R0 resections in the SC compared with the RC not subjected to pretreatment sequencing. To prove such a hypothesis, a high number of patients in two

risk-matched study populations would be required, which is beyond the capacity of a single center. Nevertheless, sequencing followed by tailored, genotype-driven therapy will most likely lead to the reduction of suboptimal TKI regimens, i.e., unnecessary switches of TKIs, and thereby reduce the time to an adequate anti-tumoral effect. We recorded a numerically lower frequency of post-operative complications in the SC compared with the RC (Table 5). It can be hypothesized, though definitely not proven, that pretreatment sequencing followed by adequate neoadjuvant therapy to some extent contributed to this result. It would be interesting if future studies could address this topic.

Others have evaluated the effect of neoadjuvant imatinib in prospective studies of GIST [7, 10, 12, 30–32]. In addition, our group has explored the feasibility of mutational analysis in pretreatment EUS-FNB, in a majority of study subjects late after diagnosis [25]. However, to the best of our knowledge, the presented, prospective work is the first study performing systematic pretreatment sequencing of GIST tissue in a high number of consecutive patients to guide neoadjuvant therapy.

There are other potential advantages of pretreatment sequencing of GISTs. First, some GISTs (<5%) are *c-KIT*-negative in immunostaining [33]. Certainly, complementary immunostaining with DOG-1 has a high sensitivity for GIST [34]. Nevertheless, the mutational analysis performed with NGS requires only a small amount of viable tumor tissue. Therefore, it may contribute to the confirmation of GIST diagnosis in biopsies with a low cellular count. In one case of the present study, a gastric GIST, the EUS-FNB sample was low in cellular count, which made the immunostaining for GIST-specific markers inconclusive and difficult to interpret. However, the cell count was still sufficient for NGS, which unveiled a *PDGFRA* exon 18 p. (D842V) mutation.

Second, the mutation profile impacts the prognosis in GIST. *PDGFRA* mutations are associated with a favorable prognostic risk [35]. Gastric GISTs with *KIT* exon 11 deletions have a more malignant course of disease compared with *KIT* exon 11 single nucleotide substitutions [36, 37]. Thus, the early detection of such mutations would support both clinicians and patients with important prognostic information.

Third, the TKI therapy response of GISTs as assessed by CT scan [38] can sometimes be misleading, as certain tumors that in fact respond to therapy do not decrease in size, but only in tumor density [39]. Hence, in such cases, there is an obvious risk that the clinician decides on TKI discontinuation despite a true anti-tumoral effect. Mutational analysis of pretreatment tumor tissue, as performed in the present study, cannot replace the evaluation by CT scan. Nevertheless, the analysis may act as an attractive complement by supporting the clinician with valuable genomic information and thereby facilitating the decision on continued neoadjuvant therapy.

