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# Evaluation of gene expression profile and gene fusions in Gastrointestinal Stromal Tumors: looking for novel predictive and prognostic biomarkers

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# Abstract

**Background:** Gastrointestinal Stromal Tumors (GISTs) are the most frequent malignant mesenchymal tumors in the gastrointestinal tract, accounting for 1% of all primary gastrointestinal tumours. Although mutational status is not currently used for risk assessment, tumour genotype has been shown to have a prognostic influence on natural history and tumour recurrence. Detection of allele frequency (VAF) levels of specific *KIT/PDGFRA* variants by next-generation sequencing (NGS) can act as a surrogate for tumour burden and correlate with the prognosis and overall survival of GIST patients, aiding the choice of adjuvant treatment. Furthermore, a growing body of evidence shows that Hippo signalling pathway is frequently dysregulated in tumours, and high nuclear expression of *YAP* and *TAZ* is closely linked to unfavourable outcomes and increased drug resistance in several cancers.

**Methods:** This was retrospective/prospective study that included fully resected GIST patients to correlate pathogenic variant (PV) codon type and position with clinicopathological features and outcome, and the prognostic role of *KIT* or *PDGFRA*-VAF. Finally, we evaluated the gene expression of *YAP*, *TAZ* and other genes involved in the Hippo pathway and the presence of gene fusions in wild type (WT) patient by NGS.

**Results:** Tumors harboring a *KIT* exon 11 deletion or deletion/insertion involving the 557 and/or 558 codons, showed a more aggressive clinical behavior compared with tumors carrying deletion/deletion/insertion in other codons, or tumors with duplication/insertion/single-nucleotide variant (SNV). When we studied the VAF normalised with neoplastic cellularity (nVAF), patients with *KIT/PDGFRA* nVAF > 50% showed less favorable Recurrence Free Survival (RFS) than patients in the group of nVAF ≤ 50%. In patients with KIT-mutated GIST, the presence of nVAF >50% was statistically associated with higher disease recurrence. From the expression analysis, the genes most frequently upregulated were *YAP*, *TAZ* and *CTGF*. Patients showing this overexpression were mainly WT and *PDGFRA* mutated, most frequently with small bowel GIST and primary tumor diameter >5 cm. Interestingly, all WT patients showed overexpression of *YAP* and/or *TAZ* and *CTGF*. In contrast, no patients were found to have overregulation of *CCDN1* and *AXL*. Finally, WT patients did not show the presence of gene fusions.

**Conclusion:** Our data confirmed the importance of the molecular characterisation of GISTs in order to improve the stratification of patients according to more detailed criteria and allow better clinical management. We demonstrated that higher levels of nVAF in patients with localised GISTs presenting *KIT* or *PDGFRA* mutations were independent predictors of prognosis and survival. These data could be relevant in the cohort of intermediate-risk patients to improve prognosis and the use of adjuvant imatinib.

Finally, gene expression analysis showed the potential role of Hippo and pathway-related genes as oncogenic mediators in GISTs, especially in WT patients and patients with disease recurrence.

CHAPTER 1

# Background

## 1.1 Generalities and epidemiology

Gastrointestinal stromal tumours (GISTs) are rare neoplasms of mesenchymal origin that may arise throughout the gastrointestinal (GI) tract. They are the most common mesenchymal tumours of the GI tract, accounting for 1% of all primary gastrointestinal tumours(1).

Based on some phenotypic similarities, GISTs are derived from interstitial cells of Cajal (ICCs) or their precursors. ICCs are responsible for intestinal peristalsis, acting as pacemakers of the muscolaris propria and regulating gastrointestinal motility(2).

Their incidence, often underestimated due to numerous subclinical forms, is estimated to be around 10-15 new cases per one million inhabitants per year, with a slightly higher frequency in the male population and an average onset between 60 and 65 years of age(3, 4).

Microscopic forms or microGISTs are much more frequent in the general population and represent preclinical forms of GISTs with a size <1.0 cm. Several studies have identified microGISTs in the GI tract of approximately 30% of unselected individuals with the presence of pathogenic tyrosine kinase variants.On the other hand, very rare are the cases occurring in childhood, termed pediatric GISTs, which represent a clinically and molecularly distinct subgroup characterized by the absence of *KIT*/*PDGFRA* alterations, frequent mutations or reduced expression of the four genes coding for the subunits of the succinate dehydrogenase (SDH) enzyme complex, a multicentre gastric onset site and possible lymph node metastases.

Finally, about 5% of cases are familial GISTs, characterised by early onset, within the age of 30(5). The following syndromes are associated with GISTs:

- Carney triad syndrome, caused by hypermethylation of the succinate dehydrogenase subunit C gene (*SDHC*) and clinically it is characterized by multifocal gastric GISTs

associated with paragangliomas and pulmonary chondroma. Onset of the first tumour occurs in adolescence with a predominance in the female(6);

- Carney-Stratakis syndrome, caused by a germline mutation in one of the subunit genes (A, B, C and D) of the SDH enzyme complex and clinically it is characteried by two typical dyad tumours, gastric GIST and paraganglioma, occurring from late adolescence to adulthood with no gender predominance(6, 7);

- Neurofibromatosis type 1 (NF1), characterised by a germline mutation in the *NF1* gene, associated with GISTs located predominantly in the small intestine, usually multifocal, with low mitotic rates(8, 9).

On the other hand, primary familial GIST syndrome, caused by autosomal dominant germline mutations in the *KIT* and *PDGFRA* genes, is very rare. It is characterized by a predisposition to the development of multiple GISTs at an early age in association with other morbidities, such as paragangliomas, skin hyperpigmentation, inflammatory fibroid polyps or intestinal fibromatosis(10, 11).

### 1.2 Clinical presentations

Most GISTs arise in the gastrointestinal tract, most frequently in the stomach (50%), followed by the small intestine (25%), rectum (5%) and oesophagus (<5%). More rarely they occur extraintestinally (<5%) originating in the mesentery or omentum(12).

Clinical manifestations depend on location and size of the tumour. Generally, small GISTs are asymptomatic and are diagnosed incidentally during diagnostic or surgical procedures for other manifestations(13). Symptomatic GISTs, on the other hand, present symptoms that are mostly nonspecific: nausea, dyspepsia, abdominal distension, anaemia(14). Larger tumours may cause, based on the type of growth, obstruction of the gastrointestinal lumen (endophytic growth) or compression of the gastrointestinal tract (exophytic growth). More severe cases include perforated neoplasms, which manifest as peritonitis or gastrointestinal bleeding leading to anaemia, melena or haematemesis(15, 16). Approximately 30% of cases present at diagnosis with locally advanced or metastatic disease. About 65% of metastatic patients have liver involvement, about 20% peritoneal involvement, while lymph node, skeletal or lung metastases are rarer(17, 18).

### 1.3 Histological features and risk stratification of recurrence

GISTs are lesions originating from the muscular tonaca propria or muscolaris mucosae of the GI tract wall, with mainly submucosal, subserosal or intraparietal development(19).

Macroscopically, they are generally well circumscribed, varying in size (from a few mm to more than 30 m), unencapsulated, and white in color(20, 21).

Based on size, small GISTSs (<5 cm) result as symmetrical masses with a homogeneous, well-demarcated surface, with sharp edges and show, with an unchanged mucosal lining and a generally intraluminal growth profile. In contrast, GISTs of intermediate size (5-10 cm) are less symmetrical and may show an intraluminal and extraluminal growth profile. Finally, large GISTSs (>10 cm) are highly irregular, show areas of hemorrhage and necrosis, the lining mucosa may ulcerate, and show extremely aggressive behavior, often resulting in liver, peritoneal, or distant metastasis(13).

