Diploma Thesis

Molecular landscape of gastrointestinal stromal tumors – a retrospective study

submitted by

Alexandra Argyropoulos

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Univ. Prof.in PDin Dr.in Bernadette Liegl-Atzwanger

PD. Dr.in med. univ. et scient. med. Iva Brcic

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Graz, am 24.01.2022 *Alexandra Argyropoulos eh.*

Danksagung

An dieser Stelle möchte ich mich bei all denjenigen bedanken, die mir bei der Erstellung und Korrektur dieser Diplomarbeit stets unterstützend und motivierend zur Seite gestanden sind.

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Glossary and Abbreviations

ABL	Abl-Kinase
AFIP	Armed Forces Institute of Pathology
AKT	Serine/threonine protein kinase B
ALK	Anaplastic lymphoma kinase
ARID	AT-rich interactive domain-containing protein
BCR	Breakpoint Cluster Region
BRAF	Rapidly accelerated fibrosarcoma Isoform B
CBL	Casitas B-lineage Lymphoma, proto-oncogene
CDK4	Cyclin dependent kinase 4
CDK4	Cyclin-dependent kinase 4
CK	Cytokeratin
Cm	Centimetres
CML	Chronic myeloid leukemia
CSS	Carney Stratakis Syndrome
CT	Carney Triad
CT	Computer tomography
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DOG1	Discovered on GIST 1
E-GIST	Extragastrointestinal gastrointestinal stromal tumor
EHE	Epithelioid hemangioendothelioma
EMA	Epithelial membrane antigen
ETV6	ETS Variant Transcription Factor 6
EUS	Endoscopic ultrasound
EUS-FNA	Endoscopic ultrasound fine needle aspiration
FDG-PET	Fluorodeoxyglucose-positron emission tomography
FFPE	Formalin fixed paraffin embedded
FGFR1	Fibroblast growth factor receptor 1
FLT3	Fms-like tyrosine kinase-3 receptor
GIST	Gastrointestinal stromal tumor
GI-tract	Gastrointestinal tract
H&E	Haematoxylin-eosin

HFS	Hand-foot syndrome
HIF-1	Hypoxic induced factor
HMB45	Human Melanoma Black
HPF	High power field
IFP	Inflammatory fibroid polyp
IHC	Immunohistochemistry
ILGF1R	Insulin-like growth factor 1 receptor
IMT	Inflammatory myofibroblastic tumor
IRS1	Insulin receptor substrate 1
LKH	Landeskrankenhaus (hospital)
LN	Lymph nodes
LS	Liposarcoma
MAPK	Mitogen-activated protein kinase
MAX	MYC-associated factor X
MDM2	Murine double minute 2
MDM2	Mouse double minute 2 (regulator of p53)
MEN1	Multiple endocrine neoplasia type 1
Mm	Millimetres
MPNST	Malignant peripheral nerve sheath tumor
MR	Magnet resonance
MUG	Medical University of Graz
NA	Not available
NET	Neuroendocrine tumor
NF-1	Neurofibromatosis type 1
NGS	Next generation sequencing
NIH	National Institute of health
NTRK3	Neurotrophin tyrosine kinase receptor 3
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PEComa	Perivascular epithelioid cell tumor
PHD	Prolyl 4-hydroxylases
PI3K	Phosphoinositide 3-kinases
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha

PKC	Protein kinase C
RB1	Retinoblastoma associated protein, tumor suppressor
RET	Rearranged during Transfection
ROS1	Repressor of Silencing 1
RTK	Receptor tyrosine kinase
SDH	Succinate dehydrogenase
SFT	Solitary fibrous tumor
SMA	Smooth muscle actin
SMT	Submucosal tumors
SOX 10	SRY-related HMG-box 10
STAT6	Signal transducer and activator of transcription 6
TKI	Tyrosine kinase inhibitors
VEGFR	Vascular epithelial growth factor receptor
VHL	Von Hippel Lindau; tumorsuppressor
WT	Wild type

Figure Legends

Table Legends

Zusammenfassung

Hintergrund: Gastrointestinale Stromatumore (GIST) sind die häufigsten mesenchymalen Tumore des Verdauungssystems und treten vor allem bei älteren Patient*innen auf. Sie sind meist gut umschriebene, solitäre Tumore, die histologisch ein spindel-, epitheloid- oder gemischt-zelliges Wachstumsmuster aufweisen können. Immunhistochemisch zeigen sie eine positive Reaktion mit CD117 und DOG1.

90 % der GISTs weisen eine Mutation im Tyrosinkinase-Rezeptor *KIT* oder im Platelet-derived growth factor receptor (*PDGFRA*) auf. GISTs ohne Mutationen in *KIT* oder *PDGFRA* werden vereinfacht als wildtype-GISTs (wt-GISTs) bezeichnet und können neben Mutationen, wie zum Beispiel in *BRAF* und *NF1,* auch mit Tumorsyndromen assoziiert sein. Die molekulare Untersuchung von GIST ist ein wichtiger Bestandteil der Therapieplanung und Voraussetzung für die personalisierte Behandlung von GIST Patient*innen.

Material und Methoden: Klinische, histologische, immunhistochemische und molekulare Daten („expanded molecular GIST panel“) von 81 Patient*innen wurden evaluiert. Zusätzlich wurden Informationen zu Behandlung, Nachsorge und Komorbiditäten erfasst. Die Ergebnisse wurden mit aus der Literatur bekannten Daten verglichen.

Ergebnisse: Die evaluierten GISTs waren am häufigsten im Magen und im Dünndarm lokalisiert und die Größe reichte von 0.8 - 20 cm. Histologisch zeigten die Tumoren in 41/81 Fällen eine spindelzellige Morphologie, in 13/81 Fällen ein epitheloidzellige Morphologie und in 27/81 Fällen eine gemischt-zellige Morphologie. Mittels Immunhistochemie zeigten 95.8% der Tumoren eine positive Reaktion mit DOG-1 und 95.2% mit CD117. Am häufigsten metastasierten die Tumoren in die Leber und das Peritoneum; Metastasen in Haut, Lymphknoten, Lunge, Ovar und Hoden wurden ebenfalls beobachtet.

In 55/81 Tumoren wurden ausschließlich Mutationen in *KIT* oder *PDGFRA* gefunden. Insgesamt gab es 45 Mutationen im *KIT*-Gen: 33 davon waren im Exon

11, acht im Exon 9, zwei im Exon 13, eine im Exon 8 und eine im Exon 17. Zehn Mutationen fanden sich im *PDGFRA*-Gen, davon eine im Exon 12, eine im Exon 14 und 8 im Exon 18. Die Exon 18 *PDGFRA*-Mutationen waren exklusiv Punktmutationen, wobei in 6 Fällen die Imatinib Resistenzmutation D842V detektiert wurde. Weiters gab es einen Fall mit einer Mutation in *KIT 11* und einer zusätzlichen Mutation in *PDGFRA 12*.

Zusätzlich gab es 2 GISTs, die eine Mutation in *NF1* aufwiesen und 5 GIST zeigten eine *SDH*-Mutation (3 *SDHA* und 2 *SDHB*). 17 GISTs wiesen mehrere Mutationen in der untersuchten Tumorprobe auf. Bei 2 Tumoren konnten keine Mutationen festgestellt werden. Das durchgeführte Archer Fusion Plex Sarkom Panel an einem Fall mit genügend Gewebematerial zeigte keine Fusion.

Schlussfolgerungen: Sporadische GISTs treten überwiegend bei älteren Menschen auf und können im gesamten GI-Trakt lokalisiert sein. Bei Kindern und jungen Erwachsenen mit GIST im Magen sind Screening Untersuchungen mittels SDHB Immunhistochemie hilfreich, um seltene syndromale GIST auszuschließen. Die immunhistochemischen Marker DOG1 und CD117 (in Verwendung in einem IHC-Panel) sind verlässliche Marker in der GIST-Diagnostik. Eine molekulare Analyse von Tumorproben ist für die Durchführung einer personalisierten Therapie erforderlich.

Abstract

Background: Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the digestive system and occur preferentially in the elderly patients. They are usually well-circumscribed solitary tumors and show either a spindle-, epithelioid- or mixed- cell morphology. The vast majority of cases express DOG1 and CD117 by immunohistochemistry.

GISTs harbor mutations in the tyrosine kinase receptor *KIT* or in platelet-derived growth factor receptor (*PDGFRA*) in approximately 90% of cases. Tumors without mutation in *KIT* or *PDGFRA* are called in a simplified way “wild-type” GISTs (wt-GISTs) and especially in children and young adults these GISTs can be associated with tumor syndromes. Nevertheless, other mutations in GIST like *BRAF, NF1* and others have been reported. Molecular analyses with an expanded molecular panel are important for selecting the adequate treatment for patients suffering from this disease.

Methods: In this study clinical, histological, immunohistochemical and molecular data of the 81 patients with GIST were collected and evaluated. In addition, treatment, follow-up, and comorbidities were recorded. The data was compared with the literature.

Results: GISTs were located most frequently in the stomach and small intestine and ranged in size from 0.8 cm to 20 cm. Histologically, they showed a spindle cell morphology in 41/81 cases, an epithelioid cell morphology in 13/81 cases, and a mixed morphology in 27/81 cases. Immunohistochemically, 95.8% and 95.2% of the tumors showed a positive immunohistochemical staining with DOG-1 and CD117, respectively. Predominantly, GISTs metastasized to the liver and peritoneum; However, metastases to skin, lymph nodes, lung, ovary, and testis were also observed.

In 55/81 tumors mutations in either *KIT* or *PDGFRA* were found. In total, 45 mutations were detected in the *KIT* gene: 33 mutations in exon 11, eight mutations in exon 9, two mutations in exon 13, one mutation in exon 8 and one mutation in

exon 17. Furthermore, 10 mutations were observed in the *PDGFRA* gene, one mutation in exon 12 and one mutation in exon 14. The other eight *PDGFRA* mutations were found in exon 18 – in six cases the *PDGFRA D842V* mutation – a known Imatinib resistance mutation was observed. Moreover, there was one case with a mutation in *KIT 11* and an additional mutation in *PDGFRA12*.

In addition, there were 2 GISTs that harbored a mutation in *NF1* and 5 GIST showed a *SDH* mutation (three *SDHA* and two *SDHB*). In 17 GISTs multiple mutations were detected. No mutations were detected in two tumors: the Archer Fusion Plex Sarcoma Panel showed no fusion in one of the samples, where enough material was evaluable for RNA-Sequencing.

Conclusions: Sporadic GISTs occur predominantly in the elderly (mean-age: 64.8 years) and are localized throughout the GI tract. In children and young adults suffering from a GIST in the stomach, the use of a SDHB-immunohistochemistry is helpful to screen for rare syndromic GISTs. Immunohistochemical markers including DOG1 and CD117 (using a panel of IHC markers) are reliable markers in GIST diagnosis. Further, molecular analysis is required for personalized treatment strategies.

1. Introduction

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors in the gastrointestinal tract with an annual incidence of approximately 10-15 cases per million (1–3). They develop from Cajal cells (specialized nerve cells) located in the Plexus myentericus between the longitudinal and circular layer of the tunica muscularis in the bowel wall. (4,5) Cajal cells are the pacemaker cells of the gut (5–8) and show positive expression for CD117 (c-Kit) by immunohistochemistry (IHC). The proto-oncogene *KIT* is an important development factor for proliferation (among others for Cajal cells) and differentiation. (5,9–12)

About 80% of GISTs show an activating mutation in *KIT* and approximately 5-10% harbour a mutation in the *platelet-derived growth factor receptor alpha* (*PDGFRA*) gene, which is also responsible for growth processes in the human body. *KIT* and *PDGFRA* mutations are mutually exclusive. (3,5,13)

Furthermore, there is a group of GISTs lacking *KIT* and *PDGFRA* mutations, referred to in a simplified way as wildtype (wt) GIST. WT-GISTs are a heterogeneous group of tumors, recently subdivided into a succinate dehydrogenase (SDH)-retained or SDH-deficient group. (1,14,15)

Before the 1990s there was no effective treatment for GIST, because these tumors were often diagnosed as smooth muscle or neural tumors and neither chemo- nor radiotherapy was successful. Surgery was the only therapeutic option. (3,16,17) In 1983 the term “stromal tumor” was introduced by Mazur and Clark. The term was based on the common expression of CD34 in this tumor type, which became a marker, however unspecific, for this tumor entity. (3,18,19) In 1998 Hirota et al. published a landmark paper demonstrating that GISTs harbour activating mutations in *KIT*, a transmembrane receptor with tyrosine kinase activity. In addition, they showed that KIT expression by immunohistochemistry can be used as a confirmatory marker for this tumor entity. (3,20) The discovery of the activating mutation in *KIT* revealed possible treatment options with tyrosine kinase inhibitors (TKIs) such as Imatinib, which was already used against BCR-ABL leukaemia (CML). (3,21) Some years later, in 2003, Heinrich and colleagues identified alternative mutations in the *PDGFRA*- gene as the second most common driver

mutations in GISTs. (3,22) Since then it has been shown that *KIT* and *PDGFRA* mutations are mutually exclusive.

In the following years GIST became a treatable disease even in a metastatic setting with terrific results, presented by Joensuu et al in 2001 (23) and Demetri et al in 2002 (24). Over the years, GISTs became the paradigm for targeted treatment and Imatinib has revolutionized the treatment for patients suffering especially on inoperable or metastatic GISTs.(3)

1.1 Epidemiology

GISTs usually occur in older adults (the average age is 60 years) but can develop at any age. In the sporadic setting men are slightly more affected. The annual incidence is 10 per million and they mainly occur in Caucasian industrialized populations. (25–27) 1-2% of GIST occur in the paediatric age group, where mainly girls are affected. (1)

The mortality rate depends on the size, mitotic rate, anatomic location and presence of tumor perforation. Generally, the 3-year-survival of GISTs is about 70% and the risk of recurrence depends on risk stratification according to Miettinen. (28–30)

The vast majority of GISTs are sporadic however, over the years GISTs in association with various syndromes like neurofibromatosis-1 (NF1), familial GISTs, Carney triad (CT) and Carney Stratakis Syndrome (CSS) have been described. (1,3)

1.1.1 GIST in association with NF1

GISTs associated with NF1 occur predominately in a younger age group with a slight female predominance. (1,28) In NF1 patients multiple tumors in the small bowel can occur. (1,3)

1.1.2 Familial GIST

Familial GISTs typically appear at a younger age (approximately around 40) and is an autosomal dominant inherited disease causing one or even multiple GISTs, especially in the small intestine or stomach. (31–33) Depending on the mutation in *KIT* or *PDGFRA*, patients with familial GIST show different symptoms. For example, mutations in exon 11 of *KIT* can show a skin hyperpigmentation. (3,32,34–36) The most frequent mutations in familial GISTs are listed in Table 1. (37)

Gene/Exon	Genetic Mutation
KIT Exon 8	p. D419del
KIT Exon 9	p. K509I
KIT Exon 11	p. V559A p. D579del p. W557R p. W557S p. L576P p. V560A p. V560G p. V560del p. Y533C
KIT Exon 13	p. K642E p. K642T p. N655K
KIT Exon 17	p. D820Y p. D820G p. N822Y
PDGFRA Exon 12	p. Y555C p. V561D
PDGFRA Exon 14	p. P653L
PDGFRA Exon 18	p. D846Y p. D846V

Table 1: Mutations in KIT and PDGFRA in familial GISTs (37)

1.1.3 Paediatric GIST

Paediatric GISTs can develop sporadically but more commonly in association with cancer syndromes like Carney Stratakis Syndrome (CSS) or Carney Triad (CT). (38) In general, most paediatric GISTs are part of the group of SDH-deficient GISTs. (1,3)

Carney Stratakis Syndrome is a tumor syndrome associated with paraganglioma and gastric GIST. Tumors in this setting usually occur during childhood and adolescence and are triggered by germline mutations in the *SDH A, B, C* or *D* subunit. (39–42)

Carney triad is a non-hereditary disease characterized by the coexistence of the following neoplasms namely paraganglioma, gastric GIST and pulmonary chondroma. CT appears mostly in young women. The CT is mainly caused due to hyper-methylation of the SDHC gene. (39–42)

GISTs can develop in both tumor syndromes CSS and CT and show genetic changes either mutations in *SDHA, B, C* or *D* (CSS) or hypermethylation in the SDHC (CT). (39–42)

GISTs in association with CT and CSS occur in the stomach. However, sporadic GISTs can occur throughout the GI-tract from the oesophagus to the rectum.

In general, GISTs most frequently appear in the stomach (60%) and small bowel (30%) whereas they are rare in the oesophagus (1%) as well as the colon and rectum (5%). On the other hand, there are reports about GISTs that might develop in extragastrointestinal location, for example mesentery or omentum, pelvic cavity or retroperitoneum. However there is an ongoing discussion in the literature if these so called extragastrointestinal GIST or E-GISTs exist. (1,25,43,44) It has been proposed that E-GISTs might develop from ectopic Cajal cells or stem cells or anomalies (like enteric duplications). (43,45) However, more likely these tumors develop from the gut wall, but the connection could not be demonstrated. (46)
Small GISTs, particularly in the muscularis propria of the proximal stomach can be found as an incidental finding during autopsy, surgery or endoscopy (Figure 1a, 1b, 1c). These small GISTs are reported to occur in about 35% of people older than 50 years and are usually asymptomatic. They are divided into mini GISTs and micro GISTs. The micro GISTs are just a few millimetres, while the mini GISTs are defined to have a size between 1 and 2 cm. (1,25,47,48)

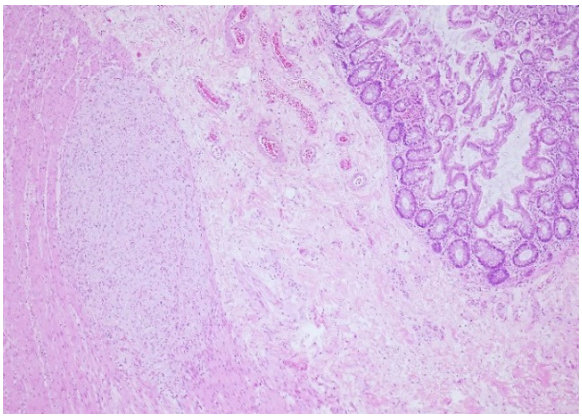

Figure 1a: Incidental micro GIST, HE, 40x, Courtesy PD Dr. Brcic

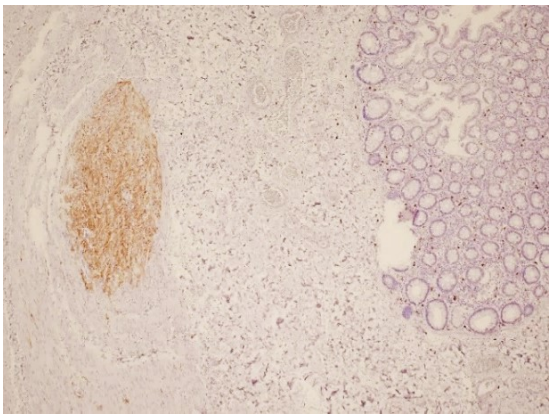

Figure 1b: Incidental micro GIST, CD117, 40x, Courtesy PD Dr. Brcic

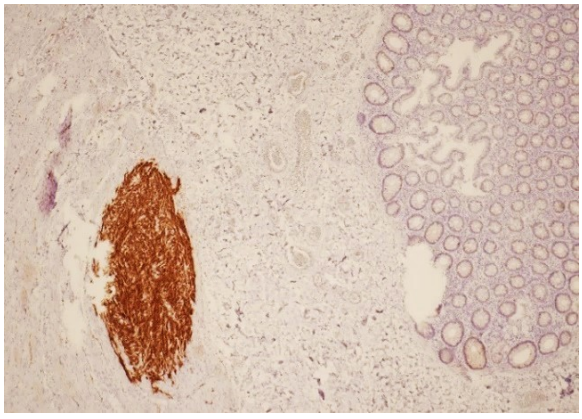

Figure 1c: Incidental micro GIST, DOG1, 40x, Courtesy PD Dr. Brcic

1.2 Clinical Management

1.2.1 Symptoms

The clinical symptoms range from totally asymptomatic (small GISTs) to fatigue, bleeding, anaemia, abdominal pain, or even an acute abdomen after tumor rupture. (25) Rarely, symptoms like obstruction, pelvic pain and dysuria or weight loss are described. (31)

1.2.2 Diagnostic

The average of GISTs are incidentally discovered as submucosal tumors (SMT) during endoscopy, other radiological procedures or during surgeries. (1,49)

Diagnostic confirmation

Usually, an endoscopic ultrasonography (EUS) and a EUS- guided fine-needle-biopsy (EUS-FNA) is done to determine the tumor entity. Depending on the location and the size of the nodule, the further diagnostic methods differ. If the tumor is smaller than 2 cm and located in the oesophagus, stomach or duodenum, they are examined with EUS and if feasible a biopsy is taken. If the tumor turns out to be a GIST it is usually excised. Depending on the patient´s age, comorbidities and tumor location, the SMT can also be just observed. Initially a short-term follow-up interval is suggested and if the tumor shows no signs of progression and remains asymptomatic, the follow-up interval can also be expanded. In contrast, rectal tumors (of any size) and tumors 2 cm or larger should be completely excised, because they have a higher risk to progress. Usually, more than one core needle biopsy is taken, if guided EUS is used for diagnostic purpose. Very rarely a percutaneous biopsy with ultrasound or CT- guidance is used for diagnostic purpose. The biopsy or the surgical preparation should be kept in 4% formalin. Unfortunately, biopsies are not always diagnostic, because the tumors are also located deeper in the gut wall and might be missed. (25,27,33,49,50) In patients with a metastatic GIST, a biopsy from a well accessible metastasis can be used for diagnosis. (27)