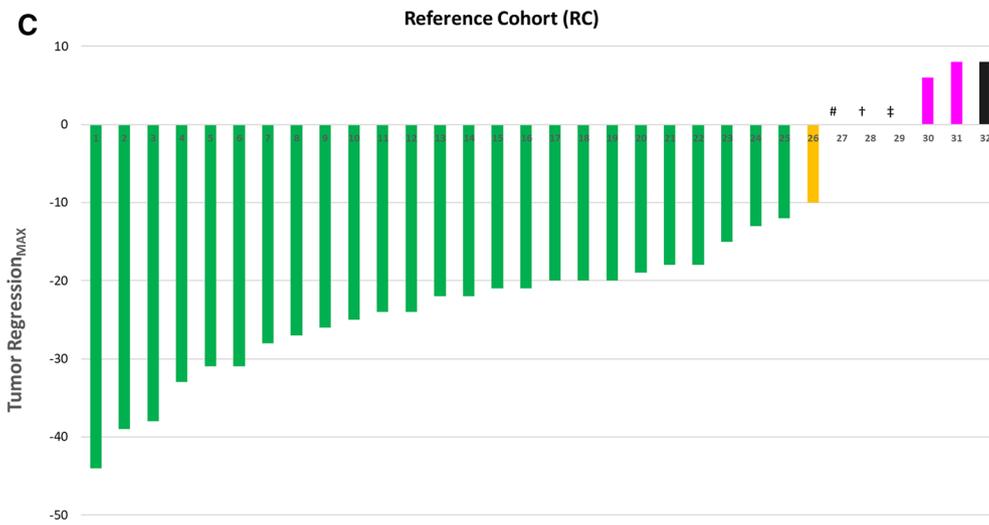
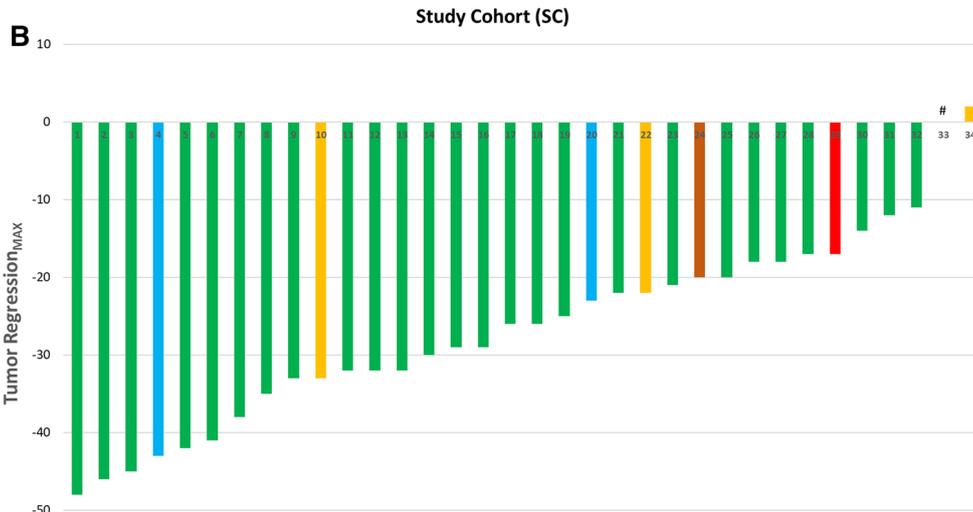
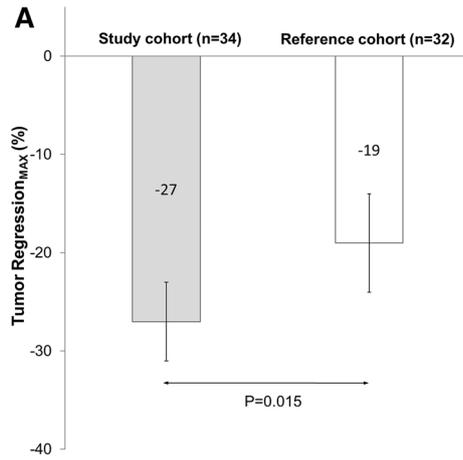


Fig. 2 a Bar chart showing the preoperative tumor size reduction (Tumor Regression_{MAX}) as evaluated by CT scan and induced by neoadjuvant TKI therapy in the study cohort (left bar in grey) and in the reference cohort (right bar in white). The error bars represent the 95% confidence interval. **b** Waterfall plot showing the preoperative tumor size reduction (Tumor Regression_{MAX}) in individual tumors of the study cohort (SC). The bar color represents the tumor genomic profile as follows: Bars in green: *KIT* exon 11-mutant (non p. L576P). Bars in blue: *PDGFRA* exon 18-mutant (non p. D842V). Bars in orange: *KIT* exon 13-mutant. Bars in brown: *KIT* exon 9-mutant. Bars in red: WT profile (case treated with neoadjuvant sunitinib and not imatinib). There was stable disease with neither tumor growth nor tumor regression in case number 33 (#): *KIT* exon 9-mutant. **c** Waterfall plot showing the preoperative tumor size reduction (Tumor Regression_{MAX}) in individual tumors of the reference cohort (RC). The bar color represents the tumor genomic profile as follows: Bars in green: *KIT* exon 11-mutant (non p. L576P). Bars in orange: *KIT* exon 13-mutant. Bars in purple: *PDGFRA* exon 18 p. (D842V)-mutant. Bars in black: *KIT* exon 11 p. (L576P)-mutant. There was stable disease with neither tumor growth nor tumor regression in case number 27 (#): *PDGFRA* exon 18 p. (D842V)-mutant; case number 28 (†): *KIT* exon 11 p. (L576P)-mutant; case number 29 (‡): *PDGFRA* exon 18 p. (D842V)-mutant

If available, the use of positron emission tomography–computed tomography (PET-CT) may also add information on the response to TKI therapy. In other studies, radiographic response on CT scan alone has been used to detect imatinib dose escalation [30] or imatinib discontinuation [10]. We believe that pretreatment mutational analysis in GISTs, at least in selected cases, should be considered in addition to the evaluation by CT scan.