Based on histologic features, however, GISTs are characterized by spindle cell (70%), epithelioid (20%) or mixed (10%) morphology. GISTs exhibiting spindle cell morphology are characterized by rich cellularity; cells are arranged in short bundles, have uniformly eosinophilic cytoplasm, and nuclei are generally uniform(22). Epithelioid cell GISTs are most often located in the stomach and are characterized by rounded cells with nest-like architecture, a clear cytoplasm, and round nuclei. In contrast, a minority of tumors exhibit mixed cell morphology, containing both cell types, fusiform and epithelioid, which may be arranged either separately or in conglomerates(23).

On an immunohistochemistry (IHC) perspective, the most widely used markers for histopathological identification of GISTs are c-kit (CD117) and anoctamine 1 (DOG1).

Expression of c-kit is quite specific for GISTs among gastrointestinal mesenchymal tumors, and 90% of GISTs show widespread cytoplasmic positivity (24).

In addition, one third of the cases show positivity to DOG1, which to date is considered a sensitive and specific marker for GISTs(25).

Other immunophenotypic markers, variably expressed but not specific for GISTs are CD34, protein chymase C, smooth muscle actin, h-caldesmon, and, rarely, desmin(26).

The histopathologic diagnosis of GIST must also include the evaluation of additional factors that contribute, in combination with the anatomic site of onset, to defining the risk of relapse and, more generally, the patient's prognosis. Among these, the description of the mitotic index, size, and definition of the integrity of the tumor capsule are relevant as they condition the biological behavior of the disease.

The first model proposed for stratification of risk of recurrence considered two parameters: tumor diameter and mitotic index expressed as number of mitoses per 50/high magnification fields (HPF) (27).

Specifically, in 2006 Miettinen et al. evaluated a cohort of 1,765 patients with GISTs in the stomach and a smaller cohort of 906 patients with primary-onset GISTs in the small intestine, demonstrating that, in addition to tumor size and mitotic index, the anatomic site

of the primary tumor is an additional prognostic factor for the relapse risk(28). This classification system demonstrates that, for the same size and mitotic count, the risk of recurrence is greater in GISTs with a primary non-gastric location than in GISTs originating at gastric sites. Of considerable importance is the definition of the integrity of the tumor capsule, as its rupture results in an absolute risk of recurrence. In addition, the same research group introduced the determination of the mitotic index as the number of mitoses in a total area of 5 mm2, a hypothesis supported by the most recent ESMO-EURACAN guidelines in which the use of mitotic count expressed in a total area of 5 mm2 is recommended rather than as an expression of the number of mitotic cells in 50HPFs(12) (**Table 1**).

Recently, the type of pathogenic variant (PV) present in GISTs has been shown to have a prognostic influence on relapse-free survival (RFS) (29, 30). Despite this, genotype is not currently included in risk stratification systems but it is a predictive parameter of response to receptor tyrosine kinase activity inhibitor (TKI) drugs.

### 1.4 Molecular classification

Molecular profiling of GISTs is essential for therapeutic personalization with targeted drugs.

Approximately 75-80% of sporadic GISTs are characterized by the presence of alterations in the *KIT* gene, resulting in constitutive activation of the cKIT receptor and proliferative signaling cascades downstream of the receptor independently of ligand binding(31). The main alterations found are point mutations, deletions, duplications, insertions or combinations thereof. These variants fall most frequently in exons 11 and 9, which are generally present at the onset of disease, while mutations involving exons 13, 14, 17 arise more frequently during progression or in the late stages of the neoplasm. Rare cases of PVs have been described at exon 8(32, 33).

In contrast, 10-15% of GISTs have PVs in the *PDGFRA* gene that most frequently involve exon 18 and more rarely exons 12 and 14(33).

GISTs with alterations in *KIT* and *PDGRFA* have similar mechanisms of tumorigenesis and progression through oncoprotein-driven signal transduction. Therefore, *KIT* and *PDGFRA* mutations are alternative and mutually exclusive oncogenic mechanisms in GISTs(34)

Ten percent of GISTs do not show any alteration in these genes and are therefore classified as *KIT/PDGFRA WT*. Molecularly, these represent a heterogeneous group, more associated with syndromic forms (**Figure 1**). In particular, about 80% of these WT GISTs show PVs in one of the 4 subunits (A, B, C and D) of the *SDH* complex and in rare cases also hypermethylation of the *SDHC* gene promoter. All *SDH* alterations lead to loss of the

SDHB protein, whose lack of expression can be easily identified by IHC(35, 36). Clinically, this subgroup of *SDH* deficient GISTs, often associated with Carney syndrome and Carney-Stratakis syndrome, occurs in the pediatric population and young adults, with female predominance. They show a predominantly gastric localization and are characterized by a predominantly epithelioid cell morphology and multifocal appearance. In addition, they have an indolent disease progression despite frequently beginning as metastatic and not responding to imatinib therapy(37, 38).

Additional molecular alterations that can be identified in the *KIT/PDGFRA WT* subgroup of GISTs involve loss-of-function mutations in the *NF1* gene, gain-of-function mutations in *BRAF* and *RAS*, or chromosomal rearrangements involving neurotrophic receptor tyrosine kinase (*NTRK*) (36).

GISTs with *NF1* and *BRAF* PVs occur almost exclusively in the small intestine. However, while GISTs with *BRAF* mutation are isolated tumors, GISTs with *NF1* alteration more commonly present as a multifocal disease, which excludes an inherited *NF1* syndrome.

To date, understanding knowledge from genomic profiling through molecular testing is an ongoing challenge for the care of GIST patients.

With the introduction of NGS into clinical practice, molecular characterization of the neoplasm has become an indispensable step in the patient's diagnostic therapeutic process (39). NGS has enabled a significant step forward for precision medicine through the detection of driver mutations and pathogenic variants inducing resistance mechanisms, through the detection of novel and rare mutations, and through the quantification of tumor mutational burden, which have made possible a new approach in cancer treatment(40).

One interesting parameter resulting from NGS analysis is variant allele frequency (VAF), which is the percentage measure of the relative frequency of a specific variant divided by the overall coverage for that genetic locus. Since genetic analysis by NGS takes a picture of the molecular background of the analyzed sample, VAF could be considered a surrogate measure of the proportion of DNA molecules present in the starting biological sample in which the variant is present (41).

Therefore, in somatic genomic testing, a high VAF value suggests that a high percentage of cancer cells carry a particular genomic alteration. In these cases, targeted therapy may be more easy to select and it is more effective (42). In contrast, low VAF values suggest the low clonality of the PV; therefore, in these patients, due to the presence of other subclones characterized by distinct molecular profiles, targeted therapy may be less effective(43, 44). Moreover, driver mutations, which occur early in tumor progression and contribute to tumor development and progression, have a higher allele frequency than late subclonal mutations(45, 46).

In literature, several studies have investigated the potential prognostic and predictive role of VAF in various malignancies, but not in GISTs. A study by Berrino et al. (47) correlated *BRAF* gene mutation rate and VAF with the clinicopathological characteristics of 345 melanoma patients at different stages of the disease and it showed that the incidence of UV radiation can result in different VAF in different portions of the body affected by melanoma. In fact, melanoma patients with primary site at the limb or face/scalp have a higher allele frequency of *BRAF* than melanomas of ocular or acral origin. Also, the metastatic site seems to play a crucial role. Indeed, in metastatic melanomas, lymph node metastases, originating from skin lesions, show higher *BRAF* VAF than secondary skin lesions. An interesting correlation also appears to exist between VAF and tumor-infiltrating lymphocytes (TILs); this could be interesting to select and stratify patients for benefit from immunotherapy treatment. In addition, thicker melanomas according to Clark levels have higher *BRAF* VAF (47). Further studies report a strong correlation between *BRAF* VAF and progression-free survival (PFS) (47-49).