Evaluation of tumor tissue by specialized pathologists is essential for personalized treatment. The tasks of pathology are to give the diagnosis, perform the risk stratification (See Prognosis and Risk Stratification) and obtain molecular analysis. The diagnostic process consists of the evaluation of morphology and

immunohistochemistry (especially CD 117 and DOG-1 expression) always in context with the clinical history. In addition, a molecular analysis can be performed to confirm the diagnosis as well as to guide treatment depending on the underlying mutation. (51–53)

Radiology

Imaging is a very important step in the diagnostic evaluation of a patient´s tumor. In case of abdominal symptoms or even an abdominal mass, a CT scan or a MRI is usually made to determine the size, place of origin, the position to essential structures (like nerves and vessels) and eventually the presence of metastases. (25)

For staging and follow-up of GISTs a triple phase contrast-enhanced CT of the abdomen and pelvis is usually done. (27) On CT GISTs usually present as an exophytic, enhancing, inhomogeneous mass and due to very large size, the origin is not always definable. Contrast application is recommended to show the bowels margins, the tumor vessels, pattern of enhancement and to better evaluate liver metastases. (25) MRI is a good alternative, especially for the preoperative staging of rectal GISTs. (51)

Additionally, a fluorodeoxyglucose-positron emission tomography (FDG-PET) can be performed to give information about the metabolic activity and it can be used to evaluate the response of the GIST to TKIs early. (25,51)

1.2.3 Surgical management

Surgical intervention is suggested whenever feasible. The aim is a complete tumor resection (achieving macroscopic and microscopic negative margins) if this is not possible due to functional consequences a R1-resection (microscopic positive margins) in low-risk GISTs may be considered. All standard surgical procedures can be performed. However, laparoscopic surgery should not be used for large tumors due to the higher risk of tumor rupture. (27) Due to exceptionally rare involvement of the lymph nodes by GIST metastases in the sporadic setting, a lymphadenectomy is usually not necessary. Exceptions are the SDH-deficient GISTs in kids and young adults, where lymph node metastases can be observed. (54)

If a GIST is classified as inoperable, recurrent/metastatic or as high risk tumor after initial surgery, targeted therapy is applied based on the results of the molecular analysis. In case of intermediate risk the interdisciplinary tumorboard discusses the

pros and cons of adjuvant medical treatment for an individual patient and the treatment strategy is afterwards discussed with the patient. (See TKI Therapy) For small GISTs, surgery is usually the only suggested treatment. (51)

1.3 Pathology

1.3.1 Morphology

Gross appearance

GISTs arise in the bowel-wall, usually as a sharply demarcated lesion in the submucosa or subserosa. Most commonly they occur as a solitary lesion. Multiple lesions are often associated with tumor syndromes, for example familial GISTs, or in association with NF1. GISTs can range in size from very small lesions (some millimetre) to up to 40 cm in diameter, the median size is approximately 5 cm. (1,43)

The majority of tumors are well circumscribed, fleshy or fibrous on the cut surface. Sometimes cystic degeneration, calcification, gelatinous area or even necrosis can be found. Syndromic SDH-deficient GISTs have a characteristic multinodular/plexiform growth pattern. (14,55,56) In case of accompanying mucosal ulceration, gastrointestinal bleeding can occur. (1,25)

Microscopic appearance

Microscopically, GISTs have a spindle cell, epithelioid or mixed morphology in the vast majority of cases. Very rarely unusual morphologies including pleomorphic/poorly differentiated and dedifferentiated GISTs can occur. The mitotic activity needs to be evaluated in an defined area of 5 mm^2 as one essential parameter for risk stratification according to the Risk Stratification Schema proposed by Miettinen et al. (1,30)

Up to 70% of GISTs show a spindle cell pattern (31). These GISTs are composed of spindle cells with elongated nuclei and vesicular chromatin, inconspicuous nucleoli and syncytial eosinophilic cytoplasm (Figure 2-4). Sometimes they show paranuclear vacuoles. The spindle cells form short fascicles or whorls and the cellularity can vary from less to highly cellular. Stroma can be hyalinized to myxoid. In addition, calcification can be found. (1,25,57)

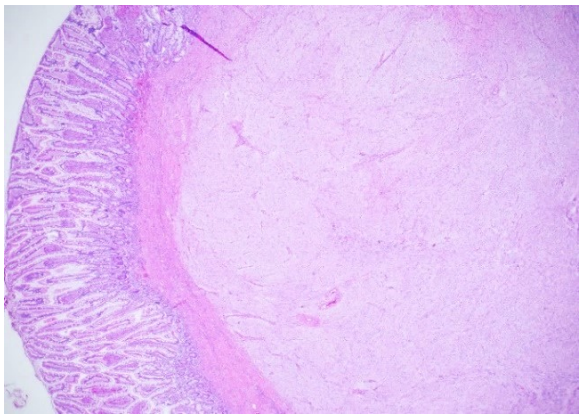

Figure 2: Small bowel GIST with spindle cell morphology, HE, Courtesy PD Dr. Brcic

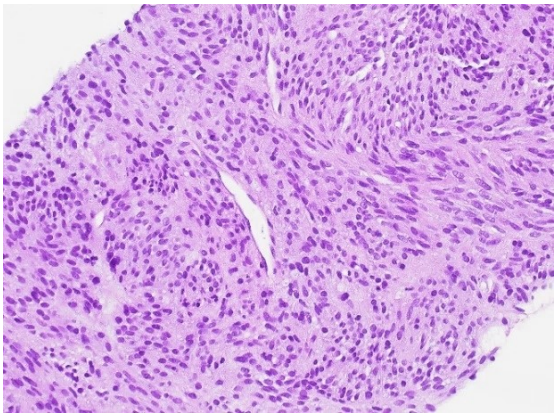

Figure 3: Biopsy of a GIST with spindle cell morphology, HE, Courtesy PD Dr. Brcic

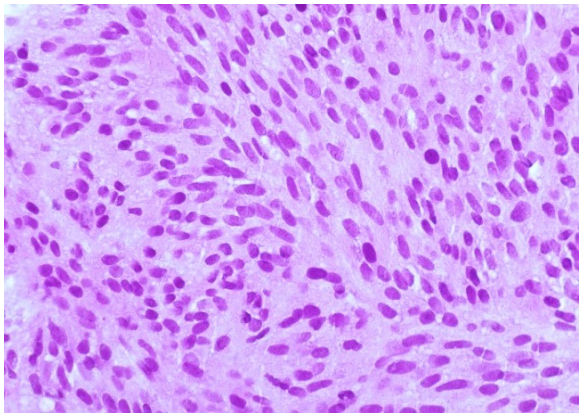

Figure 4: High power of a GIST biopsy with spindle cell morphology, demonstrating bland monotonous tumor cells with syncytial eosinophilic cytoplasm, HE, Courtesy PD Dr. Brcic

Skeinoid fibers (collagen fibres looking similar to bundles of wool) (Figure 5) and nuclear palisading can sometimes be found (the former most frequently found in the small bowel GIST) (Figure 6). (25,58)

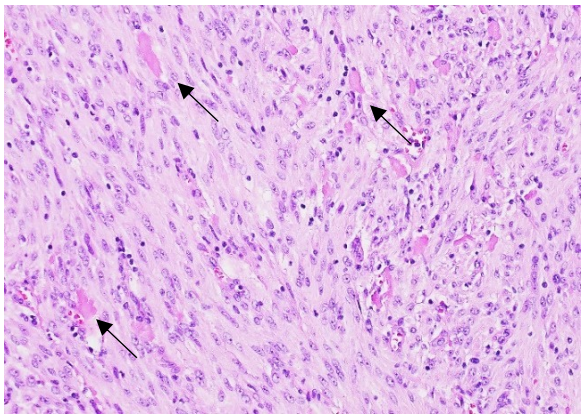

Figure 5: GIST with spindle cell morphology, skeinoid fibers, HE, Courtesy PD Dr. Brcic

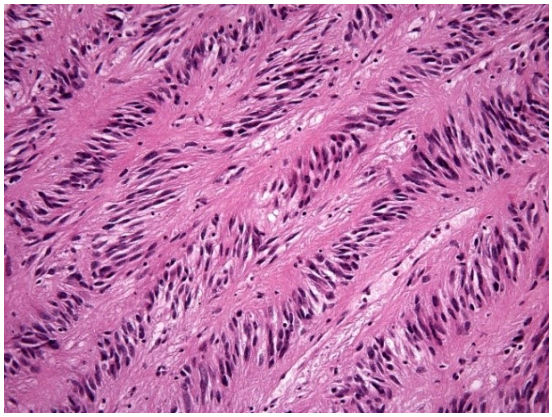

Figure 6: Nuclear palisading in a GIST, HE, Courtesy Prof. Liegl-Atzwanger

About 20% of GISTs show an epithelioid pattern. Epithelioid GISTs are characterized by epithelioid cells morphology, organized in nests or can show a diffuse pattern of growth (Figure 7-9). The cells can show more morphologic variation like plasmacytoid morphology, clear cytoplasm and vacuoles (Figure 8). Commonly, the cytoplasm is eosinophil or clear and the stroma is myxoid. (1,57,59) Interestingly, GISTs with *PDGFRA* mutations show more frequently an epithelioid pattern or mixed pattern and preferentially occur in the stomach. (60)

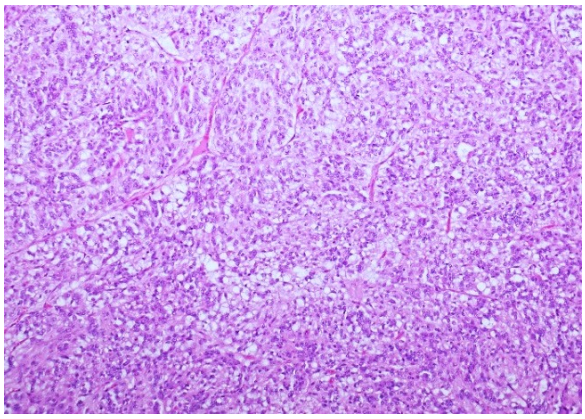

Figure 7: GIST with epithelioid cell morphology, HE, Courtesy PD Dr. Brcic

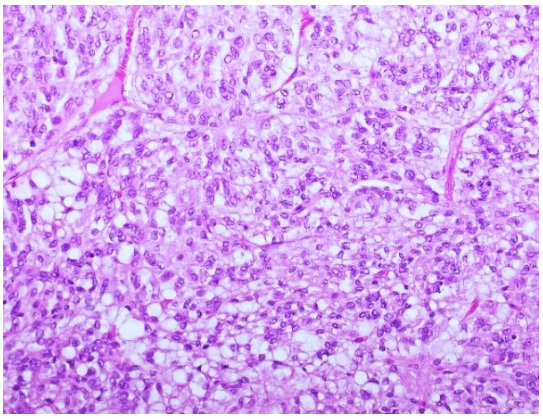

Figure 8: GIST with epithelioid cell morphology and vacuolisation of the cytoplasm, HE, Courtesy PD Dr. Brcic

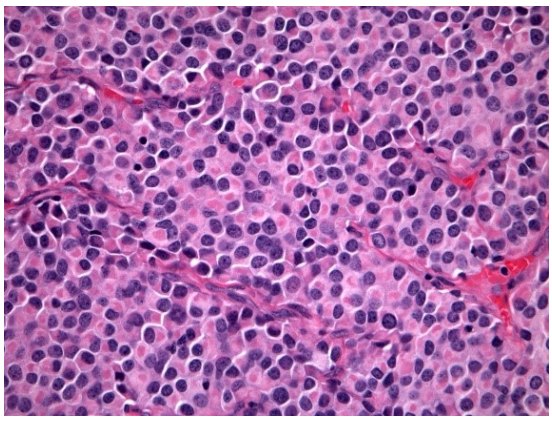

Figure 9: GIST with epithelioid/plasmacytoid cell morphology, HE, Courtesy Prof. Liegl-Atzwanger

The remaining 10% are composed of a mixture of spindle and epithelioid cells and are called the mixed type. (57,59)

Rare morphologic subtypes

Pleomorphic/poorly differentiated/dedifferentiated GIST

In general, nuclear pleomorphism is not a feature of GISTs. However, unusual morphologies like pleomorphic, poorly differentiated or even dedifferentiated GIST have been described. (Figure 10) Dedifferentiated GISTs occur very rarely. In this case an abrupt transition of a GIST with usual morphology in a highly atypical sarcomatous tumor occurs. The dedifferentiated area lacks KIT and DOG1 expression. In contrast pleomorphic GISTs are of highly atypical morphology, but express at least focal KIT by immunohistochemistry. (1)

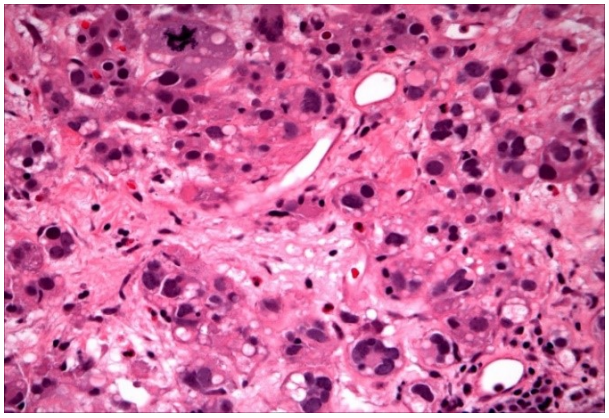

Figure 10: Pleomorphic GIST, HE, Courtesy Prof. Liegl-Atzwanger

GIST after TKI treatment

After TKI treatment the morphology of the GIST may change. For instance, the spindle cell morphology can change into epithelioid cell morphology, stroma and cellularity may change and even a rhabdomyosarcomatous de-differentiation has been described. (Figure 11) (57,61–63)

GIST with rhabdomyosarcomatous de-differentiation show a positivity for desmin and myogenin in IHC, while there is a loss of KIT expression. Tumors with rhabdomyosarcomatous de-differentiation retain the primary *KIT* mutation but lack secondary resistance mutations usually seen in GIST after treatment. Rhabdomyosarcomatous de-differentiation seems to be one rare form of resistance mechanism during treatment. In general, these GISTs show an aggressive behaviour and tend to recur more frequently and earlier. (62,64,65)

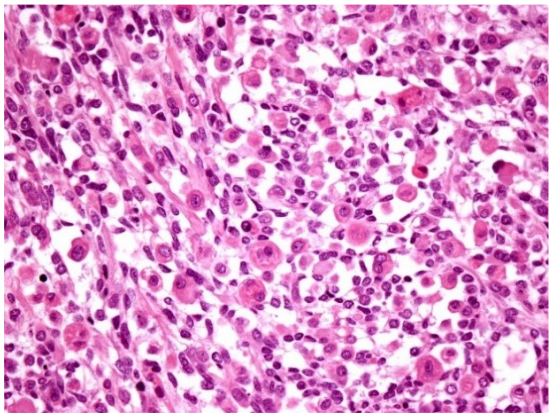

Figure 11: GIST with rhabdomyosarcomatous de-differentiation, HE, Courtesy Prof. Liegl-Atzwanger

NF1 related GISTs

GISTs associated with NF1 typically show a spindle cell pattern or sometimes even a mixed pattern with nuclear palisading combined with extracellular skeinoid fibers. Mostly they show a low mitotic rate. (3,66)

Paediatric SDHB deficient GIST

GISTs related to CT and CSS show typical morphologic features. They have a multinodular or plexiform growth pattern and are commonly composed of epithelioid cells (Figure 12-15) or a mixture of epithelioid and spindle cells with surrounding cytoplasm. (14,55,56)

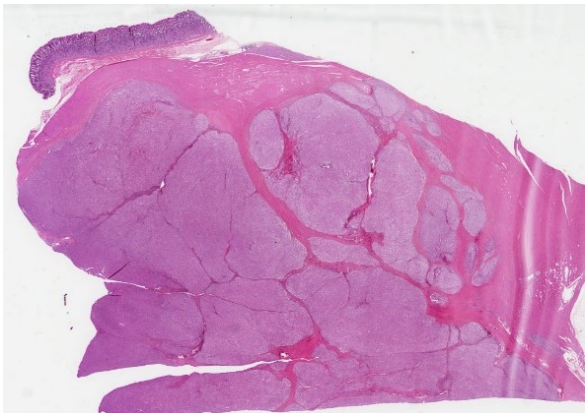

Figure 12: SDHB-deficient GIST with typical plexiform morphology, HE, Courtesy Prof. Liegl-Atzwanger

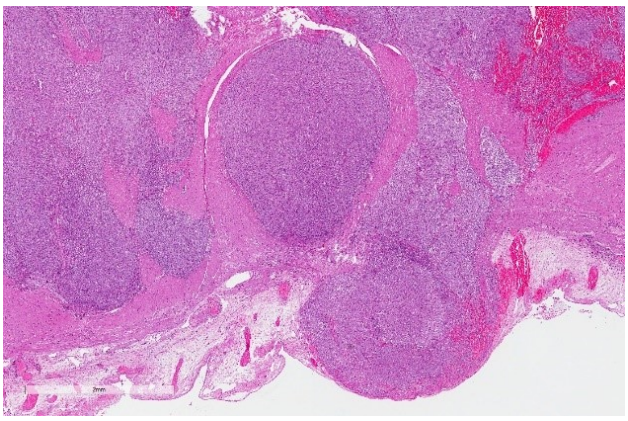

Figure 13: SDHB-deficient GIST with typical multinodular/ plexiform morphology, HE, Courtesy Prof. Liegl-Atzwanger

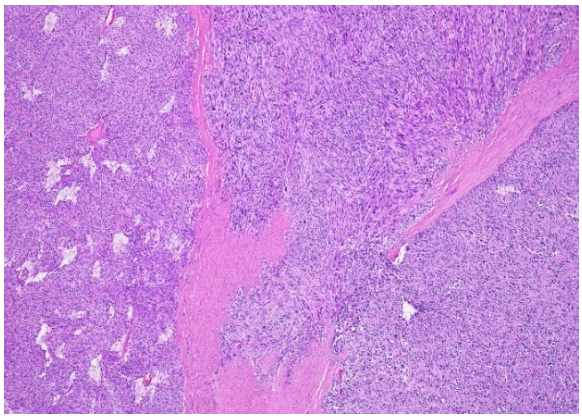

Figure 14: High power of SDHB-deficient GIST, HE, Courtesy PD Dr. Brcic

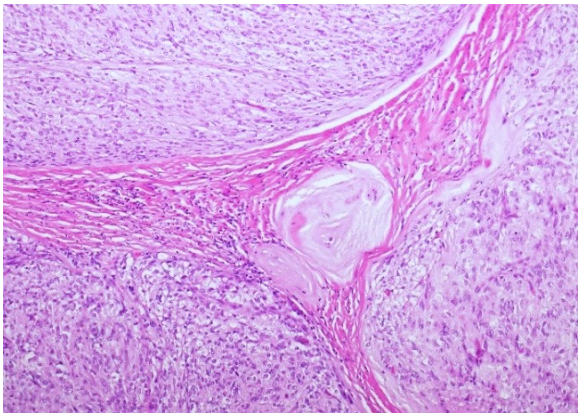

Figure 15:SDHB-deficient GIST, HE, Courtesy PD Dr. Brcic

The tumors typically lack SDHB expression by immunohistochemistry (SDHB deficient GIST). Lack of SDHB expression is associated with a genetic defect of one of the four SDH subgroups. Additional lack of SDH-A expression is highly suspicious for mutations within the SDHA subunit. (14,15,67)

1.3.2 Immunohistochemistry

Immunohistochemistry is essential in the diagnosis of GIST. The best diagnostic markers for GIST are KIT and DOG1.