The accurate pretreatment genetic profiling of GISTs is a multistep procedure dependent on an adequate sampling method, a dedicated pathologist, and a reliable sequencing method. Regarding the acquisition of the sample as such, EUS-FNB was highly accurate for GIST diagnosis in the present study, with the need for a repeated EUS-FNB only in one single case. Furthermore, all but a few tumors were indeed situated in locations within reach for oral access EUS. Therefore, the suggested work-up strategy with first priority EUS would be feasible in most large volume GIST centers.

As an alternative to EUS, we have shown that sequencing tissue acquired by the transabdominal approach (TUS-NB) is feasible in the few tumors situated in a position beyond the reach of EUS, e.g., distal small bowel GISTs. The TUS-NB procedure is safe [40]. The obvious drawback of TUS-NB is that some GISTs are small and located in deep gastric positions, making the transabdominal access demanding.

In 2016, we converted from Sanger sequencing to NGS. In the current study, we have shown that both techniques are reliable and can detect the mutations in GISTs with a high sensitivity. The advantage of NGS is that the technique requires a significantly lower amount of tumor DNA compared with the Sanger method and that it enables the simultaneous sequencing of multiple samples from different

malignancies [41]. Consequently, the widespread clinical implementation of pretreatment NGS in GISTs would most likely reduce the mean time both to the completed analysis and to the initiation of genotype-driven neoadjuvant therapy.

GISTs that may have a WT profile constitute a hazardous subgroup of tumors as there might actually be a mutation. The frequency of WT tumors reported has gradually decreased [42], most likely due to improved methods of sequencing. In the SC, we recorded only seven tumors (five resected, two nonresected) with a WT profile in the pretreatment sample. Post-treatment sequencing of the available five surgical specimens confirmed that three cases were true WT tumors, while in two cases, a primary mutation was detected—in both cases, a *KIT* exon 11 mutation. However, that potential mutations indeed exist in genes not included in the NGS panel cannot be excluded (Supplementary Table 3). Such an example is the potential existence of alterations in the SDH subunit. In the present study, the SDH status was not tested for by immunohistochemistry or by tumor genome sequencing. In addition, the low cellular count in a highly regressive resection specimen may compromise the mutational analysis and result in a false WT profile despite a true underlying mutation. Nevertheless, we believe that repeated sequencing of the corresponding surgical specimens is advisable in all cases where pretreatment sequencing has demonstrated a WT profile.

The criteria to be used to recommend neoadjuvant treatment in GIST remain to be determined [43, 44]. Treatment decisions are dependent on numerous factors, such as tumor size, tumor position, the existence of metastases, and the performance status of the patient. Unfortunately, there are few studies published on the topic. In the study by Rutkowski et al., patients with locally advanced tumors (no size criteria specified) [11] were given neoadjuvant therapy, while in the study by Kurokawa et al., tumors > 10 cm [10] were treated. As previously mentioned, in the current study, and according to the consensus at our tertiary center, more liberal criteria for neoadjuvant treatment are applied. This fact suggests that the presented results must be carefully interpreted and they might not be completely generalizable, especially in institutions that apply stricter criteria for neoadjuvant therapy.

An obvious drawback of neoadjuvant therapy in GISTs is that there is a risk for underestimation of the tumor prognostic risk (according to the NIH consensus criteria) in surgical specimens affected by long-term tyrosine kinase inhibition leading to tumor shrinkage and a decrease of the tumor proliferation rate (mitotic index) [45]. However, the Ki-67 index correlates relatively well to the mitotic rate and can potentially be used as a surrogate marker for the tumor proliferation rate [46, 47]. Consequently, in the current study, the decision on adjuvant therapy was based upon not only the surgical specimen analysis but also pretreatment data on tumor size at imaging and the Ki-67 index of EUS-FNB.