In 2021, two more research groups further supported and highlighted the interesting clinical impact of *EGFR* VAF in patients with lung adenocarcinoma treated with *EGFR* TKIs. Specifically, a significant correlation was demonstrated between a high VAF of *EGFR* exon 19 deletion and PFS and overall servival (OS) compared with point mutations at EGFR exon 21. Overall, a VAF of *EGFR* above 70% could be positively correlated with both PFS and OS, whereas lower VAF levels could be considered a surrogate for lower responsiveness to target terapie (50, 51).

However, VAF is influenced by different factors, including neoplastic cellularity of the sample, proportion of neoplastic cells carrying PVs, and the presence of copy number. To date, insufficient standardization among sequencing assays and the absence of validated VAF thresholds limit its use in clinical practice (46).

Thus, further studies are needed to better understand this parameter and its analytical and clinical validation.

### 1.5 *KIT* gene

*KIT* is a protoncogene encoding for a 145-kDa transmembrane tyrosine kinase activity (RTK) receptor located on chromosome 4q12. The KIT receptor, also known as CD117, is expressed on ICCs, mast cells, hematopoietic cells, germ cells and melanocytes(52, 53). It consists of several regulatory domains including the dimerization domain in the extracellular region (EC), the transmembrane region, the iuxta-membrane domain (JM), and the intracellular tyrosine kinase domains (TK[I] and TK[II]). Kinase activation triggers activation

by phosphorylation of a variety of intracellular signaling pathways, such as JAK-STAT3, PI3K-Akt-mTOR, and the signaling pathway involving MAPKs, which are essential in the regulation of cellular functions as proliferation, differentiation, and apoptosis.

In GISTs, all pathogenic alterations found in *KIT* result in constitutive activation of the receptor, even in the absence of its ligand, resulting in deregulation of downstream pathways(1). Mainly, mutations in the *KIT* gene involve exons encoding for functional domains of the receptor such as exons 9, 11, 13, and 17. The most frequent mutations (70-80%) found in exon 11 involve the iuxtamembrane domain and are mainly deletions/in-frame insertions within codon Gln550 and Glu560 and more rarely single nucleotide mutations. In addition, frequent deletions can also occur at codons 557-558 of exon 11 of *KIT* (28% of all GISTs) and have been associated with poor prognosis with reduced recurrence free survival (RFS) (29, 30). About 12-15% of *KIT* mutated GISTs exhibit exon 9 duplications at codons 502 and 503, which are critical for extracellular domain integrity. These GISTs have been associated with more aggressive behavior and localization in the small intestine. From a therapeutic point of view, molecular alterations at exon 9 of *KIT* are associated with greater resistance to Imatinib treatment than exon 11 alterations, requiring double dosing of the drug (54).

Alterations in the kinase domain are rare and it have been observed frequently as secondary mutations, arising after selective pressure from TKIs in GISTs, associated to imatinib resistance. These are grouped in two regions of the kinase domain: the binding pocket, encoded by exons 13 and 14, and in the activation loop, encoded by exons 17 and 18 (33).

### 1.6 *PDGFRA* gene

The *PDGFRA* gene, located on chromosome 4q12, also encodes for a tyrosine kinase activity receptor. Its activation depends on binding to its ligand, platelet-derived growth factor (PDGF), and it is involved in many physiological processes of growth and development in the human body (55). Based on the combination of monomers, five different isoforms of PDGF are distinguished (PDGF-AA, PDGF-AB, PDGF-BB, PDGF-FCC and PDGF-DD), which recognize and bind PDGFRα and PDGFRβ receptors. Normally, after ligand-receptor binding and subsequent phosphorylation of the TK domains, a series of signaling cascades (particularly RAS/RAF/MAPK, PI3K-mTOR, Jak/STAT and Notch) are activated affecting cell proliferation, apoptosis and angiogenesis (56). The presence of PVs leads to ligand-independent receptor activation, resulting in a constitutively active proliferative signal that promotes the neoplastic event. PVs in *PDGFRA* gene occur in about

10-15% of GISTs, and these are tumors mainly arising in the stomach, with epithelioid morphology (57). Molecular alterations occur most frequently at exon 18 (activation loop), followed by exon 12 (iuxtamembrane domain) and exon 14 (ATP binding site) (58). Data from cohort studies correlate the PVs in *PDGFRA* exon 18 with a favorable disease outcome compared with *KIT* point mutations on exon 9 and deletions involving codons 557 and/or 558 of exon 11(29). In contrast, it is well described that a missense mutation at exon 18, p.D842V, observed in approximately 50 to 70% of mutated *PDGFRA* GISTs, confers primary resistance to Imatinib treatment and other TKIs due to a stabilization of the kinase conformation in its active form (59).

### 1.7 Gene fusions

GISTs that do not carry PVs in *KIT*, *PDGFRA*, *SDH* or *BRAF*/*RAS*/*NF1*, called WT quadruple GISTs, show molecular and biological features still unclear (60). Recent genomic profiling studies have identified gene fusions in WT GISTs, involving genes encoding for the neurotrophic neurotrophic receptor tyrosine kinase (*NTRK*) family (*ETV6-NTRK3* and *LMNA-NTRK1*), *FGFR1* (*FGFR- HOOK3* and *FGFR1-TACC* ), *BRAF* ( *BRAF-AGAP3* and *BRAF-MKRN1* ) and the *ALK* gene (*CDC42BPB-ALK*) (61-63)

The *NTRK* gene family, including *NTRK1*, *NTRK2* and *NTRK3*, encode for the tropomyosin (TRK) receptors TRKA, TRKB, TRKC, respectively, which are involved in normal neuronal development (64). The extracellular portion is composed of three leucine-rich motifs (RRL1-3), lined by two cysteine clusters (C1-C2) and two Ig like domains (Ig1 and Ig2) linking to the transmembrane domain. The Ig2 domain, located near the trans-membrane region, is the one predominantly involved in ligand binding and it is important to determine binding specificity. At the intracellular level there is a tyrosine kinase domain that, upon receptor homodimerization, undergoes self-phosphorylation resulting in activation and signal transduction(65, 66). The main pathways consequently involved downstream are MAPK, PI3K and PKC, which ultimately converge on gene transcription of factors involved in neuronal differentiation, proliferation and survival, as well as synaptic formation and plasticity. Physiologically, *NTRK* genes are predominantly transcribed at the level of the nervous system in the adult and also during embryonic developmental stages (67).

Oncogenic activation of TRK is mainly caused by *NTRK* gene fusion, which consists of interchromosomal or intrachromosomal gene rearrangements resulting in the union of the 3' region of the *NTRK* gene with the 5' end of a fusion partner gene (68).

Currently, more than 80 fusion gene partners have been described in multiple tumor types. Although rare in the most common tumor types, *NTRK* gene fusions, on the other hand, are

recurrent in some very rare neoplasms, including salivary gland secretory carcinoma, breast secretory carcinoma, congenital mesoblastic nephroma, GIST, pediatric melanoma tumor, and childhood fibrosarcoma (69, 70).

*NTRK* fusions are considered an agnostic feature that makes tumors sensitive to TRK inhibitors, independent of the primary cancer. NTRK inhibition has been shown to be highly effective leading to durable responses regardless of patient age, tumor tissue and fusion gene partner, blocking the action of TRK proteins that promote tumor spread and growth. The identification of tumors carrying *NTRK* gene rearrangements is important for the selection of patients eligible for treatment with tyrosine kinase inhibitors (TKI), such as Entrectinib and Larotrectinib (71).

### 1.8 Hippo pathway

Tumor origin and evolution represent highly complex processes involving numerous signaling pathways. One of the pathways that has received particular interest in the past decade is the Hippo signaling pathway.

This is a highly conserved cross-species pathway involved in several biological processes including cell proliferation and differentiation, tissue regeneration, homeostasis, and organ size; therefore, it plays a vital role during embryogenesis (72).