Most of the GISTs (95%) express KIT (CD117) in a diffuse cytoplasmatic or membranous staining (rarely a dot-like (Golgi) pattern). (1,3,25) The KIT staining in spindle cell GISTs is usually more intense. (25) In contrast, *PDGFRA* mutated tumors show weak or even negative staining for KIT. (1,25,68) (Figure 16, 17)

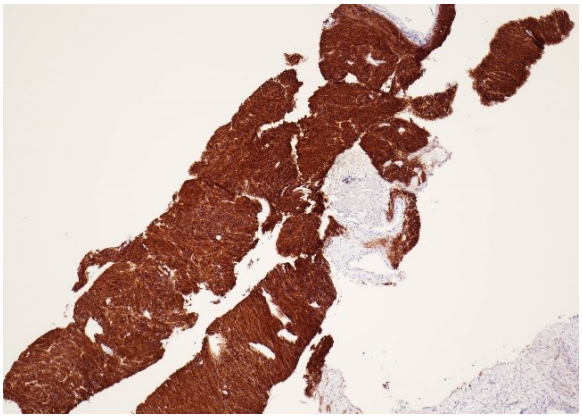

Figure 16: Immunohistochemistry with KIT (CD117), strong cytoplasmatic staining, Courtesy PD Dr. Brcic

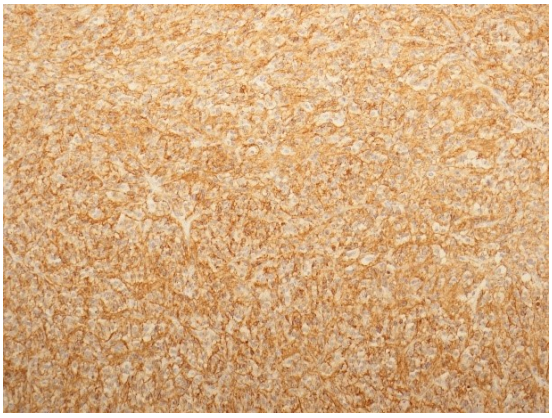

Figure 17: Epithelioid GIST showing a less intense membranous and cytoplasmatic staining with KIT (CD117), Courtesy PD Dr. Brcic

DOG1 (ANO1 or anoctamin 1), a chloride channel protein, is another diagnostic marker for GISTs expressed in approximately 98% of cases regardless of the triggering mutations and is helpful to detect KIT-negative-GISTs. (1,13,69) (Figure 18, 19)

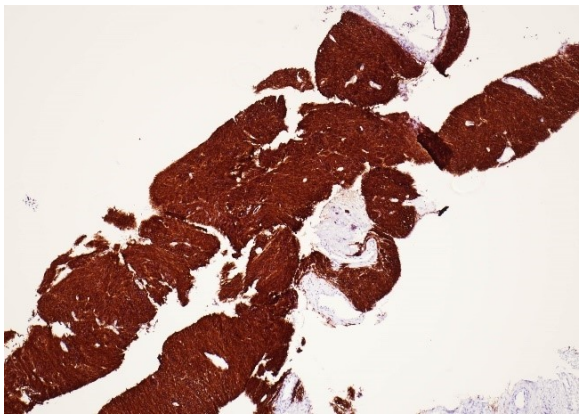

Figure 18:Immunohistochemistry with DOG1, strong cytoplasmatic staining, Courtesy PD Dr. Brcic

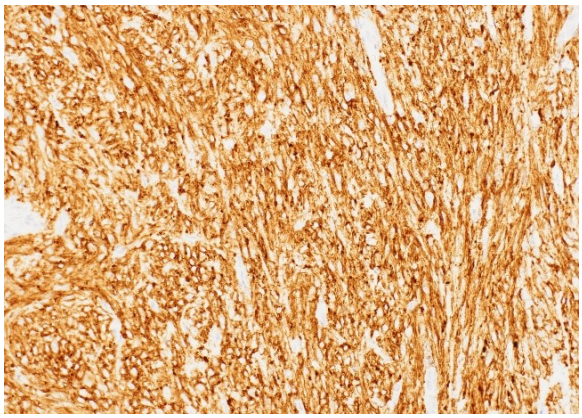

Figure 19: Membranous and cytoplasmatic staining of DOG1 in a small bowel GIST, Courtesy PD Dr. Brcic

KIT and DOG1 are very reliable markers in GIST diagnoses. However, one should bear in mind, that KIT can be expressed in epithelial cells and cerebellar neurons. Occasionally, KIT can be positive in sarcomas (particularly in Ewing sarcoma and angiosarcoma), carcinomas (such as small cell carcinomas, adenoid cystic carcinomas or thymic carcinomas), seminomas and melanomas. (70) Therefore a panel of IHC stains in context with the clinical history need to be used to diagnose GIST.

SDHB is a helpful marker to identify *SDH* mutant GIST and therefore it is used in cases of syndromic GISTs, as the loss of expression demonstrates destabilisation of any SDH subunit (SDHA-D). (14,15,67) (Figure 20).

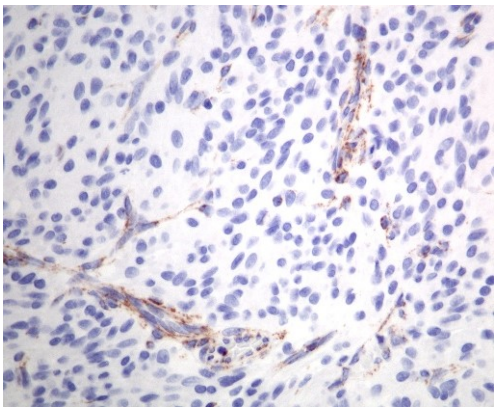

Figure 20: IHC SDHB-deficient GIST Immunohistochemistry shows SDHB loss in the tumor cells (endothelial cells stain positive and serve as an internal control). Courtesy Prof. Liegl-Atzwanger

In addition, other markers like CD34-antigen, smooth-muscle-actin (α-SMA), S100-protein and desmin have been described to be variably expressed (but are not specific). (25,67)

1.3.3 Prognosis and Risk Stratification

GISTs are classified into none, very low-, low-, intermediate-, and high-risk tumors. (17,71) Prognosis of GISTs varies and depends on the tumor size, the anatomic location, mitotic activity and presence of tumor perforation. (1,48,72) In general, each GIST has been proposed to have a malignant potential, regardless of any prognostic factors. However, small (<2cm) tumors usually show a benign clinical course and are also referred to as none risk GIST. (73–75).

In 2002, Fletcher et al. published the first risk classification system, which is called the “NIH (national institute of health) classification”. This classification depends on two parameters (1) the tumor size and (2) mitotic index. It was the first established scheme to predict the biologic behaviour of the GIST. (17,76) In 2006, Miettinen et al expanded the initial risk classification scheme by including a third parameter, namely the tumor location. The Miettinen risk stratification scheme for GISTs includes five risk groups. (25,30,76) The “modified NIH classification” is identical to the Miettinen risk of stratification and serves as a decision aid concerning the requirement of adjuvant therapy. (33,77) Another indicator for risk of recurrence is tumor rupture. Intraoperative tumor rupture puts patients into the high risk group, irrespectively of tumor size and mitotic count per $5mm^2$. (78,79) Other parameters like the Ki-67 index have been proposed, but are not frequently used. (25,67,80) Additionally, nomograms from Gold and a genomic index from Lartigue and colleagues were developed to give advice for potential treatment modalities. (25,81,82) Generally, high risk GISTs have a significant potential for recurrence. They do not tend to recur to their previous origin, instead, they appear as metastases mainly in the liver, inside the abdomen or even both. (25,28,29)

An additional prognostic factor is the type of mutation that predict the response to TKIs. In general, *KIT*-mutated GIST are more aggressive than *PDGFRA*-mutated GISTs. (25,83,84) Over the years, it became obvious that mutational analysis has a great impact on treatment response and prognosis. For example, Exon 11 mutated GISTs respond to 400 mg Imatinib whereas *KIT* exon 9 mutated GISTs need to be

treated with 800 mg Imatinib. In contrast, GIST demonstrating the *PDGFRA* mutation D842V are Imatinib resistant. Therefore, mutational analysis is essential before medical treatment is given. (25,85)

According to Rossi et al, GISTs can be divided into three risk-groups depending on their mutation. Group 1 has the lowest risk and thus the highest overall survival and contains GISTs with mutations in *KIT* exon 13 and *PDGFRA* exon 12. GISTs with mutations in *KIT* exon 17, *PDGFRA* exon 14 and 18 (D842V) are found in the second group with an intermediate-risk, while mutations in *KIT* exon 9 and 11 as well as other mutations in *PDGFRA* exon 18 show a high risk. (86,87) (See Table 2)

Risk Group	**Mutations**
Group 1 (low risk)	*KIT 13* *PDGFRA 12*
Group 2 (intermediate risk)	*KIT 17* *PDGFRA 14* *PDGFRA 18 D842V*
Group 3 (high risk)	*KIT 9* *KIT 11* *PDGFRA 18*

Table 2: Risk Groups depending on mutations (86,87)

In summary, risk stratification according to Miettinen is essential for patients´ treatment and prognosis. The evaluation depends generally on the tumor size, the anatomic location and mitotic activity. (1,30,48,72) The risk of progression varies from no risk of metastasis to a high risk to metastasize with aggressive behaviour.

Tumor Parameters		*Location in relation to tumor progression in %*			
Mitotic Index	*Size*	*Stomach*	*Duodenum*	*Jejunum/Ileum*	*Rectum*
≤ 5 per 5mm^2	≤ 2 cm	None 0%	None 0%	None 0%	None 0%
	>2 ≤ 5cm	Very low 1,9%	Low 4,3%	Low 8,3%	Low 8,5%
	> 5 ≤ 10 cm	Low 3,6%	Moderate 24%	(Insuff. data)	(Insuff. data)
	>10 cm	Moderate 10%	High 52%	High 34%	High 57%
> 5 per 5mm^2	≤ 2cm	None †	High †	(Insuff. data)	High 54%
	>2 ≤ 5cm	Moderate 16%	High 73%	High 50%	High 52%
	> 5 ≤ 10 cm	High 55%	High 85%	(Insuff. data)	(Insuff. data)
	>10cm	High 86%	High 90%	High 86%	High 71%

Table 3: Risk Stratification according to Miettinen 5mm^2 corresponding to 50HPF using old microscopes (30)

Small, usually incidental micro and mini GISTs have a very good prognosis. (48)

1.4 Molecular Classification

Up to 80% of tumors harbour activating mutations in receptor tyrosine kinase gene *KIT*, while about 5-10% are triggered by activating mutations in *platelet-derived growth factor alpha (PDGFRA*). (13) The remaining percentage is called the wild-type (WT) or *KIT/PDGFRA*-negative GISTs. (See Figure 21) The *KIT* and *PDGFRA* wt group of GIST is a heterogeneous group of tumors with different clinical phenotypes and molecular characteristics.

Recent advances in molecular pathology led to a better sub-classification of GISTs into an SDH-competent and an SDH-deficient group by immunohistochemistry, regardless of the underlying genetic changes (See Table 4). (1,14,15)

The SDH-competent tumor group includes: tumors with *KIT, PDGFRA, BRAF* and *NF1* mutations followed by rare mutations of *ARID1A, ARID1B, CBL, FGFR1, NRAS, HRAS, KRAS, MAX, MEN1* and *PIK3CA*, and recently described gene fusions, such as *KIT–PDGFRA* and *ETV6–NTRK3*. (88–92)

The SDH-deficient tumor group includes GISTs in association with CSS or CT as well as sporadic paediatric and so called “young adult” GISTs. (93–96)

Molecular Subgroup	Subgroups	Location
KIT mutation		
Exon 8		
Exon 9		small and large bowel
Exon 11		all locations
Exon 13		all locations
Exon 17		all locations
PDGFRA mutation		
Exon 12		all locations
Exon 14		stomach
Exon 18 D842V		stomach
Exon 18 others		all locations
KIT/PDGFRA "wild-type"		
SDHB IHC retained	*NF1* mutation	small bowel
(SDH-competent)	*RAS* (*KRAS*/*NRAS*) mutation	all locations
	BRAF mutation	stomach
	other mutations/fusions	all locations
SDHB IHC lost	*SDHA, SDHB, SDHC* or *SDHD* mutations(Carney-Stratakis syndrome)	stomach
(SDH-deficient)	associated with Carney triad	stomach
	sporadic pediatric GIST	stomach

Table 4: Correlation between mutation and location

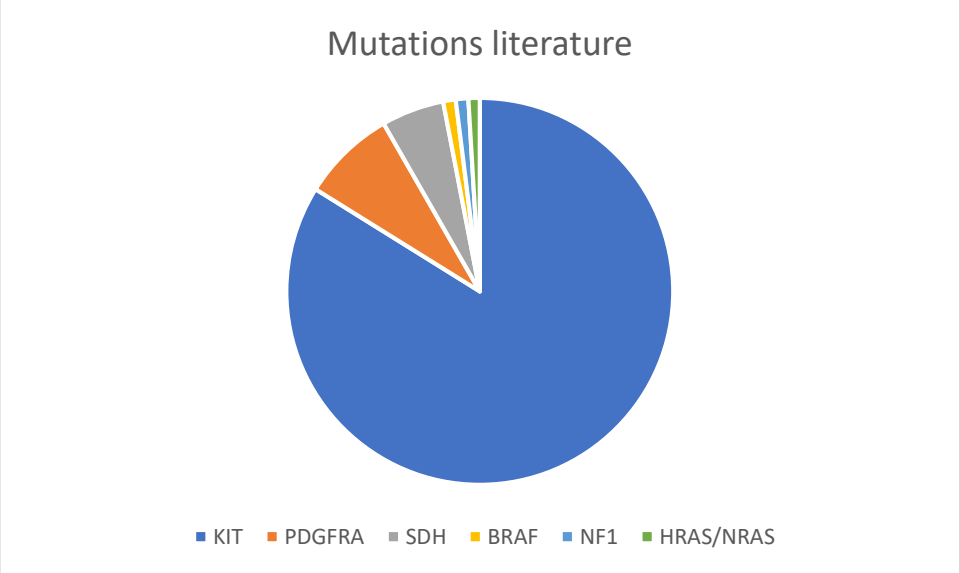

Figure 21: Mutations in GIST

1.4.1 SDH – competent group of GIST

KIT Mutations

KIT is a proto-oncogene and encodes the 145-kDA receptor tyrosine kinase (RTK) *KIT* – a transmembrane receptor with tyrosine kinase activity. It is a type III transmembrane receptor and consists of an intracellular tyrosine kinase domain, a juxta-membrane domain, a transmembrane domain and an extracellular domain (Figure 22). (15,97) Physiologically, KIT plays a role in cell proliferation and differentiation. Genetic changes in KIT are known as initiating events in tumor pathogenesis for example in GIST, acute myeloid leukaemia or acral melanoma. (15,98) Mutations can cause constant activation of the tyrosine kinase (TK) KIT. Consequently, substrate proteins are phosphorylated without the presence of the corresponding ligand, known as the stem cell factor. This influences the intracellular signal transduction cascades, such as Ras/Raf/MAPK and PI3K/AKT pathways and leads to a constant activation autonomously. (99,100) This causes an uncontrolled and increasing proliferation and inhibition of apoptosis. (15,97,98)

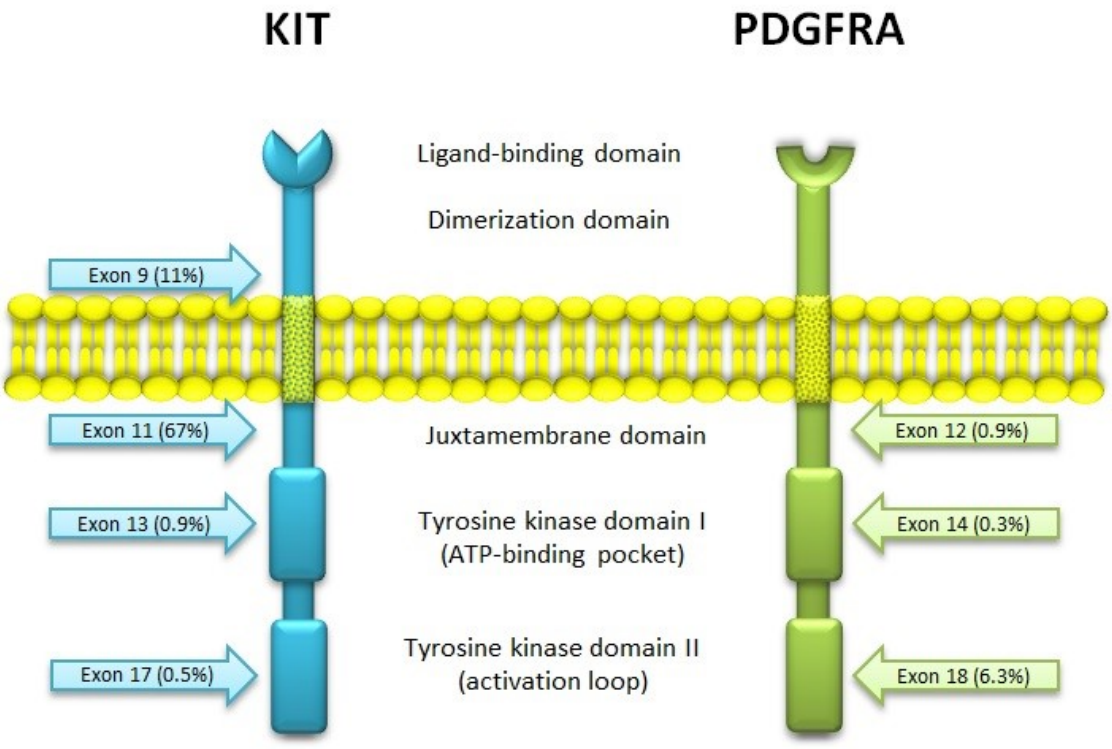

Figure 22: Mutations in KIT and PDGFRA, (adapted from (87))

Mutations in exon 11 are most frequently found and are affecting the intracellular juxta-membrane domain, while mutations in exon 9 are found in 10% and are interfering with the extracellular parts of the TK KIT (Figure 22). Exon 13

corresponds to the ATP binding packet of the tyrosine kinase domain while exon 17 corresponds to the kinase activation loop. (31,100)

Mutations in exon 11 are mostly caused by in-frame deletions within codon Gln550 and Glu560, also known as hot spot regions, missense-point-mutations mainly affecting codon Trp557, Val559, Val560 or Leu576 and duplications (especially in the 3´end). (15,31,101) Exon 9 mutations mostly arise from duplications of Ala502-Tyr503 and are commonly located in the intestine. (15,68)

Mutations in exon 13 and exon 17 are very rare. Exon 13 mutations are divided into primary (K642E) and secondary mutations (V654A and T670I). The secondary mutations are leading to resistance against the TKI Imatinib. In addition to the most common secondary mutations, additional point mutations namely R634W and N655T have been described. (102–105)

GISTs with primary mutation in exon 17 are usually caused by changes within codons 816, 820 and 823 and are located in the small intestine. Secondary mutations like substitutions in D816V and N822V are also causing Imatinib resistance. (102,106–108)

PDGFRA Mutations

The *PDGFR* is a typical tyrosine kinase receptor and together with their ligand (PDGF), they are responsible for many physiological processes of growing and development in the human body. The receptor is similarly constructed as the c-kit receptor and contains an intracellular TK domain, a transmembrane domain and an extracellular ligand binding domain. *KIT* (see above "*KIT*-mutations") and *PDGFRA* are both located on chromosome 4q11-q12. (5,109) The PDGFs are divided into five isoforms: *PDGFAA, PDGFAB, PDGFBB, PDGFCC* and *PDGFDD,* and bind to the receptors PDGFR alpha and PDGFR beta. Particularly, the PDGFR-alpha is important for lung, skin, intestine, skeleton, gonads and is an essential factor in the embryonic development. Normally, after binding to the receptor, phosphorylation activates signal cascades (Ras/Raf/MAPK and PI3K). (110,111) Due to genetic aberrations, the *PDGF* signal is active unrestrained in neoplastic cells, which leads to a ligand-independent phosphorylation and therefore uninhibited proliferation. Additionally, it plays a role in the epithelial-mesenchymal-transformation. (110)

The mutations are mostly localized in exon 18 or 14 and can rarely occur in exon 12 (Figure 22). (3,72) Nearly every *PDGFRA*-GIST shows an epithelioid pattern and is located in the stomach. (68,101)

Exon 18 codes for the activation loop in the tyrosine kinase domain and represents about 80% of the *PDGFRA* related GISTs. Mostly, these are missense mutations, that result in the substitution of Asp to Val in codon 842 (D842V). Further mutations are described in exon 14 and exon 12. Exon 14 correlates to the TK domain and exon 12 to the juxta-membrane. (68,101)

BRAF GIST

GISTs caused by *BRAF V600E* are found in about 1% of all GISTs. *BRAF V600* correlates with mutations in codon 600 – mostly somatic point mutations, which induces a change in the protein kinase B-RAF. In *BRAF V600E* the amino acid valine is replaced with glutamic acid. *BRAF* encodes for the cytoplasmic serine/threonine kinases, and is therefore responsible for cell growth, proliferation, migration, survival and differentiation. Many tumors, for example melanomas, colorectal cancer or GISTs, are associated with a *BRAF* mutation. Due to this mutation the mitogen-activated protein kinase (MAPK) pathway is ligand-independently activated, just as in mutations driven by *KIT* or *PDGFRA* – merely at a later point of the signal transduction cascade. (112–114) In particular, mutations driven by *BRAF* V600E provoke the activation of the p44/42 MAPK and AKT cascades. (112)

NF1 GIST

The GISTs assigned to Neurofibromatosis type 1 (NF-1), also known as Morbus Recklinghausen, are caused by the absence of neurofibromin 1, a tumor suppressor located on chromosome 17q11, which appears typically in neurons, glial, Schwann-cells and in an early stage of melanocytes' development. The absence of neurofibromin leads to the stimulation of the RAS/RAF/MAP-kinase pathway and consequently to unrestrained cell growth. Normally, neurofibromin stimulates the transformation of RAS into an inactive form of it, hence it inhibits the cell growth and proliferation. (66,115,116) NF1 is an autosomal dominant multisystem disorder accompanied by multiple neurofibromas, multiple café-au-lait spots and Iris Lisch nodules. Affected people are at higher risk to develop either malignant or benign

tumors concerning every organ system. (116,117) Besides, it has been assumed that sporadic wt-GISTs of adults may develop from somatic inactivating mutations of *NF1,* which is why they show no other characteristics of Neurofibromatosis. (118–120)

Usually, GISTs driven by *NF1* present a rather benign behaviour and harbour neither *KIT* nor *PDGFRA* mutations. (66,119)

Very rarely, GISTs are caused by *HRAS*, *NRAS, KRAS, PIK3CA* or other mutations. (1,91,92)

1.4.2 The SDH-deficient group of GIST

Succinate dehydrogenase (SDH) is an enzyme complex, found in the inner mitochondrial membrane of eukaryotes.
The complex is composed of 4 subunits and is responsible for the electron transport chain and essential in the Krebs and the citric acid cycle. SDHA is accountable for the transformation from succinate to fumarate, while SDHB is important for the electron transport chain and the following oxidation of ubiquinone. SDH-C and -D are responsible for the membrane- anchoring of the complex. (15,56,121,122)

Functional disruption of this complex can be caused by genetic and/or epigenetic changes, as seen in GIST. (123) Inactivity of the SDH complex causes succinate accumulation and inhibits the hydroxylation of hypoxia-inducible factor 1 alpha (HIF-1 alpha) by the prolyl 4-hydroxylases (PHD). As a consequence, HIF-1 alpha is not degraded by pVHL and this leads to an overexpression of HIF-1 alpha. HIF-1 alpha is then translocated in the nuclei and connects with HIF-1 beta to HIF-1. HIF-1 induces the transcription of vascular endothelial growth factor receptor (VEGFR) and insulin-like growth factor 1 receptor (ILGF1R) and is generally an important factor in cell growth and proliferation. (41,56,93,124–127) Especially, paediatric GISTs mostly present an overregulation of ILGF1R, probably drawn by the upregulation of HIF-1 alpha. (41,93,128) ILGF1R induces down streaming cascades like insulin receptor substrate (IRS) and phosphoinositide 3-kinases (PI3K), which increase the mitotic activity and proliferation – so when ILGF1R is overregulated, this leads to uncontrolled growth. (129–131)
Not only the inactivity of the SDH complex can be responsible for succinate accumulation also epigenetic modification plays an important role. Epigenetic

modifications are genetic transformations in the transcriptome, such as DNA methylation or posttranslational changes of histones. Accumulation of succinate has an inhibiting effect on the histone demethylase JMJD3, which leads to changes in histone H3. As a consequence of hypermethylation SDH-deficient GISTs may occur. (56,132–134)

About 30% of all SDH-deficient GISTs are attributable to mutations in *SDHA*, which are mostly associated with germline mutations and less with sporadic mutations. They typically appear in young adults in the stomach. (56,135–137)

GISTs in association with CT and CSS

CSS is associated with development of paraganglioma and gastric GIST during childhood and adolescence, triggered by germline mutations. CT is a syndrome most frequently occurring in young women and is associated with the paraganglioma, gastric GIST and pulmonary chondroma. CT is characterized by the increased methylation of the genome, but especially there is a downregulation of SDH due to epigenetic silencing and hyper-methylation of the SDHC gene. In general, in CT no *SDH* mutations are observed, hence the SDH deficiency is caused by epigenetic changes, which lead likewise to an activation of the HIF pathway. (39–42,138,139)

1.4.3 Quadruple-negative GISTs

Quadruple negative GIST is a term used for a group of GISTs lacking *KIT*-, *PDGFRA*-,*RAS*- and *BRAF*-mutations and SDH-deficiency.