Table 5 Short-term parameters related to surgery of GIST

All tumors (<i>n</i> = 57)	Study cohort (<i>n</i> = 29)	Reference cohort (<i>n</i> = 28)	<i>P</i> value
<i>Surgical procedure performed</i>			
Gastric wedge resection	15	17	
Gastric sleeve resection	2	0	
Gastrectomy (Billroth II)	4	1	
Whipple resection	3	2	
Small bowel resection	2	4	
Extensive, multiorgan resection	3	4	
<i>Post-operative complications</i>			
No post-operative complication	26	21	0.14
Yes, post-operative complication	2	7	
Repeated surgery or endoscopic intervention required	2 ^a	4 ^b	
Antibiotics required due to infection	0	3	
<i>Post-operative care</i>			
Duration of hospitalization (days), mean (SD)	11.0 (7.8)	13.3 (8.0)	0.28
Tumors < 5 cm (<i>n</i> = 21)	Study cohort (<i>n</i> = 12)	Reference cohort (<i>n</i> = 9)	<i>P</i> value
<i>Surgical procedure performed</i>			
Gastric wedge resection	7	8	
Gastric sleeve resection	1	0	
Gastrectomy Billroth II	3	0	
Whipple resection	1	1	
Small bowel resection	0	0	
Extensive, multiorgan resection	0	0	
<i>Post-operative complications</i>			
No post-operative complication	11	7	0.12
Yes, post-operative complication	1	4	
Repeated surgery or endoscopic intervention required	1 ^c	2 ^d	
Antibiotics required due to infection	0	2	
<i>Post-operative care</i>			
Duration of hospitalization (days), mean (SD)	12.1 (9.9)	14.2 (9.6)	0.63

GIST gastrointestinal stromal tumor, SD standard deviation

^aLeakage at the enteroanastomosis (*n* = 1); pyloric stenosis (endoscopic dilatation needed) (*n* = 1)

^bLeakage at the anastomosis with the development of an abscess (*n* = 1); ileus (*n* = 1); leakage at the biliary anastomosis (*n* = 1); pyloric stenosis (endoscopic dilatation needed) (*n* = 1)

^cPyloric stenosis (endoscopic dilatation needed) (*n* = 1)

^dLeakage at the biliary anastomosis (*n* = 1); pyloric stenosis (endoscopic dilatation needed) (*n* = 1)

This prospective study is strengthened by the fact that consecutive GIST patients of an entire, large region were included over a long period of time. Our center was responsible for the complete work-up process, including all clinical examinations and laboratory analyses, which ensured a standardized methodology. To the best of our knowledge, this is the first study to show the clinical and therapeutic benefits of pretreatment mutational analysis in GISTs in the setting of neoadjuvant TKI therapy.

There are some weaknesses in the presented study. For various reasons, such as discontinuation of therapy, not all

study patients could be evaluated with CT scan after the initiation of imatinib therapy. The limited number of study patients did not allow us to draw any conclusions regarding the importance of pretreatment sequencing on the outcome of surgery after neoadjuvant therapy (R0 resection). Preferably, such a study should also be designed as a randomized, controlled trial thereby disqualifying some patients from pretreatment sequencing, which could be somewhat questionable from an ethical point of view.

We conclude that the performance of high-priority EUS-FNB followed by immediate DNA sequencing of *KIT* and

PDGFRA in the acquired pretreatment tumor tissue is highly valuable to guide neoadjuvant therapy in GISTs. Therefore, ineffective treatment with standard dose imatinib in unfavorable mutants, such as the *PDGFRA* exon 18 p.(D842V) variant, can be minimized. The suggested approach is recommended to all centers responsible for the diagnostic work-up and early care of patients with GISTs.

Acknowledgements Open access funding provided by University of Gothenburg. All staff at the GEA Endoscopy unit, Sahlgrenska University Hospital.

Compliance with Ethical Standards

Funding This work was not supported by any commercial or financial interests. Research grants were obtained from the following noncommercial foundations: The Health and Medical Care Committee of the Regional Executive Board, Region Västra Götaland (Grant number: VGFOUREG-564381 and VGFOUREG-144591); Sahlgrenska University Hospital (LUA-ALF, Grant number: 73830, 74370, and 70150); The Assar Gabrielsson Foundation (Grant number: 17-20); and Magtarmfonden (Grant number: 79211).

Conflict of interest Per Hedenström, Carola Andersson, Henrik Sjövall, Ola Nilsson, Bengt Nilsson, and Riadh Sadik declare they have no conflicts to report. Fredrik Enlund reports lecture honorarium from Novartis, Astra Zeneca, Roche, Lilly, and Astellas.

Ethical approval and informed consent This study was approved by the Regional Ethical Review Board of Gothenburg. Written informed consent was obtained from all the study participants. The study was registered at ClinicalTrials.gov (NCT02360839).

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