The Hippo pathway is named after one of its key signaling components, originally identified in *Drosophila melanogaster*, the gene that encodes for Hippo protein kinase (Hpo) or STE20-like kinase 1/2 (STK4/3) that encodes for MST1/2 in humans. This pathway consists of two main ways: the central serine/threonine kinase cascade, which is regulated by several upstream signals, and the transcriptional module that induces the expression of specific target genes downstream. The central cascade, in mammals, consists of MST1/2, Salvador homologous protein 1 (SAV1), Mps One Binder Kinase Activator-Like 1 A/B (MOB1A/B), large tumor suppressor kinase 1/2 (LATS1/2), Yes-associated protein 1 (YAP), WW domain containing transcriptional regulator 1 (WWTR1 or TAZ), and (transcriptional enhanced associated domain) (TEAD) family.

The striatin-interacting phosphatase and kinase (STRN) complex (STRIPAK) acts upstream of Mitogen-Activated Protein Kinase Kinase (MAP4K) and MST1/2 and inhibits the Hippo pathway. When the Hippo pathway is activated, however, MAP4K, MST1/2 and its scaffold protein SAV1 phosphorylate and activate LATS1/2 and its scaffold MOB1A/B. The latter functionally act in a tumor suppressor manner by phosphorylating and inactivating members of the transcriptional module, YAP and its paralog TAZ, which are then retained in the cytoplasm of the cell and subsequently degraded (73). In the absence of an active kinase

cascade, YAP/TAZ, being transcriptional coactivators, translocate into the nucleus and bind to TEAD1-4, and regulate the expression of several genes such as connective tissue growth factor (*CTGF*), cysteine-rich angiogenic inducer 61 (*CYR61*), AXL Receptor Tyrosine Kinase (*AXL*), and others to promote proliferation cell differentiation and survival, apoptosis, and stem cell self-renewal (74-76).

A growing amount of evidence shows that this signaling pathway is frequently dysregulated in tumors; in particular, it is associated with tumor development and progression, tumor cell invasion and migration, regulation of the tumor microenvironment, and resistance to therapy (**Figure 2**) (77).

In NSCLC, overexpression of *YAP* has been seen to be associated with poor prognosis and disease progression, and *TAZ* exerts a similar function (78-81). In addition, *LATS2* is underregulated in 60% of NSCLC tumors, while high levels led to better prognosis and negative regulation of *YAP* (82).

Other studies have established the prognostic value of the transcriptional levels of *YAP1* and *TAZ* for colorectal cancer (CRC) patients, and the mRNA expression rates of *TAZ* and *YAP1* were positively correlated with their downstream targeted genes *AXL* and *CTGF* (76, 83).

Several evidence confirm that high nuclear expression of YAP and TAZ proteins is closely linked to poor outcomes and increased drug resistance in several cancers. Targeting YAP and TAZ in tumors with high levels of expression or activity of these proteins could be an attractive strategy (84).

To date, there are few data in the literature investigating the possible role of this pathway in GISTs.

Chen et *al.*(85) demonstrated that KIT-independent and imatinib-resistant GISTs had overexpression of cyclin D1 (*CCND1*) induced by Transcription Factor Jun and *YAP*/*TAZ*, and 'inhibition of these factors showed dramatic antiproliferative effects (85, 86).

Another gene apparently over-regulated in GISTs is Limb Expression 1 (*LIX1*), which has been identified as a marker of gastrointestinal mesenchyme immaturity. *LIX1* has been shown to induce, through Hippo's YAP effector, proliferation and differentiation of mesenchymal progenitors, and its overexpression is associated with poor prognosis. In addition, after its inactivation, in GISTs cells, YAP/TAZ activity is reduced, *KIT* is down-regulated, and the cells reacquire the phenotype of the smooth miscular lineage (87).

In vitro studies in GISTs cell lines have shown how cilostazol, a phosphodiesterase 3A (PDE3A) inhibitor, synergizes with imatinib, causing nuclear exclusion of YAP and reducing cell viability by 90% (88). Furthermore, it has been seen that GIST cell lines are highly

sensitive to the induction of iron-dependent cell death by inhibition of YAP by verteporfin (89).

Therefore, due to its involvement with regenerative and pro-cancerous functions, the Hippo pathway is an attractive target for cancer treatment challenges. Further investigation and functional studies, however, are needed to clarify its precise role in specific tumor types.

# Rationale

GISTs are the most frequent malignant mesenchymal tumors in the gastrointestinal tract and it have historically been the standard model for precision oncology and oncogene addiction (1, 90).

The OS of these patients was significantly improved with the full understanding of *KIT* and *PDGFRA* as GIST oncogenic drivers, together with the remarkable success of tyrosine kinase inhibitors (TKIs). In recent times, technological advancements and the proactive use of thorough molecular profiling have increased the molecular depth of information that clinicians can access (91).

In addition to *KIT* and *PDGFRA* alterations, which together account for approximately 85% of cases, and 9% of GISTs caused by loss of function of SDH, a variety of distinct oncogenic factors have been described in the subpopulation of GISTs originally defined as 'wild-type', including activating pathogenic variants of *BRAF* and members of the *RAS* family, and translocations of *NTRK*, *FGFR* and *ALK* (92).

Although genotypic profiling has not been incorporated into recurrence risk stratification, several scientific evidence underline the importance of tumour genotyping in clinical practice to adapt treatments according to mutational status.

Today, understanding the knowledge derived from genetic profiling through molecular testing for the treatment of GIST patients represents an ongoing challenge (39).

Particularly, the information obtained from high-performance sequencing may have clinical significance in localized and metastatic diseases. In the adjuvant setting, imatinib treatment is now recommended for localized GIST patients that are in high-risk for recurrence; however, more accurate prognostic information may improve patient selection for adjuvant therapy (12).

One interesting parameter, among the information returned by NGS analysis, is the variant allele frequency (VAF), that represents the frequency of a specific variant in the overall coverage for that genetic locus, expressed as a percentage (41).

Although the exact frequency of the allele fraction is difficult to estimate as it is influenced by several factors including the proportion of tumour cells present in the tumour sample, *KIT/PDGFRA* -VAF of the tumour tissue could represent a surrogate measure of the proportion of GIST cells harbouring the specific DNA mutation. Several studies have evaluated the impact of allele frequency on the survival of cancer patients (47).

In this study, it was hypothesised that tumour VAF might play a prognostic role in GIST patients.

Furthermore, in the context of genomic profiling, which has made it possible to obtain detailed and unique information on the individual tumour, representing a decisive advantage in the management and treatment of the individual cancer patient, the Hippo signalling pathway is attracting particular interest.

Tumour origin and evolution are very complex processes and it is now well recognised that this pathway regulates several biological processes including cell proliferation and differentiation, tissue regeneration, homeostasis and organ size (72).

A growing body of evidence shows that this signalling pathway is frequently dysregulated in tumours; in particular, it is associated with tumour development and progression, tumour cell invasion and migration, regulation of the tumour microenvironment and resistance to therapy

Several evidences confirm that high nuclear expression of *YAP* and *TAZ* proteins is closely linked to unfavourable outcomes and increased drug resistance in several cancers (77).

Based on this evidence, we wanted to investigate the role this pathway could play in GISTs, with the aim of finding new factors, such as gene expression patterns or associations of genes with molecular or clinical features, in the different molecular subgroups, that could assume a potential prognostic and/or predictive value for a better management of the cancer patient.

# Objectives

The objectives of this research project were:

- to study the effect of the exact type and codon position of *KIT* exon 11 PVs on clinical outcome and the potential association with metastatic sites in patients with relapse of disease;
- to analyze how the detection of *KIT*/*PDGFRA* PV nVAF levels can act as a surrogate of tumor burden and negatively correlate with the prognosis and overall survival of patients with localized GIST;
- search for the presence of gene fusions in patients with WT GISTs for the genes involved in the pathology;
- to study the gene expression of *YAP* and *TAZ* and genes related to the Hippo pathway as predictors of biological tumour behaviour.