1.4.4 Fusions

Recently, next generation sequencing (NGS) detected fusions in GIST. Brenca et al identified a case of rectal GIST with a gene fusion amongst exon 4 of *ETS* variant gene 6 (*ETV6*) and exon 14 of *neurotrophin tyrosine kinase receptor 3* (*NTRK3)* by transcriptome sequencing. In adjacent genes various other fusions, such as *POLA2–CDC42EP2* were detected, too. Of note, the *ETV6-NTRK3* fusion is also found in other tumors (like infantile fibrosarcomas, leukaemia or thyroid cancer). (94,95,140,141) It is assumed, that these fusions activate the IGF1R downstream cascade. (95) Detection of *NTRK* fusions are essential as NTRK inhibitors, such as Entrectinib or Larotrectinib, are available for targeted treatment approaches and have been shown to be highly effective in various tumor entities with *NTRK* 1,2,3

gene fusions. Detection of *NTRK*- fusion by RNA- Sequencing is an agnostic marker for initiation of treatment with TRK- inhibitors. (142)

1.5 Location

The most common location for GIST is the stomach where all different mutations can occur. Interestingly, GISTs harbouring *KIT* mutations in exon 9 are commonly located in the small bowel and are often classified as high-risk tumors. (3,70) GISTs with *PDGFRA* – mutations occur commonly in the stomach. (60)

While *NF1* driven GISTs and GISTs with *BRAF*-mutations tend to develop in the small bowel, SDH-deficient GISTs appear exclusively in the stomach, especially in the antrum/distal stomach. Even the location of the metastasis is depending on the mutation. Sporadic GISTs with their typical mutations in *KIT* or *PDGFRA* tend to metastasize in the liver or peritoneum (1,3,118), whereas SDH-deficient GISTs (CSS and CT) occur exclusively in the stomach and show delayed metastases to common sides like liver and abdomen but also to lymph nodes. (14,55,56,123)

1.6 Differential diagnosis

Differential diagnosis includes various benign and malignant neoplasms with spindle and epithelioid morphology listed in the Table 5. (25,67)

	Epithelioid Morphology	***Spindle Morphology***
Benign	PEComa (*)	Leiomyoma
	Glomus tumor (*)	Schwannoma
		Abdominal fibromatosis (****)
		(desmoid tumor)
		SFT (***)
		PEComa (*)
		IMTs (**)
		IFPs
		Plexiform fibromyxoma
Malignant	Metastatic Melanoma	Leiomyosarcoma
	Clear cell sarcoma	Liposarcoma
	Epithelioid leiomyosarcoma	Clear cell sarcoma
	EHE	Synovial sarcoma
	Sarcomatoid carcinoma	Metastatic Melanoma
	NET	Sarcomatoid carcinoma
	Adenocarcinomas	MPNST
	Biphasic synovial sarcoma	
	MPNST	

Table 5 Differential Diagnoses

Legend: PEComa- perivascular epithelioid cell tumor, SFT – solitary fibrous tumor, IFP. Inflammatory fibroid polyp, MPNST- malignant peripheral nerve sheath tumor; IMT-inflammatory fibroblastic tumor; EHE-epithelioid hemangioendothelioma; NET-neuroendocrine tumor; *malignant examples exist; ** intermediate category rarely metastasizing according to the WHO soft tissue and bone tumors 2020; *** risk stratification according to the WHO soft tissue and bone tumors 2020 has to be performed; **** intermediate category, recurring;

1.6.1 Benign and epithelioid morphology

Perivascular epithelioid cell tumors (PEComas) are usually benign tumors, comprised of epithelioid and spindle cells. The tumor cells are typically related to the vessel walls, and characterised by clear or granulated eosinophilic cytoplasm. (143) Typically, they are positive for α-SMA, desmin, Melan A and HMB45 and lack KIT and DOG1 staining. (67)

Glomus tumors are usually benign mesenchymal tumors of smooth muscle cells. Unusually, they occur in the GI- tract, predominately in the stomach. Their cells have nice cell borders and the stroma varies from hyaline to myxoid. Immunohistochemically, they express α-SMA. (67,144)

1.6.2 Benign and spindle morphology

Leiomyomas are benign, mesenchymal tumors with a smooth muscle differentiation and show spindle cell morphology. The spindle cells are arranged in fascicles. The nuclei of the cells are uniform, appear as cigar shaped and have an eosinophilic cytoplasm. Stroma hyalinization can be observed. Leiomyomas can occur throughout the GI-tract. Immunohistochemically, the tumor cells express smooth muscle markers (SMA, desmin, h-caldesmon), but are negative for the typical GIST-markers: KIT and DOG1. Of note, there may be some occasional KIT- positive staining due to KIT-positive mast cells. (67,145,146)

Schwannomas are the most common benign, peripheral nerve tumors in adults. They originate from the nerve sheath and are composed of Schwann-cells. In general, schwannomas are spindle cell neoplasms consisting of hypercellular areas (Antoni A) with Verocay bodies and myxoid hypocellular areas (Antoni B). (147,148) In contrast, schwannomas in the stomach typically only show Antoni A areas and are surrounded by a cuff of lymphocytes at the edge of the tumor, mimicking a lymph node tissue. On IHC, spindle cells show diffuse positive straining with S100 protein and SOX10, while KIT, DOG1, desmin, SMA and ALK are negative. (67)

Intraabdominal fibromatosis (desmoid tumor) is a proliferation of fibroblasts/myofibroblasts. This tumor has the tendency to recur but lacks metastatic potential. According the WHO Soft Tissue and Bone this tumor is put into the intermediate risk category. (149–152) Histologically, the tumor is composed of long fascicles of spindle cells with elongated vessels. Typically, the spindle cells demonstrate an infiltrative growth pattern. Keloidal hyalinisation and fasciitis like morphology can be seen. Immunohistochemically, the cells show nuclear straining for β-catenin. In addition, SMA is frequently expressed. Mutations in the CTNNB1 gene are usually seen. (67,151,153,154)

Solitary fibrous tumors (SFT) are mesenchymal neoplasms of the intermediate risk category (rarely metastasizing), that can develop in soft tissue or visceral tissue.

Histologically, the tumors are comprised of spindle cells with sparse cytoplasm and small elongated nuclei, reminiscent of fibroblasts. In malignant tumors pleomorphism with atypia and increased mitotic activity (>4/10HPF) can be seen. According to the new WHO soft tissue tumors 2020 a new risk stratification scheme has been implemented depending on age, tumor size, mitotic activity and necrosis (155). The cellularity varies from hypo - to hypercellular and is characterised by thick hyalinized vessel walls with staghorn-like configuration. IHC is almost always positive for CD34 and STAT6 (nuclear staining). The tumors show *STAT6-NAB2* fusions. (67,156–158)

Inflammatory myofibroblastic tumor (IMT) is a myofibroblastic proliferation accompanied by inflammatory cells (lymphocytes, plasma cells and eosinophilic granulocytes). They occur mostly in children or young adults. Histologically, they have a spindle cell morphology and varying morphological patterns, one with an incoherent myxoid architecture, the other with a compact structure and the third one with hyalinized stroma and less cellularity. (159,160) IMTs are typically positive for α-SMA and sometimes even for ALK, whereas the other markers, like KIT, DOG1, S100 protein and β-catenin are negative. (67,160) On molecular level, different fusions have been described, most commonly involving *ALK* and *ROS1*. (161–163)

Inflammatory fibroid polyp (IFP) a mesenchymal polyp that develops in the submucosa of the gastrointestinal tract. The infiltration of the muscularis propria is also described. IFP often harbour *PDGFRA*- mutations. (164–168) Microscopically, they consist of spindle and stellate cells infiltrated by eosinophilic granulocytes. Particularly, blood vessels are surrounded by spindle cells in the formation of an onion skin. Immunohistochemically, CD34 and α-SMA are frequently expressed. Correlation of morphology and mutation data by pathologists is essential to differentiate IFP from GIST due to overlapping mutational profile. (67,169)

Plexiform fibromyxoma also known as plexiform angiomyxoid myofibroblastic tumor are benign mesenchymal tumors located in the antrum or pylorus of the stomach. Microscopically, they are multinodular, involving the bowel wall with plexiform appearance and composed of spindle cells expressing SMA and CD10. Vascular invasion can be seen without any prognostic impact. (154,170–172)

1.6.3 Malignant with epithelioid or mixed epithelioid/spindle cell morphology

Metastatic melanoma typically appears as an isolated lesion in the proximal part of the stomach infiltrating the submucosa or muscularis propria. The mucosa can be intact, pigmented or ulcerated. (154,173–176) In the intestine, they usually present as nodules or polyps and infiltrate in the mucosa. On histology, hyperchromatic, epithelioid to spindle cells with conspicuous nucleoli are found and show positive reaction with S100, SOX10, HMB45 and Melan A. Misinterpretation might occur by KIT expression, which can be seen in a subset of melanomas. (154,177)

Clear cell sarcoma like tumor of the GI-tract occurs usually in the small bowel and consists of a mixture of spindle and epithelioid cells. They tend to metastasize in the liver or mesenteric lymph nodes. They express S100 and SOX10 and demonstrate *EWSR1 (or FUS) -CREB1* fusion gene. Fusion analysis is essential to confirm the diagnosis. (154,178,179)

Sarcomatoid carcinoma (synonym: spindle cell carcinoma or carcinosarcoma) is a carcinoma, that shows two components: one with sarcomatoid differentiation comprising of spindle cells and a better differentiated component with spindle and epithelioid cells. Nevertheless expression of cytokeratin is seen. (154,180,181)

Synovial sarcomas can be monophasic (just spindle cells) or biphasic composed of spindle cells and/or epithelioid cells admixed with epithelial component. They rarely occur in the stomach in the monophasic form (spindle cell pattern). They are focally positive for EMA and CK and are characterised by a translocation resulting in the expression of an *SYT-SSX* chimeric transcript (most commonly *SYT-SSX1/SSX2*). (154,182–184)

Additionally, epithelioid leiomyosarcoma and epithelioid hemangioendothelioma may be a differential diagnosis. (67)

1.6.4 Malignant and spindle morphology

Leiomyosarcoma is a malignant mesenchymal tumor originating from the smooth muscle tissue, with hypercellular, spindle cells with an eosinophilic cytoplasm and cigar shaped, atypical hyperchromatic nuclei and high mitotic activity. Per definition leiomyosarcomas need to express at least 2 smooth muscle markers (SMA, Desmin, Caldesmon). KIT, DOG1 and S100 protein are negative. (67,185)

Especially dedifferentiated Liposarcoma (LS) may be in the differential diagnosis of GIST. Dedifferentiated LS are characterized by a well differentiated component with abrupt transition into a spindle/pleomorphic sarcoma. In the well differentiated part recognisable adipocytes that vary in size and shape with atypical hyperchromatic stromal cells present in the fibrous septa are seen. Immunohistochemically, they are positive for MDM2 and CDK4. In contrast, pleomorphic liposarcomas can be differentiated from undifferentiated pleomorphic sarcoma only by the presence of clear cut lipoblasts because they lack MDM2 and CDK4 expression. (186)

Malignant peripheral nerve sheath tumor (MPNST) consists of spindle cells arranged in fascicles. The density of cellularity varies. The cells are atypical with high mitotic activity. Staghorn vasculature and heterologous differentiation may be observed. (152,187–189) Immunohistochemically, S100 and SOX10 are usually seen in only a subset of cells but can also be completely lost. Recently, a loss of H3K27me3 has been described as a good diagnostic marker. (190)

1.7 TKI Therapy

Years ago, the treatment of GIST was difficult and, as neither chemo-nor radiation therapy was successful, surgery was the only therapeutic option. (3,16) Nowadays, treating GISTs with cytotoxic chemotherapy is not recommended. With the insight of molecular data, GISTs became a paradigm for targeted treatment. (3,25)

Most GISTs show activating *KIT/PDGFRA* mutations and they are treated with Imatinib mesylate as a first line treatment regime in case of metastatic GIST or after complete surgical resection of an intermediate - or high-risk GIST, after molecular analysis and exclusion of the Imatinib resistance mutation *PDGFRA D842V*. Imatinib (Gleevec) contributes to recurrence-free survival. In case that the GIST is unresponsive, Imatinib is not well tolerated or Imatinib resistance mutations occur Sunitinib maleate, a multi-kinase inhibitor, is used as second-line treatment. Additionally, a third-line therapy option Regorafenib can be used. (1,3,21,51,56,72,191,192) However, Regorafenib has shown more side effects than Imatinib. (193) As already mentioned, Imatinib is used for adjuvant therapy in patients after complete excision of a high-risk GIST, for at least 3 years. (51) In addition, Imatinib can be used to reduce tumor size and the risk of intraoperative complications, like tumor rupture, by reducing the tumor size resulting in a less invasive surgical intervention. (25,191,194)

Due to the missing *KIT* or *PDGFRA* mutations and unresponsiveness to cytotoxic chemotherapy, the only effective treatment for SDH-deficient GISTs is surgical intervention. The primary tumor and metastases (if existing) should be removed, whenever possible. (56,93,136,195,196)

1.7.1 Imatinib mesylate

Normally, the daily dose of Imatinib is 400mg for 3 years according to a study of Heinrich, et al (197). There is no benefit of increasing the dose up to 800mg when treating a *KIT*-exon-11-mutation or even a WT GIST. In contrast, when treating a GIST with *KIT*-exon-9-mutation, an increased dose of 800 mg has positive effects on the outcome and on the time of tumor progression. 800 mg of Imatinib is now used as a standard treatment in *KIT* exon 9 mutated GISTs. (51,85,197)

Imatinib is metabolized by CYP 3A4 and therefore, the blood concentration may be influenced by CYP 3A4 inhibitors (grapefruit juice or ketoconazole) and inducers (rifampicin or phenytoin) and should be controlled carefully, if there is a suspicion of interference. (25)

Typical side-effects of Imatinib are muscle cramps, edema accompanied by ascites or pleura effusion, leukopenia and gastrointestinal complaints such as nausea, diarrhoea, abdominal pain and fatigue. Furthermore, there is a low risk for tumor haemorrhage associated with large tumors, (25,198)

1.7.2 Sunitinib malate

Sunitinib inhibits *KIT*, *PDGFR, VEGFR*, *fms-like tyrosine kinase-3 receptor (FLT3)* as well as the *rearranged during transfection (RET)-receptor*. The standard dosing for Sunitinib is 50mg for 4 weeks with a 2-week break. However, due to the side effects often a dose of 37.5 mg is given continuously instead. The treatment decision is based on the individual. (51,81,199,200) Like Imatinib, Sunitinib is metabolized by CYP 3A4 and therefore the blood concentration varies if any CYP 3A4 inhibitors or inducers are present. Undesirable side effects are gastrointestinal complaints as well as hand-foot syndrome (HFS), hypothyroidism, myelosuppression, and mucositis. Additionally, through the inhibition of *VEGFR*, Sunitinib may cause hypertension, hence patients with a history of coronary artery disease should be controlled constantly. (25,201–203)

1.7.3 Regorafenib

Regorafenib is the third-line therapy for GISTs used if there is a tumor progression under Sunitinib therapy. Generally, it inhibits *KIT, PDGFR, VEGFR* and *BRAF*. The daily dose is 160 mg and is given orally in a 3 week- cycle with one week pause. Side effects are mainly hypertension, oral mucositis, HFS and diarrhoea. (27,33,193,204)

1.7.4 Resistance

Various GISTs subtypes show different response to therapy. In addition, primary and secondary resistances against therapy with Imatinib have been reported. (15) If patients show clinical progression during the first 6 months of Imatinib treatment, it is allocated to primary resistance. (25) Nowadays, with expanded molecular profiling the group of patients with primary resistance is small. As patients with *KIT*

exon 9 mutations are from the beginning treated with 800 mg of Imatinib and *PDGFRA* D842V mutated GISTs are not treated with Imatinib.

The treatment for GISTs which harbour *PDGFRA* mutations causes difficulties, as the most common *PDGFRA* mutation the D842V shows resistance against Imatinib. Mutations located in exon 18 concerning codon D842, such as D842V, RD841-842KI, DI842-843IM - excluding D842Y - are mainly drug-resistant, while other mutations in exon 18 tend to be Imatinib sensitive. (68) A therapeutic option for GISTs harboring mutations in *PDGFRA* exon 18 codon D842V is a new TKI called Avapritinib. (205)

Secondary resistance is associated with an initial response to Imatinib and subsequently tumor progression, resulting from specific secondary point mutations in *KIT* or *PDGFRA*. (25,101) While the primary mutations still respond to Imatinib, these new mutations are resistant. Secondary mutations cluster in hot spots namely *KIT* exon 13 as well as exon 17 and 18. Genomic amplifications of the *KIT* receptor have also been described as a possible cause for Imatinib resistance. (25,206–209) Similar resistance mechanisms have been shown in *PDGFRA* mutated GISTs. (210)

1.7.5 NTRK Treatment

Based on the fact that GISTs rarely demonstrate *NTRK*-fusions, NTRK inhibitors like Larotrectinib or Entrectinib are potential treatment options in this setting. Both inhibit NTRK 1, 2 and 3 and thus the MAPK, STAT3, PKC and PI3K-AKT pathways are downregulated. (142,211–213) Entrectinib additionally inhibits the activity of *ALK* and *ROS 1*. (142,214)

Acquired resistance against the first-generation NTR-inhibitors is caused by point mutations in NTRK. Newer second line TRK inhibitors, like LOXO-195, TPX-0005 or ONO-5390556, show good results to overcome this resistance in vitro. (142,215–217)

In general, first-generation TRK inhibitors are well tolerated, but typical undesirable side-effects are dizziness, weight gain and paraesthesia. (142,218,219)

Larotrectinib and Entrectinib are excellent TRK inhibitors and demonstrate excellent results in the adult and paediatric patients harbouring tumors with a NTRK-fusion. (88,142,212,218)

1.7.6 Monitoring

It is very important to monitor the therapy success of targeted therapy. The TKI response in GISTs is assessed on the “Response Evaluation Criteria in Solid Tumors” and should be done every 3 to 6 months with CT scans. CT is a good method to detect any tumor progression or resistance to TKI therapy. Suspicion of tumor progression (for example new lesions, no change or even increase in size or nodules within the tumor showing contrast enhancement), should lead to more frequent controls and performance of imaging methods like MR or contrast-enhanced ultrasonography. (25,33,220,221)

Patients who are responding to TKI therapy are showing changes within 1-2 months, the maximal response is usually accomplished after 6 to 12 months. Sometimes the size of the tumor is not decreasing but increasing because of haemorrhage or myxoid degeneration inside the tumor. However, the GIST turns hypodense and homogenous on the CT scan and the tumor vessels start to vanish (pseudoprogression). Furthermore, a FDG-PET scan can be used to evaluate the tumor response at an early stage. (25,51,221,222)

Generally, after successful surgical treatment, a follow-up should be done – including appropriate imaging procedures. Normally, recurrence happens in the first years after surgery, therefore follow-up should be done regularly - frequency and duration depend on risk stratification and adjuvant therapy. Longitudinal CT of the abdomen or pelvis is suggested. In young patients or in ones with a low-risk GIST MR is a method of choice due to less radiation. Unfortunately, the ideal interval between follow-ups does not yet exist. Generally, patients with high-risk GISTs should be monitored over years: in some institutes they are scheduled every 6 months under adjuvant therapy and after discontinuing the adjuvant therapy follow-up is every 3-4 months for 2 years and then once or twice a year for approximately 10 years. For patients with low-risk GISTs after adjuvant therapy a CT/MRI scans every 6-12 months (over 5 years) are recommended. (33,51,223,224)

2. Materials and Methods

2.1 Patients and Tumor Characteristics

In total 81 cases analysed from 2017 to 2019 at the Diagnostic and Research Institute of Pathology, Medical University of Graz, Austria, were included in the study.