In the first phase, the identification of gene alterations in GIST patients was carried out. In WT patients, who did not show the presence of pathogenic variants, gene fusion analysis was performed. Both analyses were performed on neoplastic tissue sections by multigene panel in Next Generation Sequencing (NGS).

Subsequently, the information obtained by molecular analysis in NGS was correlated with clinico-pathological data, in order to improve the molecular characterization of the tumor for better stratification of patients and to investigate the potential prognostic role.

During the second phase, however, expression analysis of Hippo pathway-related genes was conducted on a cohort of patients selected based on the molecular alterations detected. Analysis was performed on RNA extracted from neoplastic tissue by digital droplet-PCR.

The aim of the work was to investigate the molecular basis of this rare tumor and to find new factors, such as patterns of gene expression or associations of genes with molecular or clinical features, that could potentially have a potential prognostic and/or predictive value for better management of the cancer patient.

CHAPTER 2

# Patients and Methods

## 2.1 Study Population

The following studies were carried out at the ‘SicilianRegional Center for the Prevention, Diagnosis and Treatment of Rare and Heredo-Familial Tumors’ of the Section of Medical Oncology of University Hospital Policlinico ‘P. Giaccone’ of Palermo.

All the patients provided written informed consent for the collection of the clinical, pathological, and genetic information required. All data were anonymously recorded.

The studies (Protocol ‘G-Land 2017’ and Protocol ‘EVA GIST Project’) were approved by the ethics committee (Ethics Committee Palermo 1; approval number: 01032017; 04-130422) of the AOUP ‘Paolo Giaccone’ University Hospital of Palermo.

### 2.1.1 Analysis 1

In this retrospective analysis all patients with localized GISTs completely resected referred to the centre between January 2005 and December 2020 were included.

Patients with primary metastatic or inoperabile GISTs, or patients previously treated with neoadjuvant imatinib or adjuvant imatinib therapy were excluded.

To study the clinico-pathological characteristics of patients with GISTs according to mutational status, information on sex, age, primary tumour site of origin, primary tumour diameter and mitosis, Miettinen-Lasota risk category classification (28) and *KIT* or *PDGFRA* PV was collected. The effect of *KIT* exon 11 mutations, particularly *KIT* exon 11 deletions or deletions/insertions involving codons 557 and/or 558, on RFS was evaluated.

Following surgery, staging was performer using contrast-enhanced computed tomography of the chest, abdomen and pelvis. Periodic evaluations included abdominal computed tomography and blood biochemical analyses.

Tumor metastatic sites, such as the liver, peritoneum, or both the liver and peritoneum,

were reported in *KIT* exon 11-mutant patients with relapsed tumors after curative surgery (93).

### 2.1.2 Analysis 2

Multicentre, hospital-based, retrospective cohort study to investigate the prognostic role of *KIT* or *PDGFRA* -VAF of GIST in patients with localised disease and radically resecate tumours.

Patients with metastatic GIST at diagnosis, or lacking molecular test information and clinical follow-up data, were excluded from the analysis.

The histological subtype, primary tumor diameter, mitotic count, and primary tumor origin site collected on primary GIST.

All included patients had a tumor molecular profiling testing result by using a targeted NGS panel for the presence of GIST hotspot mutations. Mutational analysis was locally assessed at each participating center as part of routine clinical care. The clinical data on GIST surgery, the type and duration of adjuvant treatment, and tumor recurrence were abstracted from the clinical records. RFS and OS were calculated. The association between VAF (%) and the clinical outcomes wase valuated (94).

### 2.1.3 Analysis 3

Patients with histologically confirmed diagnosis of primary localised and metastatic GISTs at diagnosis were included in this prospective study.

Clinico-pathological data concerning sex, age, site of origin of primary tumours, primary tumour diameter, mitotic index were collected.

Molecular analysis was performed to assess mutational status and gene fusion analysis was performed in GISTs from patients that didn't show the presence of pathogenic variants. Both analyses were performed on neoplastic tissue sections by means of a multi-gene panel in NGS.

Subsequently, a cohort of patients with GIST was defined on the basis of the molecular alterations presented. On the last cohort, gene expression of *YAP*, *TAZ* and Hippo pathway-related genes was assessed using digital droplet-PCR (ddPCR).

## 2.2 Sample collection, DNA and RNA extraction and quantification

GIST's tissue samples were accessibile as exploratory biopsies or neoplastic tissue obtained by surgery and availableas Formalin-Fixed Paraffin-Embedded (FFPE). Tissue samples were sectioned at 10 µm with >20% malignancy from the pathology laboratory of the same hospital agency.

The extraction of DNA and RNA from FFPE tissue was obtained using a commercially available extraction kit according to the manufacturer's protocol.
This extraction method involves the use of silica membrane columns for the isolation and purification of nucleic acid. The first step involves an initial de-paraffination phase followed by a lysisphase. This is continued with digestion using proteinase K, which partially removes the formalin bond with the released nucleic acid, improving the yield and performance of downstream analyses. The DNA and RNA obtained was quantified with a fluorometer and its quality was assessed.
This quantisation system allows the measurement of fluorescence emission using a fluorophore thats pecifically binds double-stranded DNA (dsDNA). In addition, itis a more sensitive system than quantification based on UV absorbanceas the latter does not distinguish between dsDNA, single-stranded DNA (ssDNA), RNA and nucleotides.

### 2.3 Direct automated sequencing: Sanger method

The evaluation of the presence of pathogenic sequence variants at exons 9-11-13-17 of the *KIT* gene and exons 12-14-18 of the *PDGFRA* gene by Sanger sequencing was carried out using forward and reverse oligonucleotides drawn on the target regions of interest (**Table 2**) and with the BigDyeTM Terminator v1.1 CycleSequencing kit on the SeqStudio Genetic Analyzer platform (Thermofisher), according to manufacturer’s protocols.

### 2.4 Next-Generation Sequencing analysis

NGS analysis was performer using the Ion Torrent S5 platform with a multigene panel (“Oncomine Focus Assay Panel” Thermofisher) that allows the identification of variants in 52 relevant genes in solid tumours. The test allows simultaneous analysis of DNA and RNA to detect multiple variant types simultaneously, including hotspots, single nucleotide variants (SNVs), indels, copy number variants (CNVs) and gene fusions (**Figure 3**). The Invitrogen™ SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) was used for reverse transcription.
The Ion AmpliSeq Library kit 2.0 (ThermoFisher) was used to prepare the libraries following the instructions given in the protocol.
The first step is the creation of a library of amplicons obtained through a series of amplification reactions using one or more pools of primers designed on the target sequences of interest. The amplicons will then undergo a partial fragmentation reaction by endonucleases, that cut the DNA/cDNA at specific palindromic sequences, creating

cohesive ends that will be exploited in the subsequent ‘ligation’ phase of adapters and barcodes. Barcodes, in particular, are nucleotide sequences of knownlength and sequence that, being recognised by the software during analysis, allow each library and thus each individual patient to be uniquely identified. The adapter, on the other hand, enables the binding of the library fragment to a solid phase (in the specific case of the platform used, these are marbles) on which subsequent amplifications will take place in the so-called ‘template preparation’ phase. Subsequently, the ‘barcoded’ libraries will be quantified by fluorimetry or by real-time PCR (Ion Library TaqMan®Quantitation Kit), brought to an equimolar concentration and merged.

Subsequently, an emulsion PCR was set up to achieve target enrichment and ssDNA template preparation. The final step is sequencing with the NGS Ion Torrent S5 platform (Thermofisher Scientific).

Ion Torrent sequencing chemistry is based on the sequential addition of nucleotides, interspersed with each other by a series of washes, defining the so-called ‘flow’. Nucleotide in corporation will result in a pH change due to the release of an H+ ionas a reaction by-product. This change will be detected by the sensing layer of the chip so that the chimica signal reaches the silicon substrate, which will send the data to a bioinformatics system that will allow it to be converted into an electrical signal.