The analysis included clinical data (age, gender, symptoms, location, treatment, and follow-up), tumor size, histological and molecular findings. Haematoxylin & eosin (HE) slides from formalin-fixed, paraffin-embedded (FFPE) paraffin tissue blocks were evaluated (AA, IB, LAB). This retrospective study was approved by the Institutional Review Board of the Medical University of Graz, Graz, Austria (29-205 ex 16/17).

Tumors were classified according to the “Risk of stratification” from Miettinen/NCCN guidelines 2006 (See Table 3). Risk stratification is based on 3 main parameters (a) tumor size, (b) anatomic location and (c) mitotic activity (mitoses in an area of 5 mm^2 corresponding to 50 HPF according to Miettinen) the GISTs were classified in none risk, very low, low risk, intermediate risk and high risk of disease progression.

Diagnosis of the GISTs was established on the basis of surgical specimen (50/69, 72.5%) biopsies (18/69, 26.1%) and cytological material (1/69, 1.4%). Patients with biopsies underwent surgery in average 43 days after initial diagnosis (range 5 - 402 days).

2.2 Immunohistochemistry

For immunohistochemistry, FFPE tissue-blocks were cut in 4 µm thick whole-tissue-sections. Immunohistochemistry was performed on a Dako autostainer with the detection Kit Dako REAL Envision Plus, K5007. Sides were stained with CD117 (KIT), a polyclonal antibody (c-kit; clone A4502, 1:1000 dilution; Dako, Glostrup, Denmark), DOG1, a monoclonal antibody (Clone: SP31, 1:100 dilution, Thermo Fischer, Waltham, MA), SDHA, a monoclonal antibody (clone 2E3GC12FB2AE2, 1:750, Abcam, Cambridge, MA), and SDHB, a monoclonal antibody (clone 21A11AE7, 1:1000 dilution, Abcam, Cambridge, MA).

2.3 Molecular analysis

2.3.1 DNA Processing

The DNA was isolated from up to 8 unstained,10 µm thick FFPE sections of a selected tumor area. On an H&E-stained slide, a tumor area with high tumor content was marked and microdissected with a needle. DNA was extracted using the Maxwell RSC DNA FFPE kit according to manufacturer's instructions and was then quantified by picogreen fluorescence. 20ng DNA were needed for multiplex PCR reactions applying a custom Ion Torrent AmpliSeq panel covering specific genes.

2.3.2 Targeted next-generation sequencing

For mutational analyses next-generation sequencing (NGS) (Ion AmpliSeq technology MUG GIST Panel - searching for mutations in *KIT, PDGFRA, PDGFRB, SDHA, SDHB, SDHC, SDHD, H-RAS, N-RAS, K-RAS, BRAF, NF1, TP53, RB1* gene and *CDKN2A*) was used in the Laboratory for Diagnostic Genome Analysis, Institute of Pathology, Medical University of Graz, Austria. With the Ion Torrent Ampliseq Library Kit 2.0 adapter ligation and purification were completed and on Ion Torrent Proton or Ion Torrent S5 with the Ion PI Hi-Q Sequencing 200 kit NGS libraries were sequenced. Following, reads were geared to the human reference genome (hg19). Variants were called with the Torrent Variant Caller v5.6 and were checked to remove artefacts. Afterwards, they were annotated with an open-source-software (SNPeff and ANNOVAR). All analyses of each DNA-samples were accomplished as technical duplicate with separate NGS libraries.

3. Results

3.1 Clinical Data

Clinical Data are summarised in Table 6.

The patients´ average age was 64.8 years (median=66 years) with a range of 26 to 88 years. 61.73% were male (n=50) with an average age of 64.5 years (median=66years) and 38.27% were female (n=31) with an average age of 65.3 years (median= 67 years).

The exact tumor location was available for 70 cases. Most primary tumors were located in the stomach (37/70, 52.9%) and in the small intestine (28/70, 40%) including: 7 in the duodenum (10%), 4 in the jejunum (5.7%), 2 in the ileum (2.9%); for the remaining 15 small intestine-GISTs the detailed information about the exact anatomic location was not available. Lastly, 2/70 (2.9%) GISTs were located in the colon and 3/70 (4.3%) in the rectum.

21/43 patients, where detailed clinical information was available, showed metastatic disease (see Table 7), including metastasis in the liver (n=13; cases #27, #29, #32, #35, #37, #42, #47, #56, #64, #65, #71, #77 and #81), peritoneum (n=7; cases #29, #39, #40, #41, #42, #53 and #63), skin (n=2; cases #39 and #64), lung (n=1; case #32), right ovary (n=1; case #29), autochthonous back musculature (n=1; case #64) and the testicle (n=1; case #64). Lymph node metastasis occurred in four patients (cases #11, #14, #57 and #64). Moreover, five patients showed multiple metastases (cases #29, #32, #39, #42 and #64).

3.2 Gross findings

The average tumor size was 8.32 cm (median=7.5) and ranged from 0.8 cm to 20 cm. The tumors were most frequently well circumscribed, nodular, grey-whitish, solid, with a beige-fleshy pink cut surface. Some tumors had areas of necrosis and haemorrhage.

3.3 Histological findings

Histological findings were assessed in 81 cases. 41/81 (51%) GISTs had a spindle cell morphology, 13 tumors (16%) were epithelioid, and in 27/81 (33%) cases mixed morphology was observed. The tumors were composed of round to elongated nuclei syncytial eosinophilic cytoplasm. In rare cases cytologic atypia or pleomorphism was present. Tumor cells were arranged in fascicles, in strands, cords, or nests. Multinodular and plexiform growth pattern was observed in 4 cases. In addition, myxoid degeneration was found in eight cases, skeinoid fibres in one small bowel GIST (case #15), and three cases showed intracytoplasmic vacuoles.

Mitotic activity was available for 76 cases and ranged from 1 to 50 mitoses / 5mm^2. In 39 GISTs ≤5 mitoses / 5mm^2 were found, and in 37 cases >5 mitoses / 5mm^2 were observed.

Risk stratification could be performed on 62 cases. 28 (45.2%), 14 (22.6%), 17 (27.4%) and 3 (4.8%) tumors were classified as high-risk, intermediate-risk, low-risk and none-risk, respectively. In the other cases only selected tumor tissue was available for mutational profiling.

Characteristic	N	Percentage (%)
Patients	81	100
Gender (male/female)	50/31	61.7/38.3
Age (range, median)	26-88, 66 years	
Size (range, median)	0.8-20cm, 7.5cm	
Location		
Stomach	37	52.9
Duodenum	7	10.0
Jejunum	4	5.7
Ileum	2	2.9
Small intestine *	15	21.4
Colon	2	2.9
Rectum	3	4.3
Information NA	11	
Mitotic activity		
≤ 5 M/ 5mm2	39	51.3
>5 M/ 5mm2	37	48.7
Morphology		
Spindle	41	50.6
Epithelioid	13	16.0
Mixed	27	33.3
Risk Stratification		
High	28	45.2
Intermediate	14	22.6
Low	17	27.4
None	3	4.8

Table 6: Clinical data; (*) no detailed information available

Metastasis	*Primum*	*N*	*Detail*
Liver only	Stomach	6	
	Small Bowel	3	
Peritoneal only	Stomach	1	
	Small Bowel	3	
Lymph Node	Stomach	1	
	Duodenum	2	
Multiple			
	Stomach	1	-Liver, peritoneum, ovary
	Rectum	1	-Liver, lung
	Small Bowel	3	-Peritoneum, skin
			-Liver, peritoneal
			-Liver, stomach, skin, LN, autochthonous back musculature, testicle

Table 7: Location of metastatic disease

3.4 Immunohistochemical findings

The immunohistochemical findings are shown in Table 8.

Immunohistochemical stains were evaluated for 62 patients. A diffuse cytoplasmatic staining for CD117 was found in 95.2%, in one case a weak staining was observed. 95.8% showed a membranous or cytoplasmatic reaction with DOG-1. Additionally, 63.6% were positive for CD 34, 40.7% for SMA, 9.4% for S-100 and 4% for desmin. 2 GISTs (cases #11 and #40) showed a loss of SDHB expression.

Marker	***Percentage (%)***
CD117	95.2
DOG1	95.8
CD34	63.6
SMA	40.7
S100	9.4
Desmin	4

Table 8: Immunohistochemical staining profile

3.5 Molecular genetic findings

The findings of molecular genetic analysis are listed in Table 9 and Table 10 and presented in Figures 23 (summary of detected mutations) and 24.

In total 58 patients (71%) had *KIT* or *PDGFRA* mutation. 47 mutations (58%) were detected in the *KIT* gene including: exon 8 (n=1), exon 9 (n=8), exon 11 (n=33), exon 13 (n=2), and exon 17 (n=1). Case #42 had a mutation in *KIT* exon 11 and developed a secondary resistance mutation in *KIT* exon 13. Case #79 showed *KIT*-mutations in both exon 13 and exon 17. 10 GISTs (12%) harboured *PDGFRA* mutation in: exon 12 (n=1), exon 14 (n=1) and exon 18 (n=8; six out of them in codon D842V).

One patient - Case #38 presented with both: *KIT* exon 11 and *PDGFRA* exon 12 mutation – the *KIT* mutation has an uncertain pathogenicity according to Varsome, whereas the *PDGFRA* mutation is likely benign. (225) Five (6%) patients showed exclusively mutations in the *SDH*-complex: *SDHA* (n=3) and *SDHB* (n=2). Two (2.5%) cases demonstrated only *NF-1* mutations.

14 patients (17%) showed multiple mutations: in 7 patients with *KIT* exon 11 mutation, additional mutation(s) were found, such as *SDHA* (n=2; cases #6 and #63), *SDHB* (n=2; cases #5 and #40) , *SDHD* (n=2; cases #53 and #60), and *KIT* mutation in exon 13 and an additional *NF-1* mutation. According to Varsome, both *SDHA* mutations are classified as “uncertain pathogenicity” and both *SDHB* and *SDHD* mutations as benign. Furthermore, 7 patients harboured additional mutations in *CDKN2A* (n=4; three of them benign (cases #5, #19, #24) and one likely pathogenic (case #62)), *TP53* (n=4; cases #19, #27, #55, #71 – all of them classified according to Varsome as likely pathogenic or pathogenic) and *RB1* (n=2; cases #19 (classified as “of uncertain significance”) and #51 (classified as “pathogenic”)) (225) (see Table 10)

Two patients (2.5%) were wild type and showed no mutations (cases #26 and #37), indicating that with the use of an expanded mutation panel the wt group is very small.

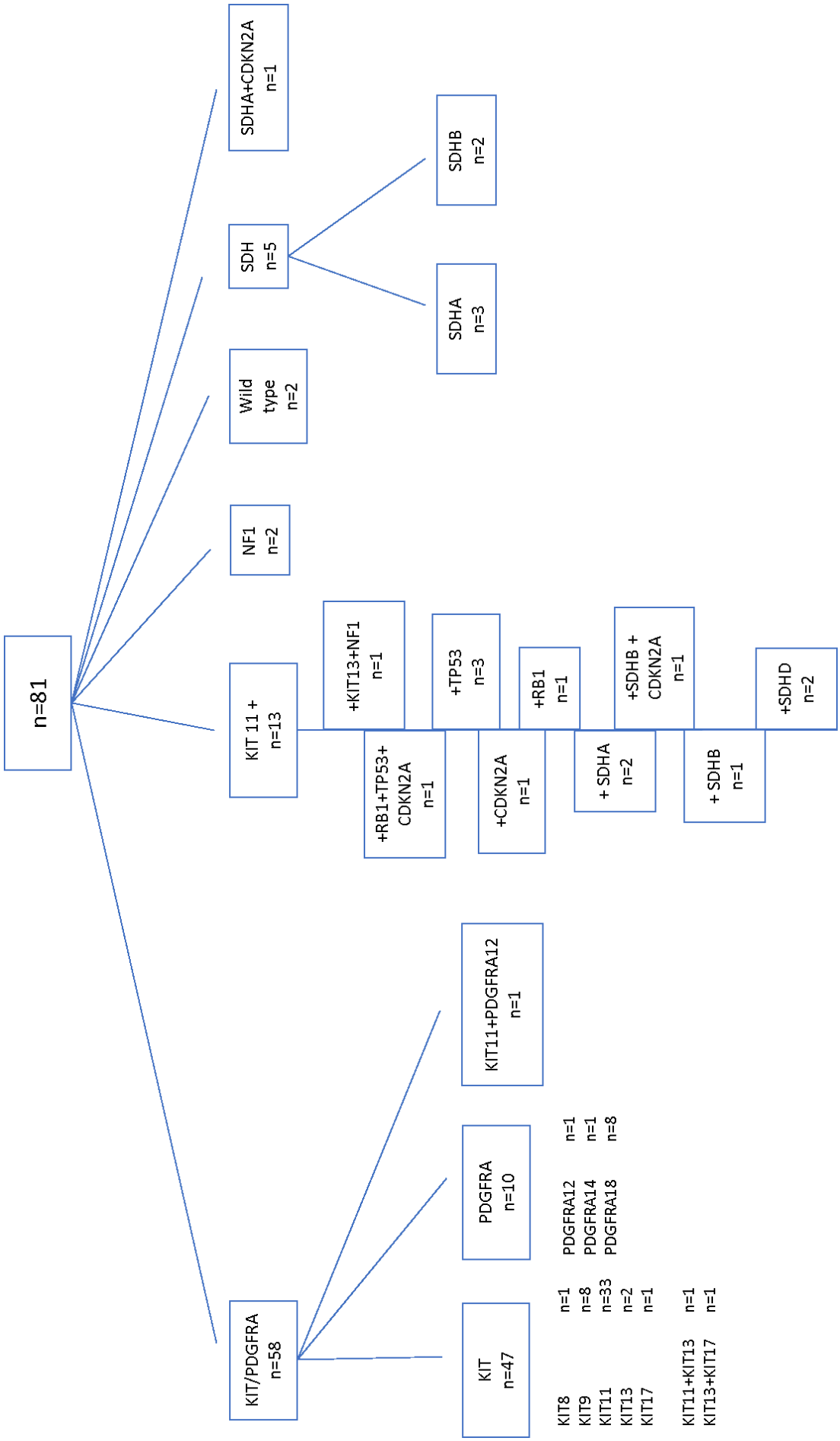

Figure 23: Summary of detected mutations

Gene	Genetic Mutation	N
KIT Exon 8	p.417_419delinsY	1
KIT Exon 9	p. 502-503dup AY	5
	p. Y503dupAY	1
	p. 503_504insAY	2
KIT Exon 11	p. W557R	5
	p. V560 D	3
	p. V559 D	4
	p. D579 del	3
	p. 573_588 dup	1
	p. 551-559 del	1
	p. 557_559 delins C	1
	p. 552_557del	1
	p. 559_566del	1
	p. L576 P	2
	p. W557_K558del	2
	p. 1571_D579dup	1
	p. 556_561del	1
	p. M552T	1
	p. V559G	4
	p. V559A	1
	p. 550-555delinsL	2
	p. 556_558del	3
	p. 557_558delWK	1
	p. W557G	1
	p. 555_559del	2
	p. 559_559del	1
	p. Y553N	1
	p. 578_580dupYDH	1
	p. 570_780del	1
	p. 559_573delinsA	1
	p. K550fs	1
	p. V560E	1
KIT Exon 13	p. K642 E	3
	p. V654A	2
KIT Exon 17	p. N822H	1
	p. N822K	1
PDGFRA Exon 12	p. V561D	1
	p. 566_571delinsR	1
PDGFRA Exon 14	p. N659Y	1

PDGFRA Exon 18	p. 842_845del	1
	p. D842V	6
	p. 843_846 del	1
SDHA	p. P95R (exon 3)	1
	p. Q170L (exon 5)	1
	p. A221P (exon 6)	1
	p. S445L (exon 10)	1
	p. C467R (exon 10)	1
	p. P658L (exon 15)	1
SDHB	p. S163P (exon 5)	2
	p. R217H (exon 7)	1
SDHD	p. G12S (exon 1)	2
NF1	p.M1R	1
	p.E1384K	1
	p.R69Xfs	1

Table 9: Molecular genetic data

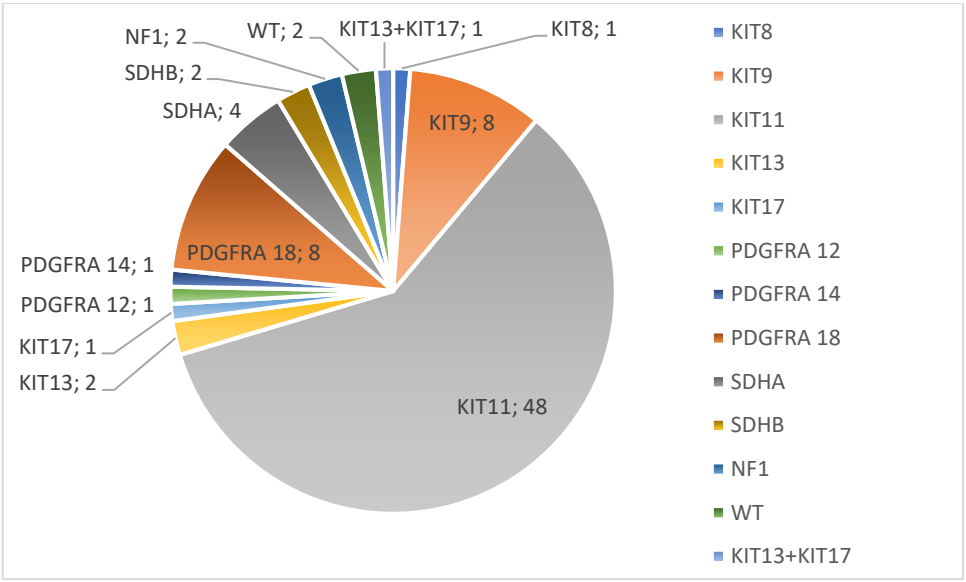

Figure 24: Molecular genetic findings

Mutations	MAF	Pathogenicity*
***SDHA*:**		
KIT 11 (p. D579 del)+SDHA (p.Q170L)	SDHA (p.Q170L): 42.75 and 43.47%	SDHA: uncertain significance
KIT 11 (p. 550_555del)+SDHA (p.P658L)	SDHA (p.P658L): 57.84 and 53.85%	SDHA: uncertain significance
***SDHB*:**		
KIT 11 (p. V559A)+SDHB (p.S163P)	SDHB (p.S163P): 87.35 and 87.18%	SDHB: benign
KIT 11 (p. V560D)+SDHB (p.S163P)+CDKN2A (p.A148T)	CDKN2A (p.A148T): 44.08 and 43.67% SDHB (p.S163P): 75.74 and 81.88%	SDHB: benign CDKN2A: benign
***SDHD*:**		
KIT 11 (p. 555_559del)+SDHD (p.G12S)	SDHD (p.G12S): 45.87 and 48.25%	SDHD: benign
KIT 11 (p.557_558delWK)+SDHD (p.G12S)	SDHD (p.G12S): 52.23 and 53.65%	SDHD: benign
***NF1*:**		
KIT 11 (p.W557_K558del)+KIT 13 (p.V654A)+NF1 (p.E1384K)	KIT 13 (p.V654A): 83.33 and 71.15% NF1 (p.E1384K): 32.21 and 27.30%	NF1: likely pathogenic
CDKN2A, TP53, RB1:		
KIT 11 (p. Y553N)+CDKN2A (p.L62Q)	CDKN2A (p.L62Q): 53.23 and 59.74%	CDKN2A: likely pathogenic
SDHA (p.C467R)+CDKN2A (p.A148T)	SDHA (p.C467R): 74.25 and 75.38% CDKN2A (p.A148T): 45.81 and 52.34%	SDHA: uncertain significance CDKN2A: benign
KIT 11(p.V560D)+TP53 (p.R273H)	TP53 (p.R273H): 43.20 and 40.82%	TP53: pathogenic
KIT 11 (p.556_558del)+TP53 (p.L32fs)	TP53 (p.L32fs): 54.54 and 63.22%	TP53: pathogenic
KIT 11 (p. 556_558del)+TP53 (p.R273C)	TP53 (p.R273C): 13.31 and 8.70%	TP53: pathogenic
KIT 11 (p.579del)+RB1 (p.841fs)	RB1 (p.841fs): 9.87 and 10.47%	RB1: pathogenetic
KIT 11 (p.W557R)+RB1 (p.L218fs)+TP53 (p.Q52X)+CDKN2A (p.A148T)	RB1 (p.L218fs): 36.35 and 33.30% TP53 (p.Q52X): 77.68 and 83.48% CDKN2A (p.A148T): 45.59 and 48.97%,	RB1:uncertain significance TP53: likely pathogenic CDKN2A: benign

Table 10: MAF of additional mutations and (*) pathogenicity according to Varsome (225)

3.6 Treatment and follow-up

The results are presented in Table 11.

45/58 (77.6%) cases underwent surgery depending on location, namely: gastric resections (n=14; 12 partial resection and 2 total resection), laparoscopic gastric wedge resection (n=1), partial resections of the small bowel (n=14), Whipple resection (n=3) and colectomy and sigma resection (n=1 each) was done. For 11 cases no detailed surgery information was available. 39/58 (67.2%) patients received adjuvant therapy with Imatinib (Glivec) 400mg per day, one case with *KIT*-mutation in exon 9 received double dose – 800mg per day (case #43). Two patients got a neoadjuvant therapy with Imatinib followed by resection of the primary tumor (cases #18 and #53). Six patients stopped Imatinib therapy because of adverse side effects or allergic reaction (cases #3, #7, #13, #53, #57 and #61). Four patients switched to Sunitinib (cases #39, #42, #43 and #56) and one to Regorafenib because of tumor progression (case #32).

Nine patients received palliative care (cases #17, #29, #37, #38, #43, #53, #63, #65 and #72) because of second tumors, progressed disease and highly reduced general condition.

Information about comorbidities and pre-existing diseases were available for 48 patients. 7 patients had different neoplasms previously or after the GIST diagnosis, including: two patients with colon carcinoma, one with a basal cell carcinoma pretibial, two with lymphomas (follicular and MALT-lymphoma), one with prostate carcinoma and pancreas carcinoma, each. In Case #11, a pulmonary chondroma and a mediastinal tumor were found, compatible with Carney triad. One patient (case #14) had amyloidosis.

Detailed follow-up information was available for 48 patients. Clinical follow-up differed depending on location, risk stratification and therapy of the tumor and included imaging procedures (MRI, CT, PET-CT, EUS), blood analyses and endoscopy. Initially the interval between the clinical follow-ups ranged from 1 to 3 months.

In 9 patients the tumor recurred within 1-15 years (mean=7.4 years).

Characteristic		*N*	*Detail*
Treatment			
Surgery			
	Gastric resection	14	Partial resection (n=12) Total resection (n=2)
	Laparoscopic gastric wedge resection	1	
	Small bowel resection	14	Partial resection (n=14)
	Whipple resection	3	
	Colectomy	1	
	Sigma resection	1	
Targeted Therapy			
	Imatinib 400mg/d	41	Adjuvant (n=39) Neoadjuvant (n=2)
	Imatinib 800mg/d	1	
	Sunitinib	4	
	Regorafenib	1	
Palliative care		9	
Comorbidities			
	Other neoplasms	7	Colon carcinoma (n=2) Lymphomas (n=2) Basal cell carcinoma (n=1) Prostate carcinoma (n=1) Pancreas carcinoma (n=1)
Follow-up		48	Initially follow-up: 1-3 months with imaging procedures, blood analysis and endoscopy
Recurrence		9	1-15 years (mean=7.4 years)

Table 11: Treatment, Comorbidities and Follow-up

4. Discussion

In this retrospective study we summarize the advanced molecular profiling of GISTs samples diagnosed at the D&R institute of Pathology in a three-year period.

The epidemiologic data of our cohort was mainly in line with the published literature (25,26). However, the lower frequency of KIT mutations can be explained by a reference centre bias. Our patients´ cohort consisted mainly of adults with an average age of 65 years including 38.3% of female and 61.7% of male patients. A 26 year old woman showed typical characteristics of the Carney triad by developing a SDHB-deficient gastric GIST and pulmonary chondroma (39–42). Patients under the age of 18 years were not included in this study.

According to the literature (1,30), up to 70% of GISTs show a spindle cell pattern, approximately 20% an epithelioid pattern and the remaining a mixed pattern. In our cohort, half of GISTs consisted of a spindle cell pattern, 16% showed an epithelioid pattern and one third had a mixed morphology. GISTs with SDH-deficiency by immunohistochemistry showed the typical multinodular/plexiform growth pattern and either an epithelioid or mixed pattern, as previously described. The *PDGFRA* mutated GIST showed a predominately epithelioid morphology in line with the literature (14,55,56).

Immunohistochemically, 95% of the tumors were positive for CD 117 (KIT) and approximately 96% showed a positive staining for DOG1. This confirms the effectiveness of the two widely used IHC markers in GIST diagnostics (1). The majority of GISTs were positive for both markers, in rare cases only one of those markers was expressed. Immunohistochemical markers like CD34, SMA and S-100 showed variable expression in GIST and desmin was only expressed in 1 case. These findings nicely demonstrate that these markers are not specific for GIST. However, a panel of different IHC markers need to be used to confirm GIST or to rule out other tumors in the differential diagnosis of GIST.

The molecular profiling was overall in line with previously published data (1) (See Figure 25 and 26).The majority of GISTs harboured mutations in either *KIT* or *PDGFRA*. In total 76.5% cases harboured a *KIT* mutation - with 3 cases showing a secondary resistance mutation and additional 14 cases with *PDGFRA 12, KIT 13* and *NF1, RB1* and *TP53* and *CDKN2A, TP53, CDKN2A, RB1, SDHA, SDHB* and

CDKN2A, SHDB and *SDHD* mutation in addition to the primary pathogenic *KIT 11* mutation. 46 of the *KIT*-mutations (74.2%) were in exon 11 (intracellular, juxtamembrane domain). 8 *KIT*-mutations (13%) were localized in exon 9 (extracellular parts of the transmembrane receptor) – a mutation that is sensitive to an increased dose of Imatinib (800mg/d) (85). 13.6% harboured *PDGFRA* mutations: the most frequently detected mutation in *PDGFRA* was localized in exon 18 D842V, known as a Imatinib resistance mutation (68). However, recently a novel TKI avapritinib, has been shown to be an potent drug for GIST especially for patients harbouring a *PDGFRA* D842V mutation (205). The *SDH*-complex was mutated in 5 cases, the most commonly affected subunit was the subunit A, which is predominately induced by germline mutations (56), followed by the subunit B.

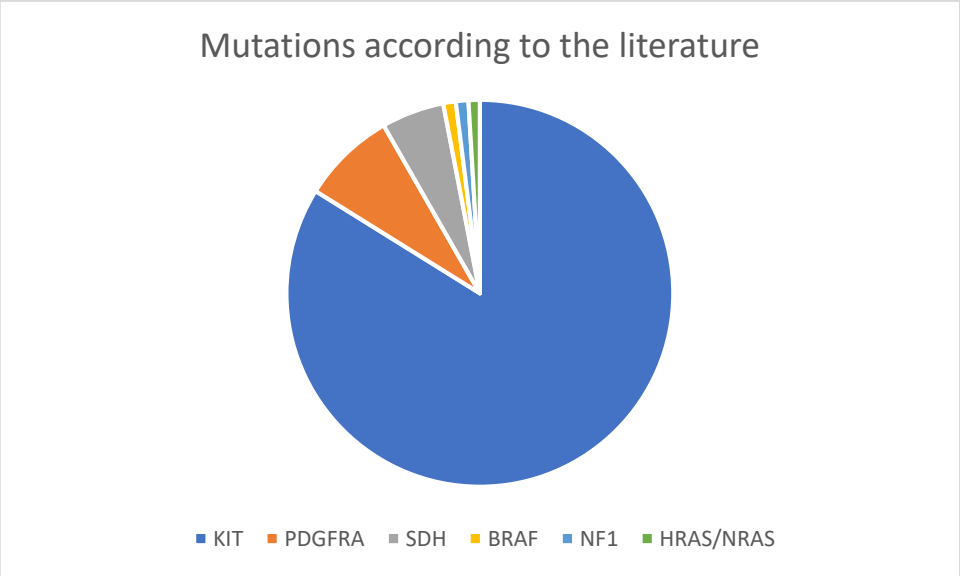

Figure 25: Mutations in GIST according to the literature (1)

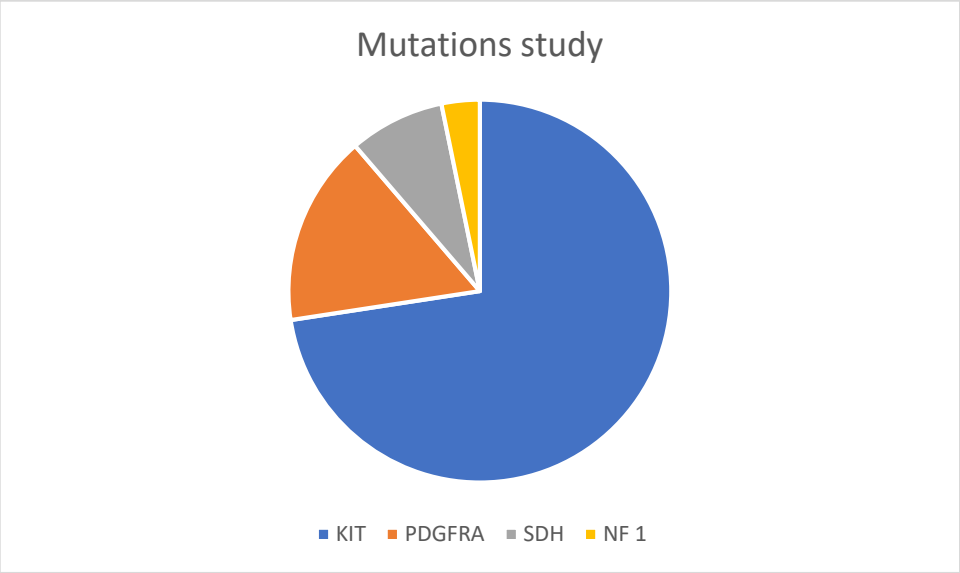

Figure 26: Distribution of Mutations in the presented retrospective study

Two tumors were wild type without detected mutations, so we performed Archer Fusion Plex Sarcoma Panel to exclude the fusions (like *NTRK*) by RNA-sequencing. In one case, no fusion could be detected. Unfortunately, there was not enough tissue available in the second case. (94,95)

In our study collective, we observed two cases with a *NF1* mutation, one case showed clinical features in concordance with the literature. However, one case was very exceptional (case #64 a small bowel GIST with a *NF1* mutation (p.R69Xfs)). In this case, in 2013, the patient underwent a small bowel resection – according to the external pathology reported a high-risk GIST (33 Mitosis/50HPF). He was treated in a peripheral hospital with adjuvant Glivec without performing mutational analysis and showed progressive disease. Therefore, the patient was switched to Sutent, also with progressive disease. He developed liver metastases and was treated by liver resection in 2014. Then he was treated with Sutent for 3 years. When he developed additional metastases in the liver and a tumor in the stomach, surgical resection was done in March 2017. Between 2017 and 2020 he was treated with Sutent, Regorafenib, Nilotinib and Dasatinib and developed further metastases in the liver, skin, lymph nodes, autochthonous back musculature and testicle during this time. He progressed on the mentioned TKIs and was switched to Sorafenib with

currently good response (last follow up July 2021). This case is a very unusual presentation of a *NF1* mutated GIST. *NF1* mutated GISTs are usually small, maybe multifocal in the small bowel and are commonly of low-risk.

Furthermore, according to the literature (1), GISTs in association with *NF1* occur mainly in younger women. Interestingly, in our cohort the two patients (cases #30 and #64) harboring *NF1* mutations were older than 60 years. Recent studies (118–120) have suggested, that in some cases somatic mutations in *NF1* occur. This finding could explain the advanced age and the absence of other characteristics of NF1-disease in our patients with *NF1*-mutations.

Additionally, case #29 showed a primary *KIT* exon 11 mutation with a secondary mutation in *KIT* exon 13 and additionally the patient harboured a *NF1* mutation, which is considered as likely pathogenic according to Varsome.

Different studies have shown, (3,60,70) that most of the mutations found in GIST are typical for certain sites. For example, GISTs with *KIT* exon 9 mutation are mostly found in the duodenum, GISTs with *PDGFRA*-mutation occur mainly in the stomach and GISTs with SDH-deficiency are located exclusively in the stomach (120). In our study, more than half of GISTs were detected in the stomach and more than one third in the small bowel. Only rare cases were found in the colon or rectum.

Metastatic disease was predominately observed in the liver and peritoneum (1,3). However, although our tumor collective was small, we observed metastases to skin, lung, ovary and even testicle. Lymph node metastases were observed in one SDH-deficient GIST associated with the CT, in one *NF1*-mutated GIST (case #64) and in two *KIT*-mutated GISTs. An explanation of some of these unusual findings might be the consultation bias including the fact, that patients were referred to the University Hospital for molecular analysis and evaluation of further treatment options.

The prognosis and the aggressiveness of the tumor are also associated with certain mutations. (25,83,84). In our study, GISTs with *KIT*-mutation developed more metastasis and showed a more aggressive behaviour than *PDGFRA*-mutated GISTs. High-risk GISTs – according to risk stratification of Miettinen - were mostly associated with mutation in *KIT* exon 11 (mutations: W557R, W557G, V559 G,

V560D, V560E, L576P, 551-559 del, 557_559 delins C, 1571_D579dup, 550-555delinsL, 556_558del, 570_780del, 559_573delinsA) and 9 (mutations: 502-503dup AY, Y503dupAY, 503_504insAY). Intermediate and low risk GISTs were predominately observed in *PDGFRA*-mutated or *SDH*-mutated GIST. Tumors with mutations in *KIT 11* and additional *SDH* mutation were found in the low and high risk group. All identified *SDH* mutations in addition to *KIT 11* mutation were classified as benign or of uncertain significance according to the Varsome database.

In 2 patients with *KIT 11*-mutation and an additional mutation in *CDKN2A* the tumors were classified as low-risk GISTs. By using Varsome for classification of the detected mutations, the *CDKN2A* mutations were classified as benign in cases #5, #19 and #24 (see Table 10) and in case #62 as likely pathogenic. In the rest of the cases (#19, #27, #55 and #71), patients with additional *RB 1*, *TP53* and *CDKN2A* mutations were seen only in high-risk GIST in line with the progression model. (43)

In total, 6 GISTs harboured a mutation in *KIT exon 11* with an additional mutation in the *SDH*-complex – referring to Varsome, the two cases with additional *SDHA* mutation (cases #6 and #63) have an uncertain significance concerning their pathogenicity and the additional mutations in *SDHB* (cases #5 and #40) and in *SDHD* (cases #53 and #60) are benign.

The high allelic frequency suggests germline mutation. Especially in primary SDH mutated GIST a genetic counselling should be considered.

As already mentioned, treatment is depending on the molecular profile of the tumor. Molecular profiling is a state-of-the-art procedure before treating patients with TKI. Surgical intervention is performed if a complete resection is possible (33). Hence, in our retrospective study, more than three quarters of patients underwent surgery - depending on tumor size and anatomical location, partial or total resection of the affected organ is necessary. According to the literature (191), half of the patients with high risk GIST develop metastasis or recurrence within 5 years after surgical treatment, therefore adjuvant therapy is recommended. Depending on the type of a mutation, different targeted therapies are used. GISTs harboring *KIT*-mutations are treated with Imatinib. In the second line, if the tumor is unresponsive or the medication is not tolerated, Sunitinib is applied (1). Furthermore, Sunitinib is very

effective against mutations in *KIT* exon 9, 13 and 14 (72). In wt GIST Sunitinib might also be a therapeutic option.

In our cohort, more than half of the patients received adjuvant therapy with Imatinib 400mg/d, a patient with *KIT* exon 9 mutation received, as recommended (85), 800mg/d and two patients received neoadjuvant therapy with Imatinib with a 400mg dosage. If the tumor progressed under Imatinib therapy, second line treatment with Sunitinib was given and associated with treatment response.

Secondary resistance was observed in one patient, due to new mutations in the *KIT*-tyrosine kinase domain I (exon13). This finding explained the initial tumor reduction, that was followed by further tumor growth during follow-up and is in line with the known resistance theory in GIST (226). Changing the patient´s medication to Sunitinib (37.5mg/d) was successful and resulted in tumor reduction.

Follow-up in our patient's collective was scheduled depending on the risk stratification and therapy. According to guidelines (25,220), most patients had their follow-ups every three months, using imaging procedures, especially CT or MRI, to exclude tumor progression or even resistance to targeted therapy. Some patients with high-risk GISTs were initially monitored monthly, and afterwards in a 3-month-intervall and later every 6 months. After the initial phase, patients were generally followed as recommended (33,224). Follow-ups of patients (who underwent surgery and received adjuvant therapy) with low- or intermediate-risk GISTs were scheduled in longer time intervals.

5. Conclusion

Sporadic GISTs occur predominately in patients older than 60 years of age, at any site of the GI-tract. If occurring in younger patients, a syndromic GIST should be suspected and excluded. Well established IHC markers for GIST, namely DOG1 and CD117, are essential for confirming the GIST diagnosis. In addition, SDHB is a new and very helpful marker to stratify GIST in different molecular subgroups. Molecular analyses have greatly improved our understanding of GIST development and identified targetable genetic alterations and are part of the state-of-the-art diagnostic work up. Elucidating the molecular pathology of GIST had a great impact on treatment and prognosis of GIST patients and lead to a better survival for the majority of patients suffering from this disease. By performing comprehensive NGS based molecular testing the group of wt-GIST can be drastically reduced (in our collective 2.5%). Due to therapies with various TKIs, GISTs have a good prognosis even in metastasised stage of disease. Further therapeutic options are likely to emerge in the future, as our understanding of the GIST molecular changes increases, and further technical possibilities become available.

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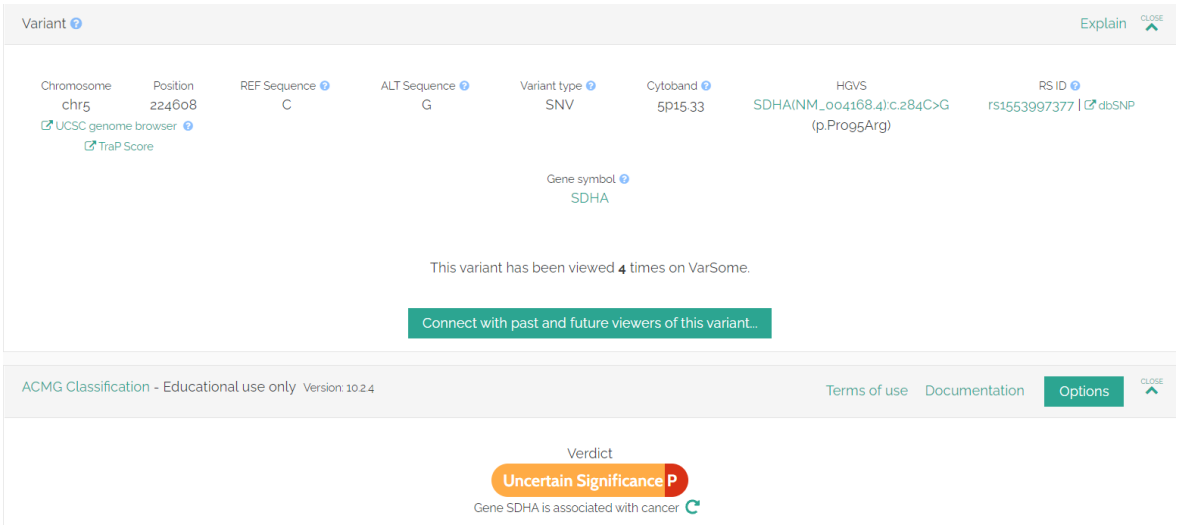
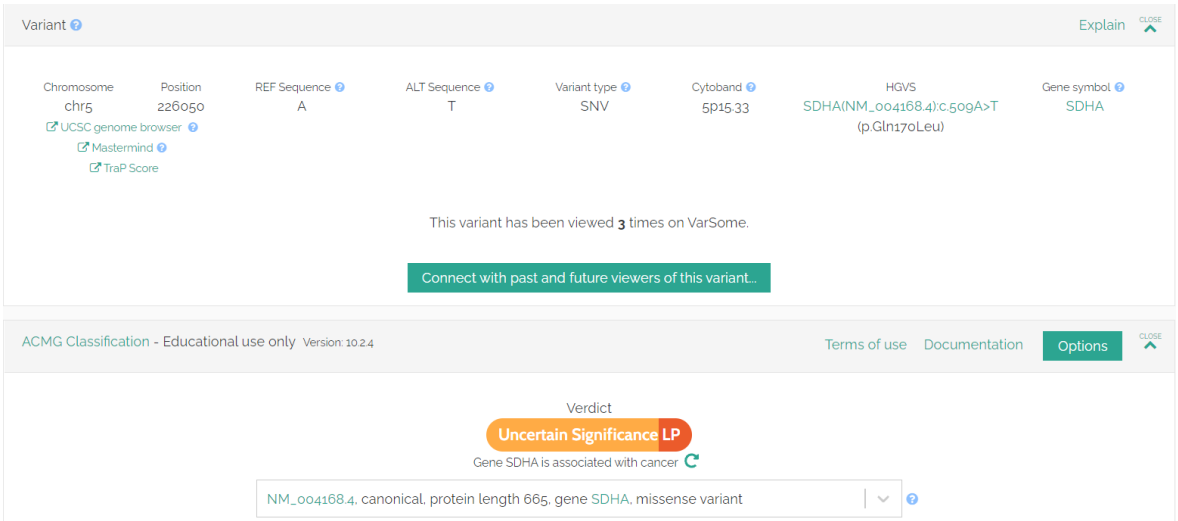
Attachments

Screenshots of the search results of Varsome (225):

SDHA:



Attachment 1: SDHA P95R SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/SDHA%20P95R?annotation-mode=germline