Therefore, an ionogram is generated in which each of the individual peaks corresponds to an embedded nucleotide. The Ion S5 system processes the raw data displayed in the ionogram in the form of files called fastQs that will be converted by dedicated software into interpretable data. In particular, the NGS analysis parameters can be visualised using the Torrent Suite software, which returns information on the quality of the analysis (ISP density, chip loading, enrichment, readlenght, usable reads, average coverage and others). The actualcalling of variants and related genetic and pathogenic information isdone via the Ion Reporter Software.

The classification of the variants was performed by consulting the databases “Catalogue of Somatic Mutations in Cancer” (COSMIC), Varsome tool, and ClinVar.

### 2.5 Reverse Transcription - Droplet Digital Polymerase Chain Reaction (RT-ddPCR)

Restrotranscription and gene expression analysis was performer using the One-Step RT-ddPCR Advanced Kit for Probes (Bio Rad), following the instructions given in the protocol.

This kit simplifies the reaction setup by combining the first-strand cDNA synthesis (reverse transcription) and qPCR in one mixture. It also greatly reduces the possibility of

contamination by eliminating the cDNA to PCR operation step. One-step RT-qPCR can use only a limited number of probes per sample, but because it amplifies the whole sample, the sensitivity is greatly enhanced.

The optimized enzyme blend enables partitioning of RNA samples into droplets while keeping the enzymes inactive until the reverse transcription reaction is performed at 50°C. This enhances the specificity and efficiency by ensuring full enzyme activation for primer-mediated cDNA conversion. The supermix contains RNase inhibitor that protects the RNA throughout the entire workflow.

The genes specifically investigated are: *YAP*, *TAZ*, *CTGF*, *CCDN1*, *LIX1*, *NFKB*1, *AXL*. *GAPDH* was used as the housekeeping reference gene.

Hydrolysis assays including a fluorescently labeled sequence-specific oligonucleotide probe (TaqMan Hydrolysis Probes) in addition to the sequence-specific primers were used. TaqMan assays exploit the 5' exonuclease activity of certain thermostable polymerases. The hydrolysis probe is labeled with a fluorescent reporter at the 5' end and a quencher at the 3' end.

When the probe is intact, the fluorescence of the reporter is quenched due to its proximity to the quencher. The amplification reaction includes a combined annealing/extension step during which the probe hybridizes to the target and the dsDNA-specific 5' to 3' exonuclease activity of Taq cleaves off the reporter. As a result, the reporter is separated from the quencher, resulting in a fluorescence signal that is proportional to the amount of amplified product in the sample.

The reaction mix was prepared for the number of reactions needed according to the guidelines shown in the **Table 3**. Each sample was applied in duplicate, with a concentration of 20ng for each reaction.

Subsequently 20 µl of reaction mix was transferred into a sample well of a DG8™ Cartridge for QX200™ Droplet Generator, followed by 70 µl of Droplet Generation Oil for Probes into the oil wells. After gene rating droplets with the QX200™ Droplet Generator, they were carefully transferred to a clean 96-well plate, which was sealed using PX1 PCR Plate Sealer.

We proceeded with the thermal profile described in the **Table 4** and subsequent reading of droplet in the QX200 Droplet Reader.

After reverse transcription, the resulting cDNA is amplified for target detection usingTaqMan hydrolysis probes. After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target RNA was present or not present after partitioning. Each droplet provides an independent digital measurement.

Positive and negative droplets are counted and QuantaSoft™ software calculates the concentration of target RNA as copies/µl. Only reactions that had a total number of events (corresponding to the number of droplets generated) greater than 10,000 were considered. The relative expression value of each gene was calculated as the ratio of droplet numbers for the studied gene compared the reference gene.

### 2.6 Statistical analysis

Clinico pathological variables, VAF and different gene expression values were evaluated for genetic subgroups of localized GIST patients. Comparisons between subgroups were performed with Fisher's exact test, Pearson's chi-square test and ANOVA test.

RFS was measured between the date of surgery and the date of first documentation of GIST recurrence or death, censoring patients who are alive with out recurrence on the date of the last follow-up. OS was calculated from GIST diagnosis to death by any cause or last follow-up (censored patients).

The receiver operating characteristic (ROC) curves analysis was used to determine the optimal cut-off for VAF and nVAF, to classify “low” vs. “high” values. The optimal cut-off of *KIT/PDGFRA*-VAF was 45% (AUC = 0.86; 95% CI, 0.72-0.99; *P*-value< .01). The optimal cut-off of nVAF, normalized to the percentage of neoplastic cellularity, was 50% (AUC = 0.84; 95% CI, 0.68-0.99; *P*-value< .02). The analysis of RFS and OS between groups was compared using the Kaplan-Meier method and log-rank test. To identify independent prognostic factors for RFS and OS, univariate and multivariate Cox proportional hazard regression models were performed. All tests were performed with a significante level of $P < .05$. Statistical analyses were conducted using IBM SPSS Statistics for Windows Version 27.0 (IBM Corporation).

CHAPTER 3

# Results

## 3.1 Cohort 1

### 3.1.1 Clinicopathological characteristics of *KIT* exon 11-mutated patients according to critical mutations

We chose to investigate patients with *KIT* exon 11 mutations because they represent the largest molecular subgroup of GIST and are characterized by wide variability in PV types and clinical behavior.

Between January 2005 and December 2020, 96 GIST patients with localized disease carrying a *KIT* exon 11 PV, were included in the study.

The patients were classified based on the *KIT* exon 11 PV as follows: (a) *KIT* exon 11 deletion or deletion/insertion involving 557 and/or 558 codons (named 'Del-557/558'); (b) deletion or deletion/insertion in codons other than 557 and/or 558 (named 'Del-No-557/558'); (c) duplication, insertion, or SNV (named 'No-Del').

A total of 36 patients were in the Del-557/558 group (37.5%), 26 patients in the Del-No-557/558 group (27.1%), and 34 patients in the No-Del group (35.4%) (**Figure 4**).

To highlight the prognostic impact of mutational status on the natural history of GISTs, we analyzed the clinicopathological characteristics of patients with GISTs according to *KIT* exon 11 critical mutations (**Table 5**)

The patients with 557 and/or 558 codon deletion or deletion/insertion, were mainly men (60.6%), more frequently with gastric GISTs (75%), primitive tumor diameter >5 cm (72.2%), and mitotic index >5/50 HPF (63.9%). The patients with other *KIT* exon 11 PVs less frequently showed GISTs of gastric origin (Del-No-557/558: 46.1%; No-Del: 55.9%; $p = 0.03$), but also a lower number of large primitive tumors with baseline diameter >5 cm (Del-No-557/558: 38.5%; No-Del: 55.9%; $p = 0.02$), and high median mitotic rate (Del-No-557/558: 42.3%; No-Del: 32.4%; $p = 0.02$).

Therefore, only 15.4% of patients with deletions in other *KIT* exon 11 codons and 44.1% of patients with duplication, insertion, or SNV were classified as high-risk GISTs (p = 0.008). Instead, patients with tumors carrying 557 and/or 558 codon deletion or deletion/insertion were classified as high-risk GISTs in 55.5% of cases.

### 3.1.2 Outcome analysis

The out come investigated was RFS, which presented a 7-year rate of 69.8% [median 134.7 months; Confidence Interval (CI), 118.6–150.8]. During follow up, a total of 29 RFS events (recurrence or death) were observed (30.2%): 18 events in 36 Del-557/558 patients (50%); 7 events in 26 Del-No-557/558 (26.9%), and 4 events in 34 No-Del patients (11.8%).
Patients with GISTs with deletion or deletion/insertion of codons 557 and/or 558 had a worse prognosis than anyother *KIT* exon 11-mutated patient when RFS between the three groups was examined.
At the same time, however, patients whose GISTs harbored a *KIT* exon 11 deletion or deletion/insertion in codons other than 557 and/or 558, had less favorable outcomes than patients with duplication, insertion, or SNV [Del-557/558 versus Del-No-557/558 versus No-Del: 7-year RFS, 50% versus 73.1% versus 88.2%; median RFS (mRFS), 86.9 months (95% CI, 60.1–13.7) versus 148.02 months (95% CI, 121.8–74.2) versus 155 months (95% CI, 137.7–172.2), respectively; p < 0.001] (**Figure 5**).
To determine the independent prognostic factors for RFS, univariate and multivariate Cox proportional hazard regression models were constructed.
The following factors were found to be statistically significantly associated with RFS in univariable analyses: diameter of primary tumor >5 cm [Hazard Ratio (HR): 4.72; 95% CI, 1.91–11.66; $p < 0.001$]; mitosis >5/50 HPF (HR: 3.34; 95% CI, 1.57–7.10; $p = 0.002$); no gastric site of origin (HR: 2.13; 95% CI, 1.03–4.43; $p = 0.03$); risk categories (HR: 4.67; 95% CI, 1.89–11.54; $p = 0.001$), and *KIT* exon 11 deletions or deletion/insertion involving codons 557 and/or 558 (HR: 0.19; 95% CI, 0.08–0.42; $p < 0.001$).
Tumour diameter (HR: 2.76; 95% CI, 1.01-7.57; p = 0.04), site of origin (HR: 2.49; 95% CI, 1.13-5.48; p = 0.02) and *KIT* exon 11 PV type (HR: 0.23; 95% CI, 0.1-0.53; p = 0.001) remained statistically significant in the final multivariable Cox regression model. The results of the analysis of univariable and multivariable prognostic factors for RFS are shown in the **Table 6**. Instead, the **Figure 6** shows the plotted RFS curves according to each independent prognostic factor.

### 3.1.3 Metastatic sites and PV classification in relapsed tumours after curative surgery

A total of 29 of 96 patients with *KIT* exon 11 mutations (30.2%) showed tumour recurrence at a median follow-up of 92 months. In this population, the tumour metastasis sites (liver, peritoneum or liver and peritoneum) were described and were classified according to the presence/absence of deletion or deletion/insertion in codons 557 and/or 558. In patients relapsed with GIST presenting with deletion or deletion/insertion 557 and/or 558, 72.2% of the metastatic spread involved the peritoneum (13/18 patients), 16.7% the liver (3/18 patients) and 2 patients had peritoneal and liver metastases at tumour recurrence (11.1%). In patients relapsed with PV of tumour exon 11 that did not involve 557/558 deletions, 54.5% had peritoneal metastases (6/11 patients), 27.3% had peritoneal and liver metastases (3/11 patients) and 18.2% had only liver metastases (2/11 patients) ($p = 0.5$) (**Table 7**).

Subsequently, it was shown that of the 18 patients with tumours carrying deletions or deletions/insertions at codons 557 and/or 558, 14 (77.8%) had GISTs with deletions involving codons 557 and 558 simultaneously.

Furthermore, only 4 of the 18 relapsed patients (22.2%) showed tumour relapse (n.2, p.K558_V559delinsN; n.1 p.K550_W557del; n.1 p.K558_V559del) (**Figure 7**).

### 3.1.4 The impact of PV in stratifying risk of recurrence

We evaluated the prognostic effect of PV on intermediate-risk versus high-risk GIST patients' outcomes.

A total of 74 patients were included in the analysis, of which 35 were intermediate-risk and 39 high-risk and were grouped according to the presence/absence of deletion or deletion/insertion of codons 557/558.

When the RFS between the four groups was compared, patients with intermediate-risk GISTs and Del-557/558 showed a worse out come similar to the group of high-risk patients with PV-bearing tumours without Del-557/558 (duplication, insertion or SNV) (**Figure 8**).

## 3.2 Cohort 2

### 3.2.1 Study population

In this study were included 200 patients with localised GIST afferent between 2015 and 2022 at 6 Italian Oncology Centres of the European Reference Network on Rare Adult Tumours (EURACAN).

Using ROC curve analysis, the optimal *KIT/PDGFRA* nVAF threshold of 50% was determined, nVAF ≤ 50% (termed 'low nVAF') vs nVAF>50% (termed 'high nVAF').

To assess whether the nVAF of *KIT* and *PDGFRA* somatic mutations can influence clinico-pathological features, it was correlated with the characteristics of GIST patients (**Table 8**).

In our study, 111 patients were male (55.5%) and 89 female (44.5%). The median age was 58 years [interquartile range (IQR), 21-87 years] in the nVAF ≤50% group and 62 years [IQR, 30-81 years] in the nVAF>50% group.

There were variations in the age groups' median, nVAF were more frequently >50% in the group of 153 patients (76.5%) aged >50 years than in the group of 45 patients (22.5%) aged ≤50 years (P =.03).

Interestingly, in patients with a non-gastric site of origin compared to those with a gastric GIST (P = .04), primary tumour diameter >5 cm versus basal diameter ≤5 cm (P = .02), the nVAF was significantly higher.

While there were no statistically significant differences in primary mitotic count and histology.

The impact of the mutational status on tumor nVAF low vs. high was then evaluated.

Of the 200 patients, 159 (79.5%) had a GIST harboring a *KIT* PV, and 41 patients (20.5%) showed a *PDGFRA* PV.

Patients with *KIT* PVs had a considerably higher nVAF than patients with *PDGFRA*-mutated tumors (median *KIT* vs. *PDGFRA* nVAF, 55% vs. 46%; P =.04).

However, there were no statistically significant variations in low or high nVAF respect to *KIT* exons (exon 11 or other exons; P =.5) in the group of patients with *KIT*-mutated GISTs. In order to examine the effects of different *KIT* exon 11 PV types, patients were classified according to the presence of deletions (del) or deletions/insertions (delins) of *KIT* exon 11 involving codons 557 and/or 558, compared to other mutations, where the other mutations were deletions or delins in codons other than 557 and/or 558, or duplications, insertions or single nucleotide variants (SNVs) of *KIT* exon 11.

### 3.2.2 Outcome Analysis

We evaluated the survival, RFS and OS outcomes of patients with localised GIST in relation to nVAF. Data were available for n.178 patients with localised GIST and the median follow-up time was 24 months (range, 6-116 months). The 2-year RFS rate was 80.9% (median RFS 62 months; 95% CI, 38.5-85.5).

A total of 34 RFS events (recurrence or death) (19.1%) were observed during follow-up, 5 of which occurred in the group of patients with tumours showing a tumor nVAF ≤ 50% (6.9%), while 29 events occurred in GIST patients with a tumor nVAF> 50% (27.3%).

Comparing the RFS between the 2 groups, GIST patients with tumor nVAF> 50% showed a less favourable RFS than patients in the group with nVAF ≤ 50% (2-year RFS, 72.6% vs. 93%, respectively; P = .003; **Fig. 9F**).

Examining the main prognostic factors in localised GISTs (primary mitotic count, tumour size and tumour site), primary mitotic count, tumour size and tumour site all were significantly associated with RFS (tumor mitosis >5/mmq vs. ≤ 5/mmq: 2-year RFS, 63.2% vs. 93.1%, P < .001; primary tumour diameter >5 cm vs. ≤ 5 cm: 2-year RFS, 70.7% vs. 93.8%, P = .002; non-gastric vs. gastric site of origin: 2-year RFS, 70.5% vs. 91.1%, P = .007; **Fig. 9A-9C**).

Concerning the impact of *KIT* exon 11 PVs on RFS, in the subset of patients with deletions or deletions/insertions in codons 557 and/or 558 of *KIT* exon 11 was unfavourable compared to the rest of GIST patients (2-year RFS, 59.5% vs. 89.5%, P < .001; **Fig. 9D**).