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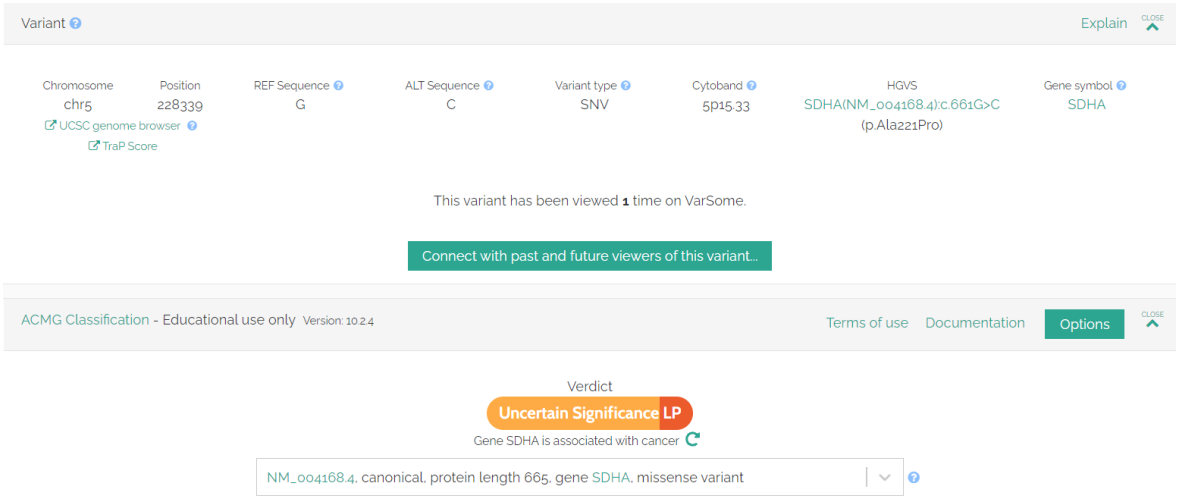
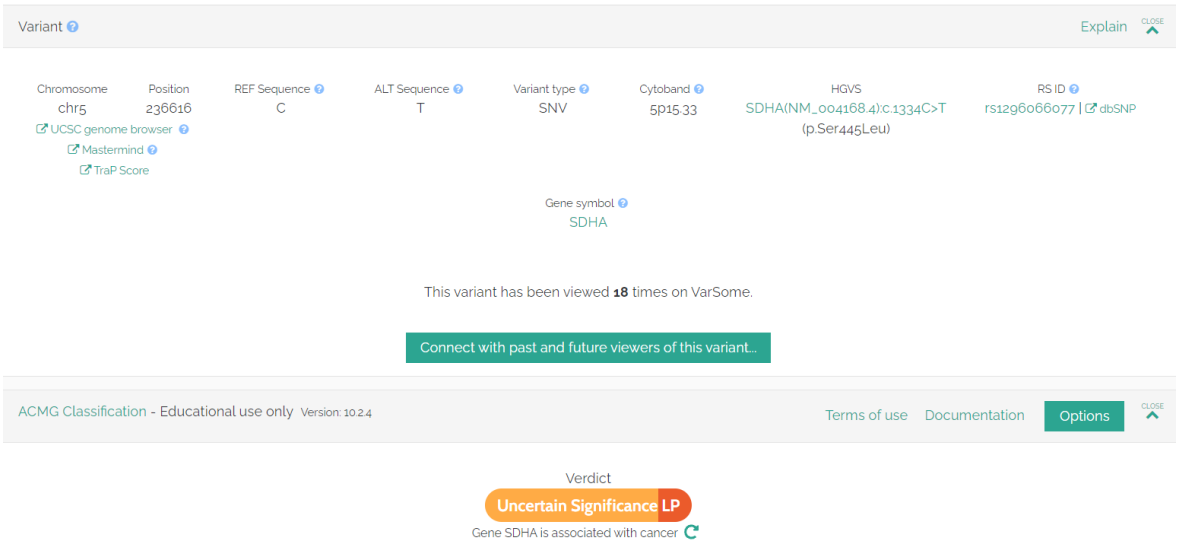


Attachment 3: SDHA A221P SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/SDHA(NM_004168.4)%3AA221P?annotation-mode=germline



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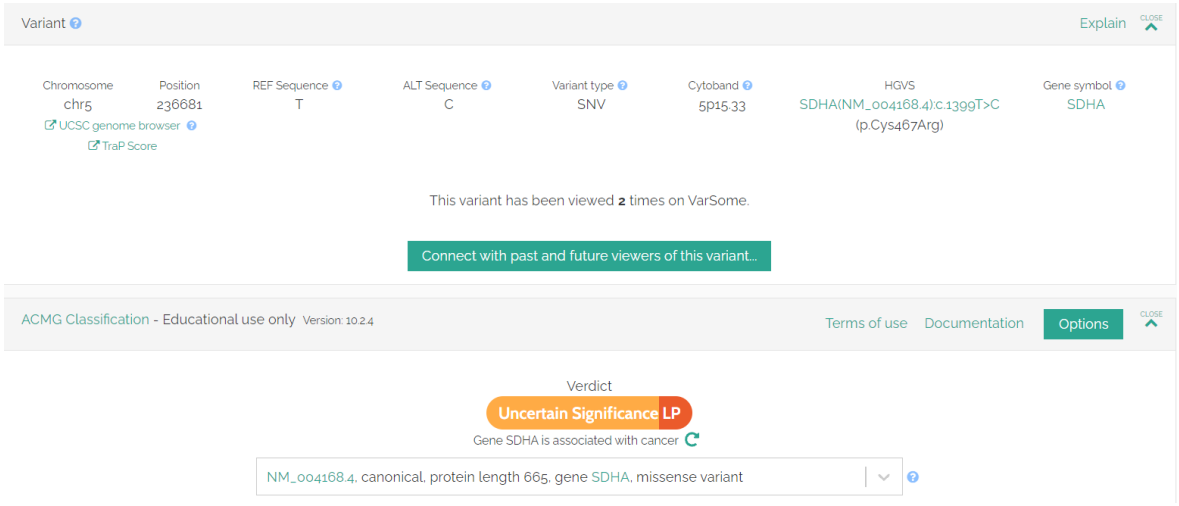

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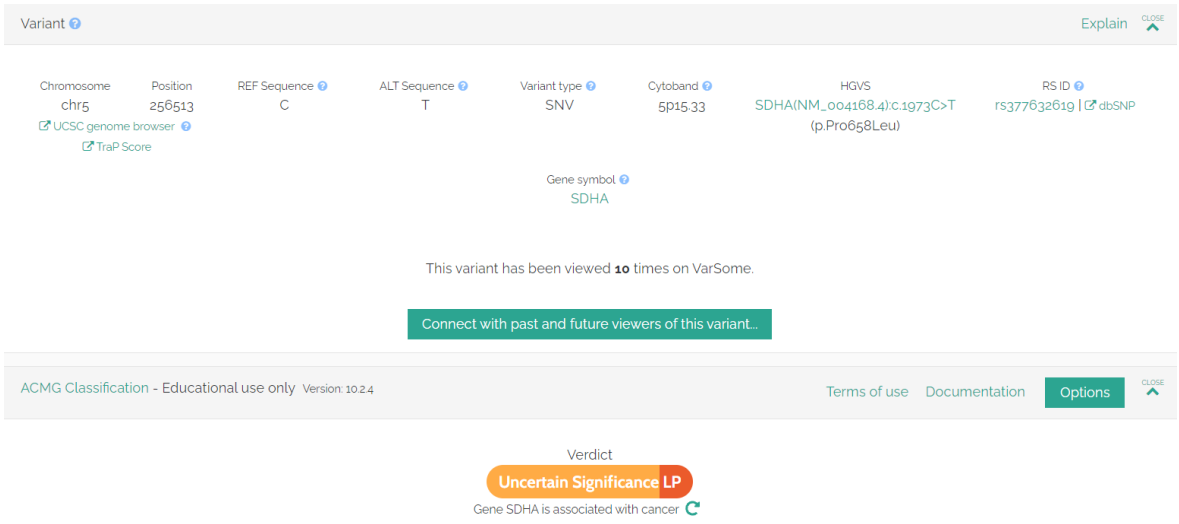

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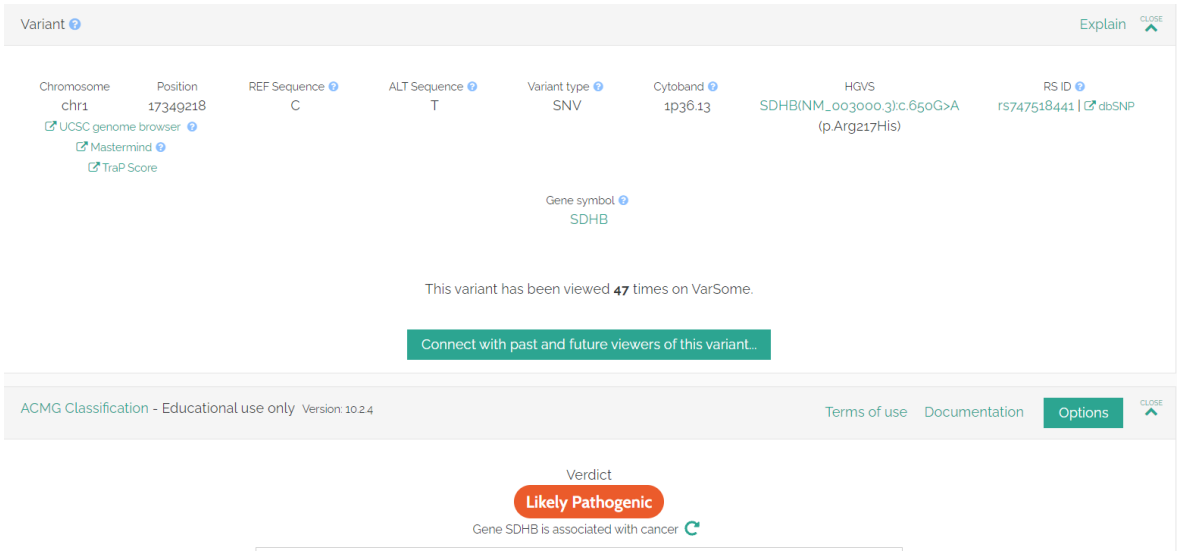
SDHB:



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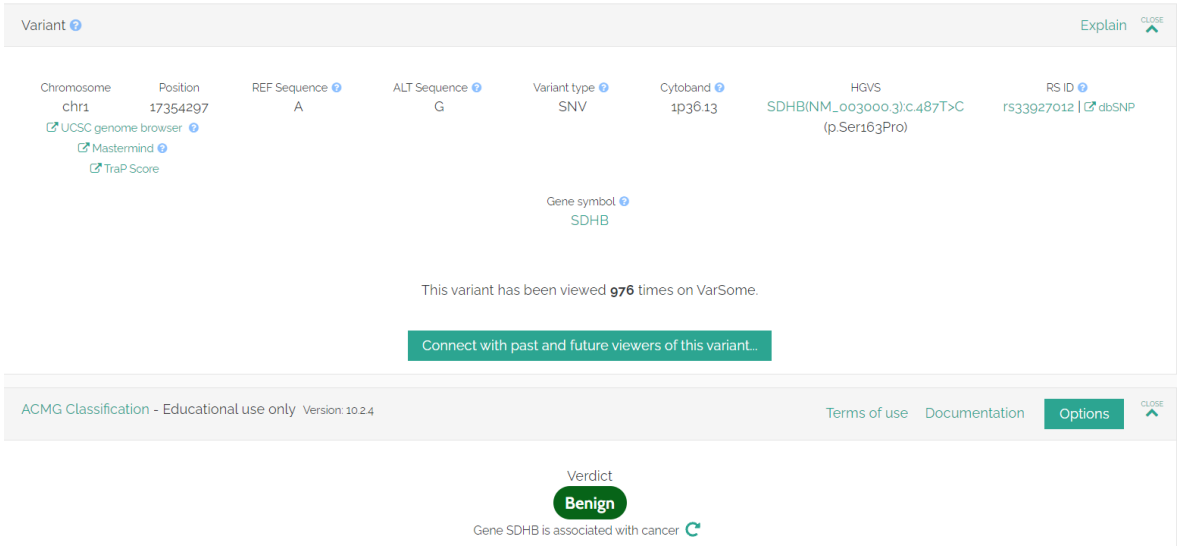

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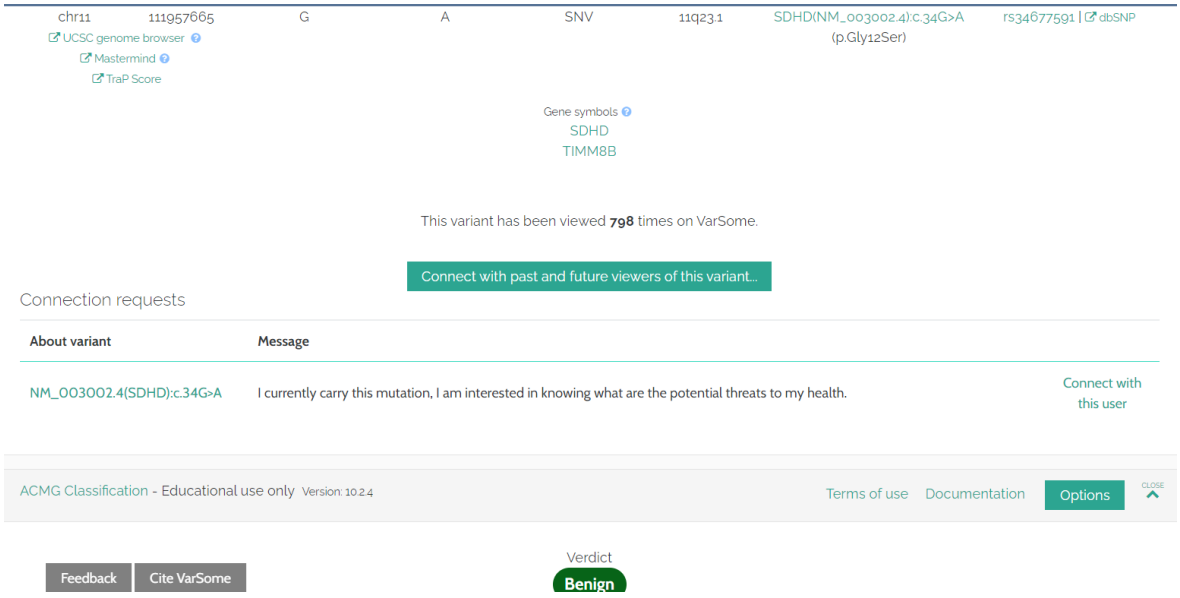
SDHD:



Attachment 9: SDHD G12S SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/SDHD%20G12S?annotation-mode=germline

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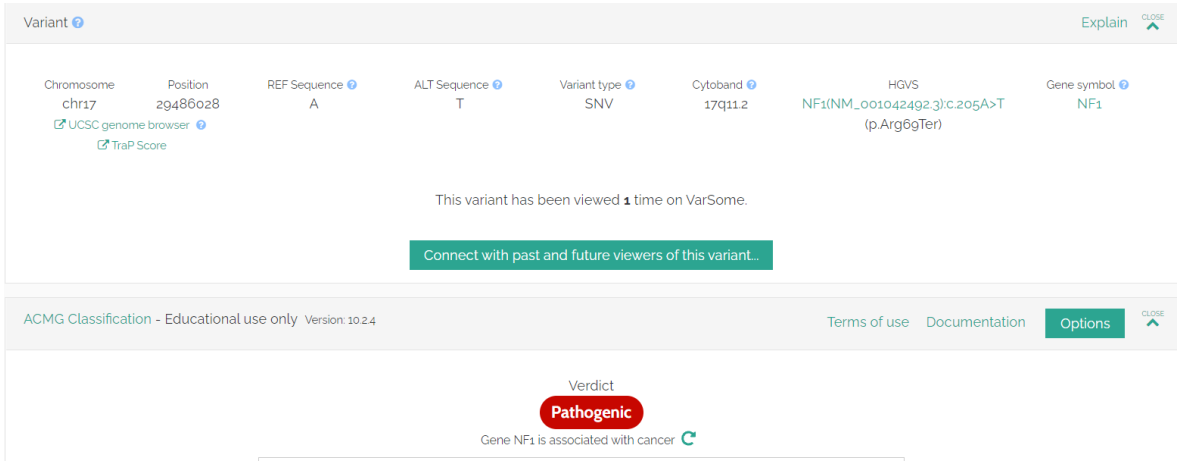

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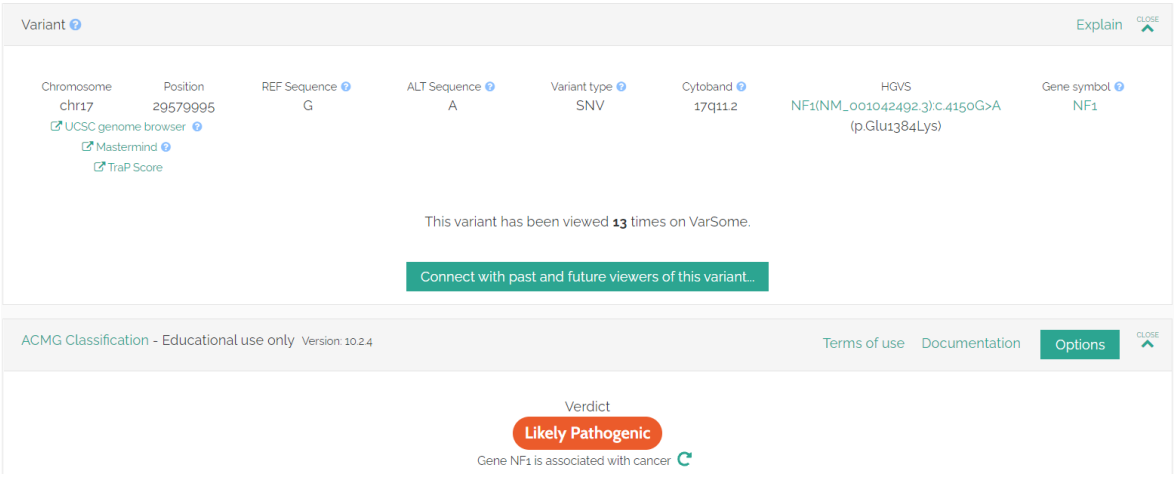

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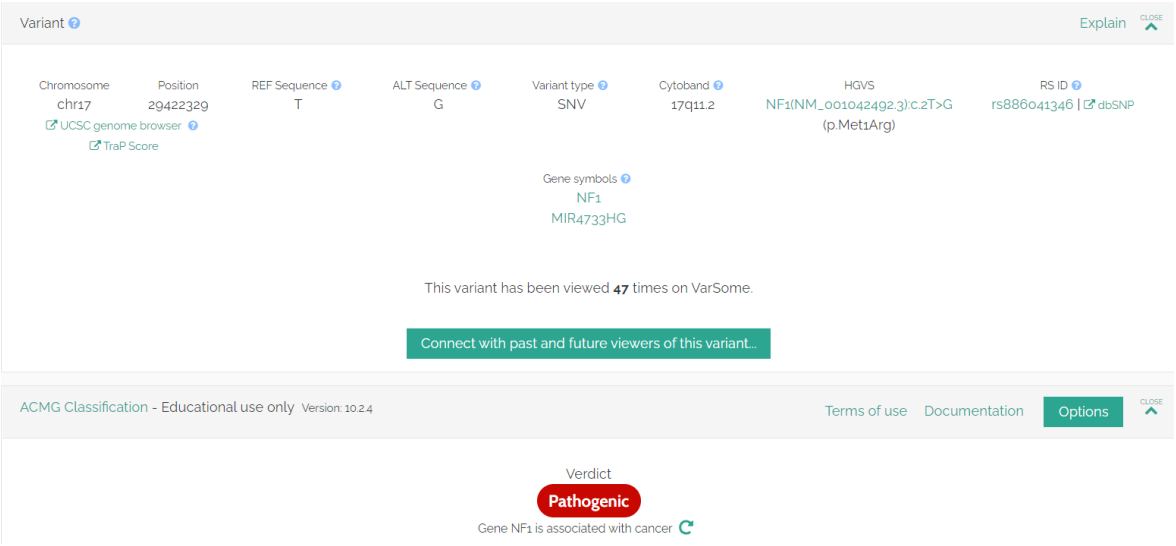
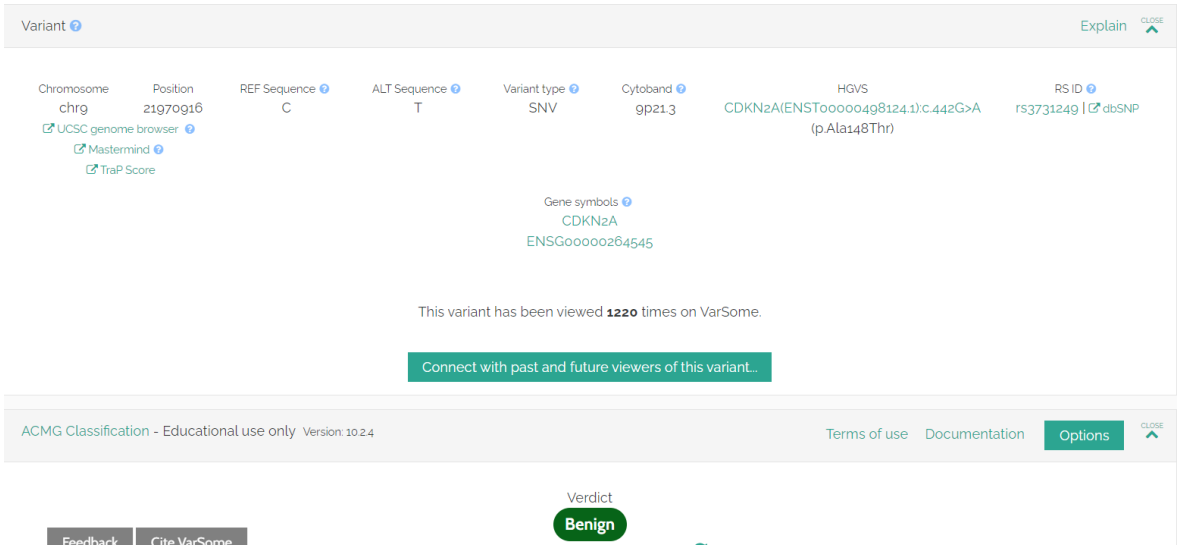


Attachment 12: NF1 M1R SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/NF1(NM_001042492.3)%3AM1R?annotation-mode=germline

CDKN2A:



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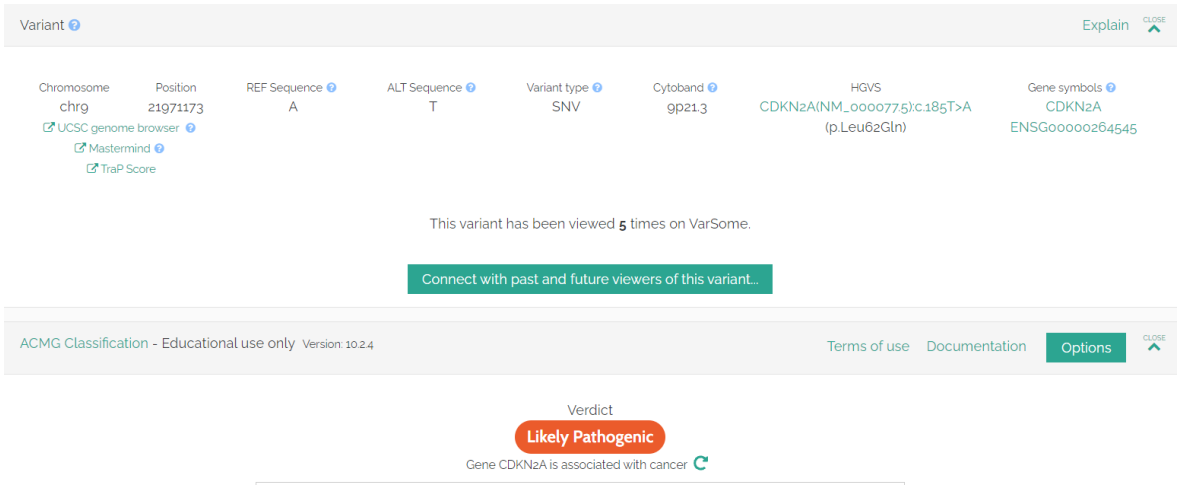
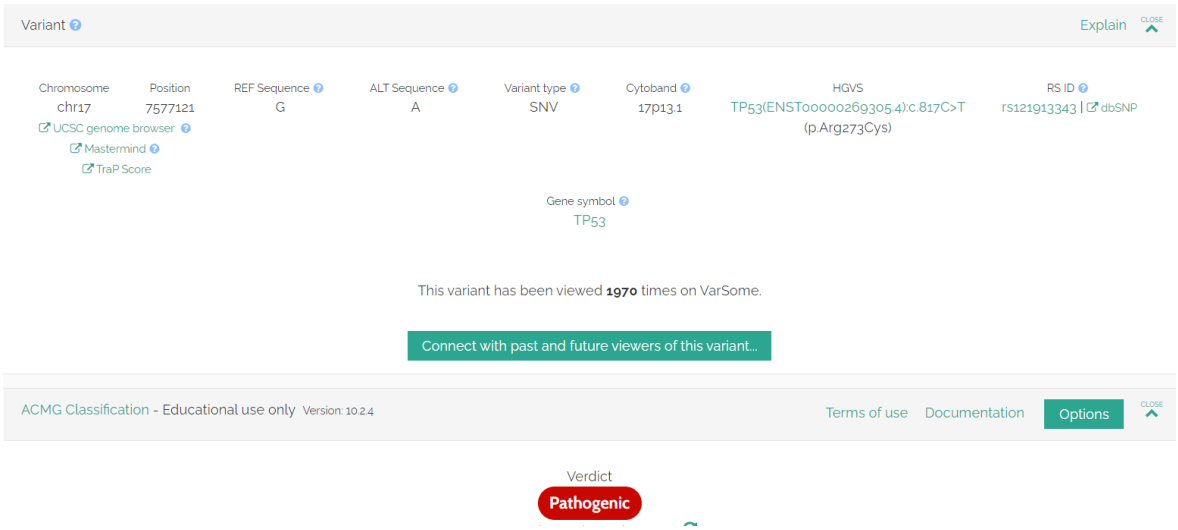
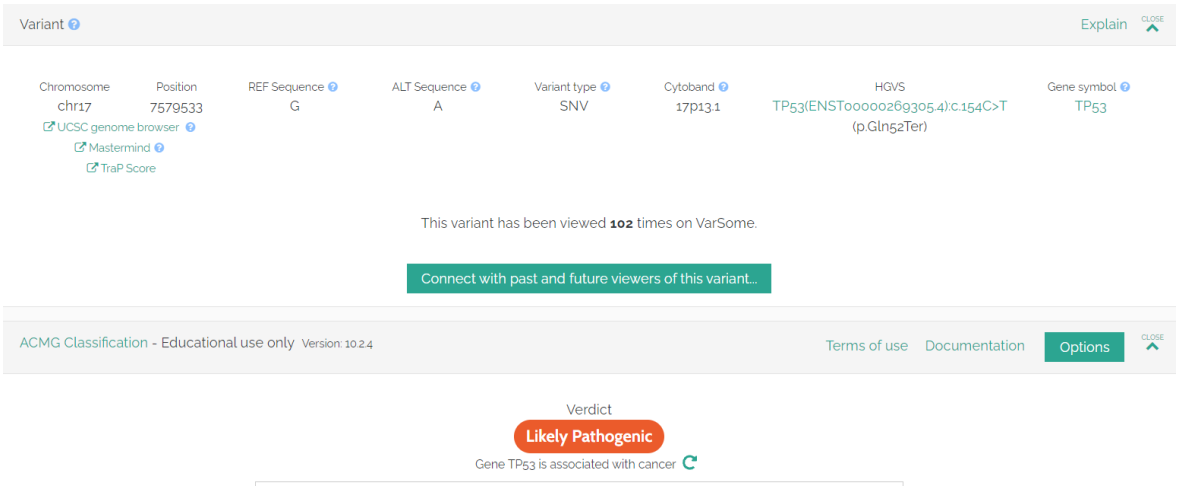


Attachment 14: CDKN2A L62Q SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/CDKN2A(NM_000077.5)%3AL62Q?annotation-mode=germline

T53:



Attachment 15: TP53 R273C SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/TP53%20R273C?annotation-mode=germline



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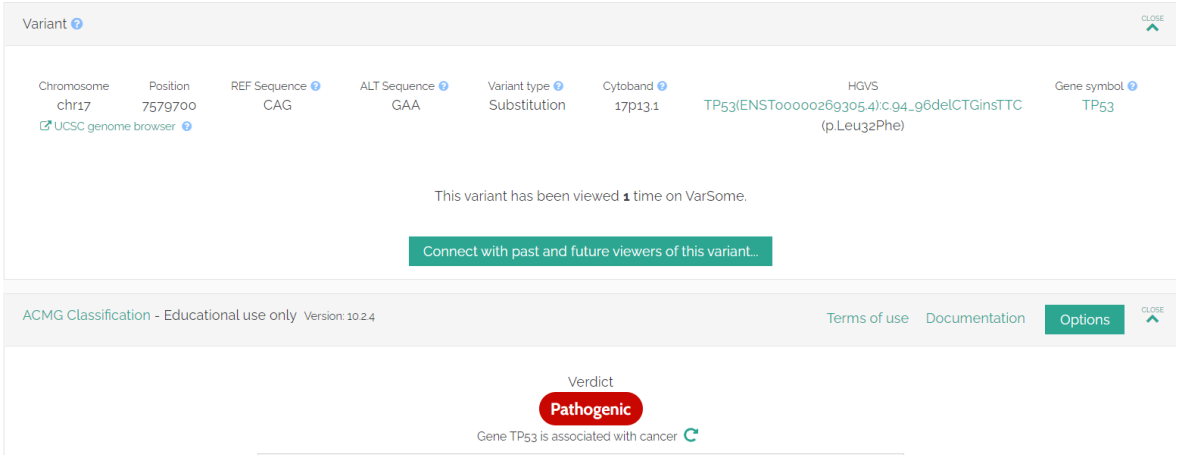
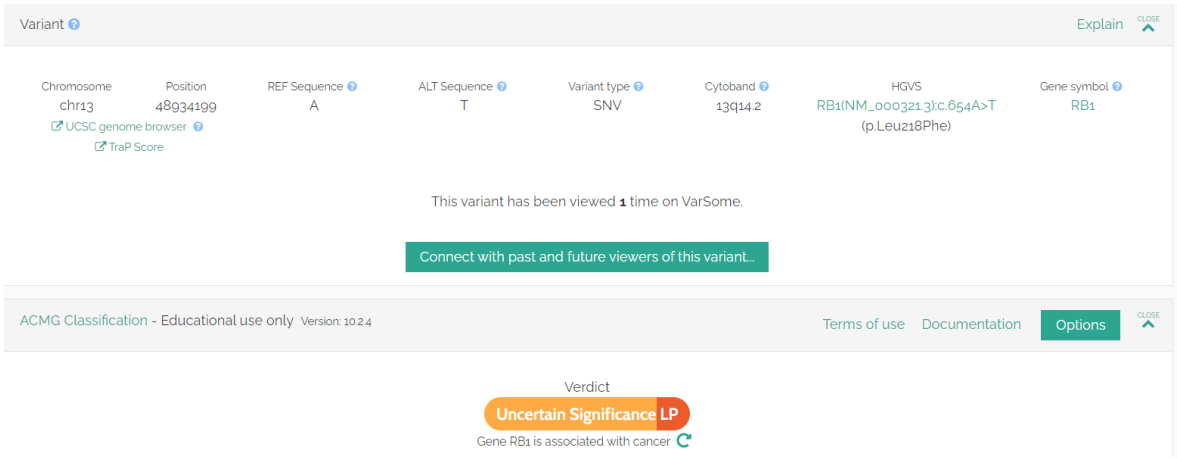
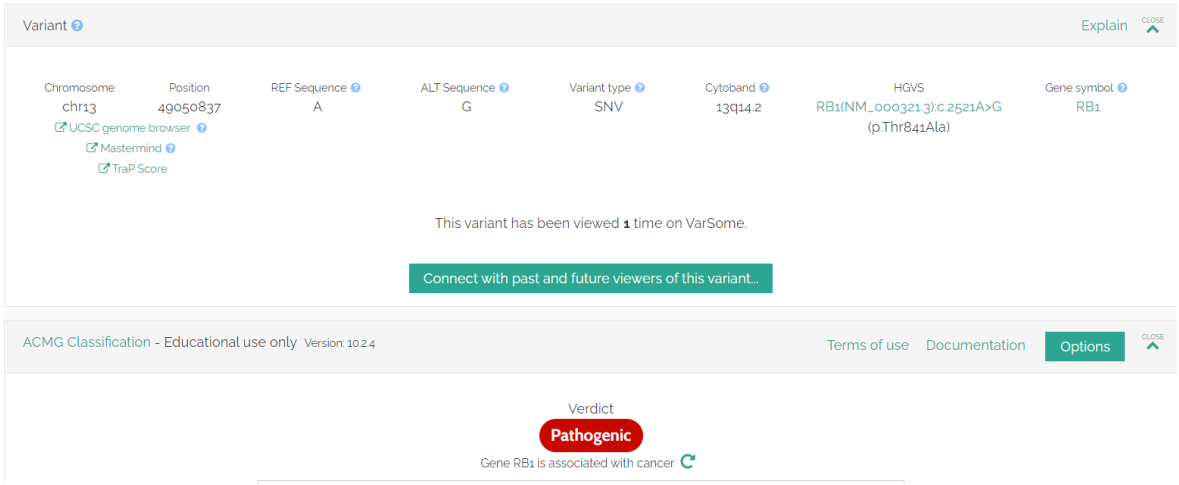


Attachment 17: TP53 L32F SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/TP53%20L32F?annotation-mode=germline

RB1:



Attachment 18: RB1 L218F SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/RB1%20L218F?annotation-mode=germline



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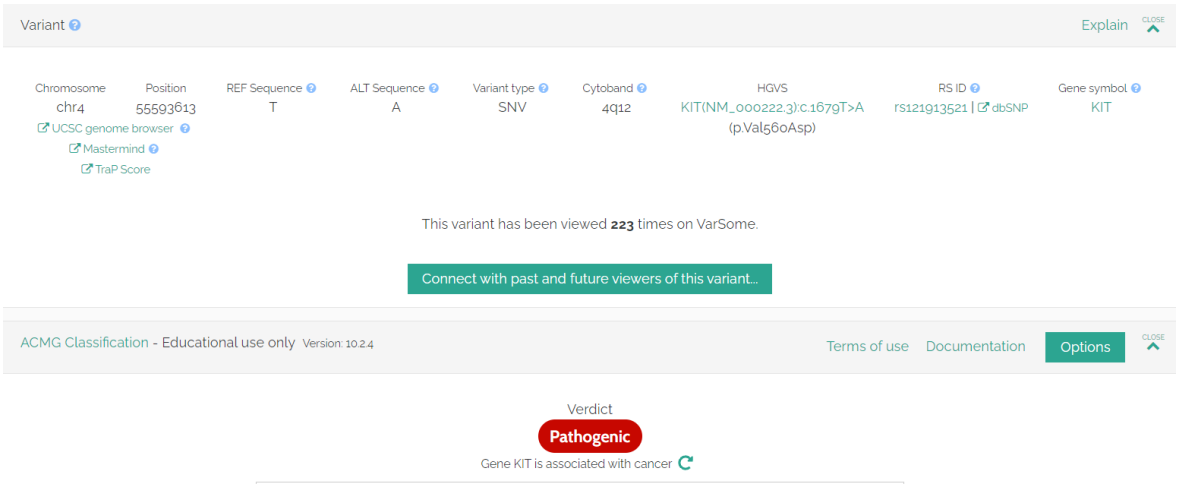
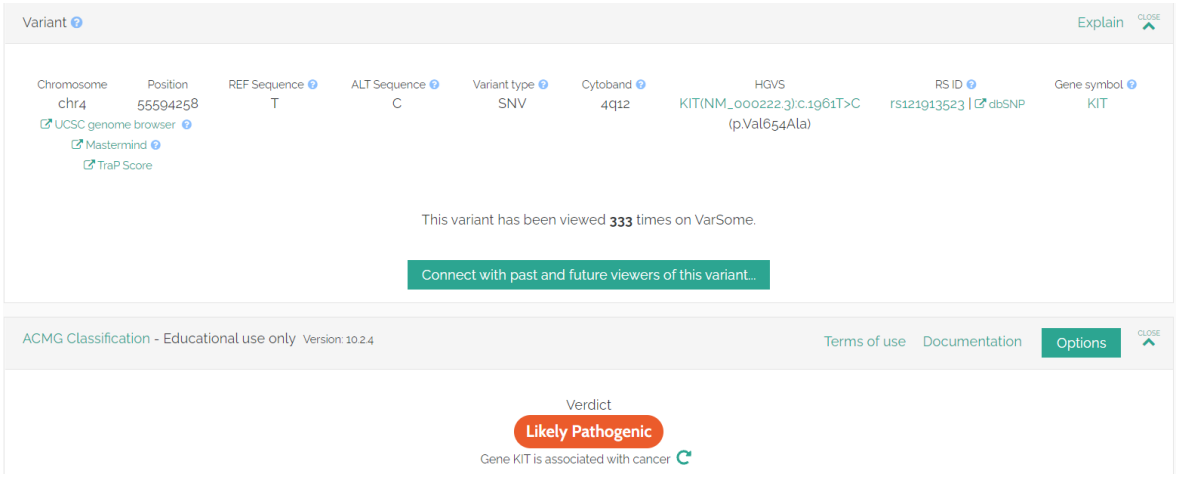
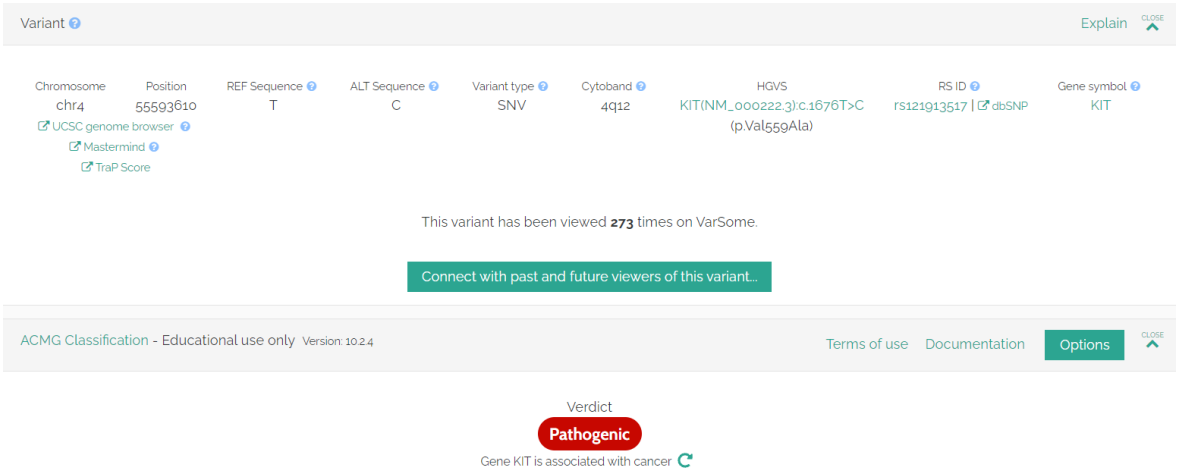
KIT:



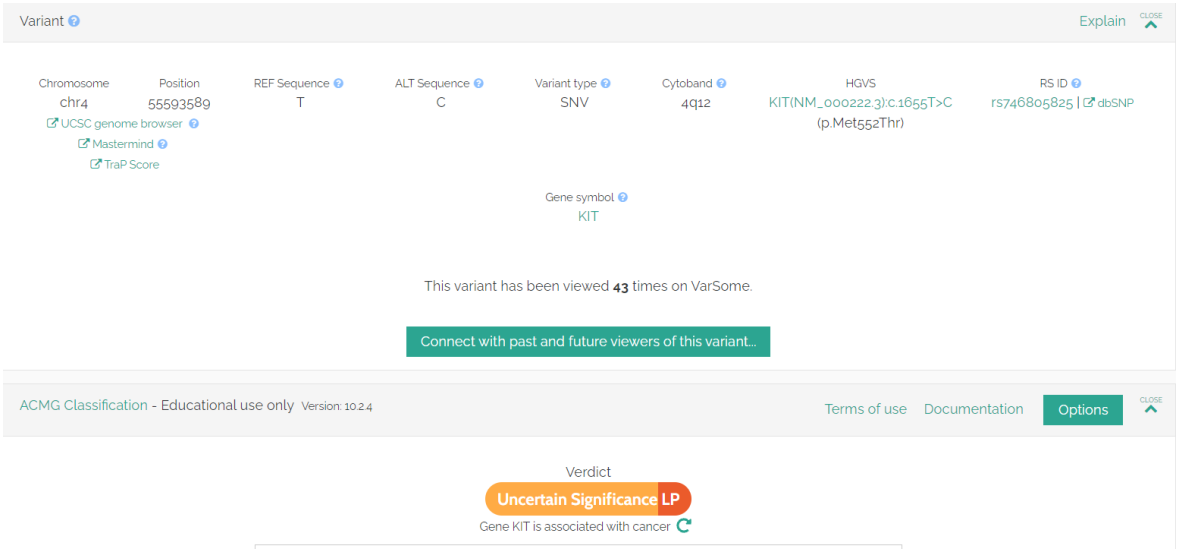
Attachment 20: KIT V560D SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/KIT(NM_000222.3)%3AV560D?annotation-mode=germline



Attachment 21: KIT V654A SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/KIT%20V654A?annotation-mode=germline

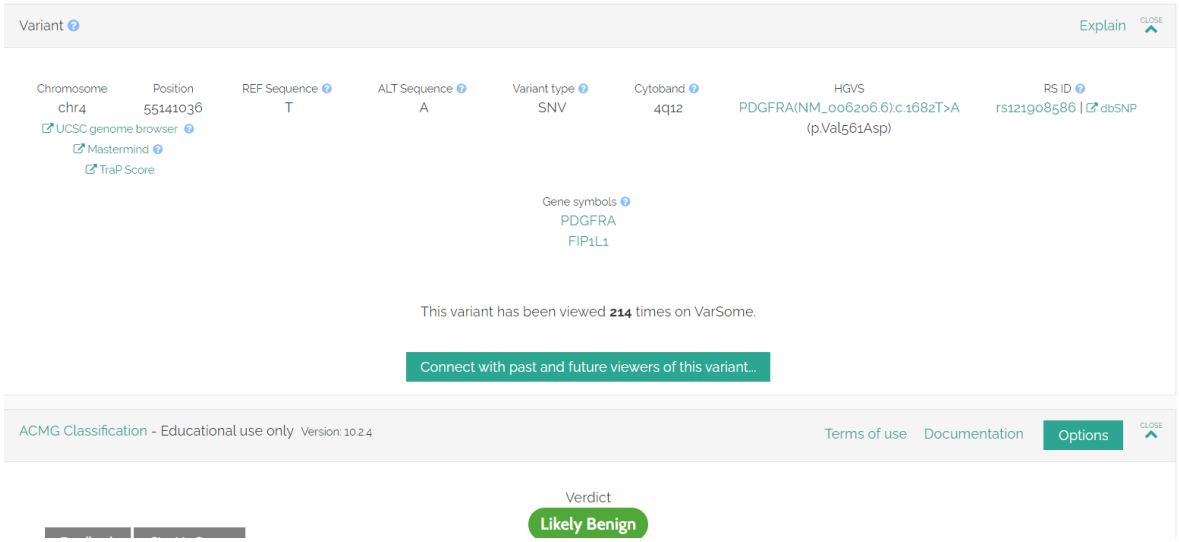


Attachment 22: KIT V559A SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/KIT%20V559A?annotation-mode=germline



Attachment 23: KIT M552T SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/KIT%20M552T?annotation-mode=germline

PDGFRA:



Attachment 24: PDGFRA V561D SNV | hg19 [Internet]. [cited 2021 Oct 11]. Available from: https://varsome.com/variant/hg19/PDGFRA(NM_006206.6)%3AV561D?annotation-mode=germline