The overall median OS was not reached. A total of 8 deaths (4.5%) occurred in the group of patients with nVAF>50% (P = .04; **Fig. 10F**); in contrast, no events were observed in the group with nVAF ≤50%.

Univariable and multivariable Cox proportional hazard regression models were performed to investigate whether the prognostic value of nVAF for PFS and OS was independent of other known factors.

Factors statistically significantly associated with RFS in uni variate analyses were: primary tumour diameter >5 cm , mitosis >5/mmq, non-gastric site of origin, *KIT* exon 11 deletions or deletions/insertions involving codons 557 and/or 558 and nVAF> 50%. In the final multivariable Cox regression model, mitosis, *KIT* exon 11 PV type and nVAF remained statistically significant.

Regarding OS, only *KIT* exon 11 deletions or deletions/insertions involving codons 557 and/or 558 were statistically significantly associated with OS in univariable analyses (**Table 9**). We also investigated survival outcomes compared to non-normalised VAF. The optimal cut-off for *KIT/PDGFRA* -VAF, determined by ROC curve analysis, was 45%.

Although VAF was also statistically associated with RFS and OS, the statistical significante was lower than VAF normalised to individual neoplastic cellularity.

### 3.2.3 The impact of nVAF in intermediate-risk patients with *KIT* mutations

We performed an additional analysis to evaluate the ability of nVAF to identify a subpopulation of intermediate-risk patients with a higher risk of disease recurrence, who may benefit from adjuvant treatment. We therefore included 66 patients with GIST at intermediate risk of recurrence, with *KIT* mutations and not treated with adjuvant imatinib.

A total of 10 RFS events (relapse or death) were observed during follow-up (15.1%). The RFS rate was 74.4% for the high nVAF group and 100% for the low-risk group. As can be

seen in the **Figure 11**, patients with intermediate-risk GIST and high nVAF showed poorer RFS than the low nVAF group (P = 0.01).

## 3.3 Cohort 3

### 3.3.1 Molecular characterization of GISTs

From November 2020 to June 2023, 73 patients with histologically confirmed diagnosis of GISTs, both localized and metastatic, afferent to the 'Sicilian Regional Center for the Prevention, Diagnosis and Treatment of Rare and Heredo-Familial Tumors' of the Section of Medical Oncology of University Hospital Policlinico 'P. Giaccone' of Palermo, were recruited.

Molecular analysis was performed on tumor tissue for evaluation of mutational status by multigene panel in NGS.

Of the 73 patients, 59 (80.8%) showed molecular alterations in the *KIT* gene including 51 (86.4%) in exon 11, 2 (3.4%) in exon 13 and 6 (10.2%) in exon 9. The most common site of mutations at *KIT* exon 11 occurred between codons 550-560. Seventeen out of 51 patients (33.3%) had deletions involving critical codons 557 and/or 558 or as part of larger deletions that included mutation positions upstream of codon 557 and downstream of codon 558.

Variants were identified in the *PDGFRA* gene in only 7 (9.6%) of the patients and most of the mutations were identified in exon 18. The most frequent variant was the known primary resistance mutation to Imatinib p.D842V (n.5 patients, 6.8% of all patients; 71.4% of all *PDGFRA*-mutated GISTs), a SNV involving the kinase domain of the receptor and resulting in a single substitution at position 842 of an aspartic acid (D) for a valine (V). Finally, a rare deletion/insertion (p.S566_E571delinsR) was identified at exon 12 of *PDGFRA*.

Finally, one patient showed a pathogenic variant in *BRAF* and one in the *MAP2K1* gene (**Table 10**).

In this analysis, 5 patients (6.8%) showed no alterations in the investigated genes. On the latter, to assess the presence of fusion transcripts, the analysis was performed again by multigene panel in NGS from tissue RNA.The results obtained showed that none of the patients under investigation had gene fusions.

### 3.3.2 Gene expression analysis

To assess the implication of *YAP*, *TAZ* and other genes involved in this signaling pathway, we performed gene expression analysis by ddPCR.

Twenty-seven patients were included for this study, stratified according to the molecular characteristics presented. Specifically, 18 (66.7%) had mutations at exon 11 of *KIT*, 5

(18.5%) at the *PDGFRA* gene, and 4 (14.8%) had no alterations in the genes investigated by panel in NGS in the previous analysis.

From the expression analysis, the software calculates the target RNA concentration in copies/µl. The relative expression value of each gene was calculated as the ratio of the concentration of the investigated gene to the target gene. Thus a value greater than one will indicate that the investigated gene is more overexpressed than the housekeeping gene, in contrast, a value below unity reflects an downregulation of the gene investigation.

In the **Table 11-12** we can see the clinicopathological characteristics of patients according to the expression value of each gene analyzed.

Among the genes investigated, those most frequently upregulated were *YAP*, *TAZ* and *CTGF*.

Patients showing this overexpression were mainly WT and *PDGFRA* mutated, most frequently with small bowel GIST (*YAP* 46.2%, *TAZ* 45.4%, *CTGF* 40%) and primary tumor diameter >5 cm (*YAP* 76.9%, *TAZ* 72.8%, *CTGF* 66.7%) (**Figure 12**).

Interestingly, all WT patients showed overexpression of *YAP* and 3 (75%) of *TAZ* and *CTGF*.

In contrast, no patients were found to have over regulation of *CCDN1* and *AXL*.

Of the 27 patients, 5 (18.5%) were metastatic at diagnosis and among them the most overexpressed gene was *CTGF* ( 3 patients, 60%).

During follow-up, median of 6-24 months, 3 (11%) recurrence events (progression or death) occurred. These patients showed overexpression in most genes (*YAP* 100%, *TAZ* 100%, CTGF 66.7%, *LIX1* 66.7%, *NFKB1* 100%) and also, in this group, they included the only patients with an overexpression of *LIX1* (**Figure 13**).

From the statistic alanalysis, however, no value was found to be statistically significant, most likely because the number of the cohort under study was small.

CHAPTER 4

# Discussion

Over the last two decades, molecular profiling of GISTs has greatly improved the understanding of the development of these tumours, bringing significant value to the management of cancer patients in clinical practice. Indeed, the characterisation of specific molecular subtypes has changed therapeutic approaches, making GISTs an ideal model for the development of precision medicine in cancer treatment (95).

Furthermore, several studies have shown that the type of pathogenic variant has a relevant prognostic impact in completely resected (96, 97), although the genotypic profile has not been incorporated into the stratification of risk of recurrence.

To date, understanding the insights from genomic profiling through molecular testing for the treatment of patients with GISTs is an ongoing challenge.

Although several clinical pathological factors have been established as predictors of clinical tumour behaviour and patient survival, further insights are needed to further refine prognosis.

In this PhD thesis, patients with localised and/or metastatic GISTs were subjected to in-depth molecular analyses in order to improve the understanding of the molecular mechanisms underlying the classification of GISTs for a better management of the disease.

In the first part of this project, the clinicalpathological features of GIST patients were studied according to mutational status, and the effect of exon 11 *KIT* type mutations on RFS was assessed.

It was seen that patients with *KIT* exon 11 deletions or deletions/insertions involving codons 557 and/or 558, compared to other exon 11 PVs, more frequently presented gastric GISTs, with larger primary tumor diameter and a higher mitotic index. In this group, 55% of patients were stratified at high risk of recurrence.

Furthermore, the impact on RFS was evaluated and the data showed worse outcome for patients with GIST having deletion or deletion/insertion of codons 557 and/or 558 compared to patients with any other *KIT* exon 11 PV.

Multivariate analysis confirmed the independent prognostic value of this result.

These data are consistent with previous studies that confirm the malignant behavior of the tumor presenting this type of pathogenetic variants (96-98).

Furthermore, 14 of 18 patients with relapse had deletions simultaneously involving codons 557 and 558, underscoring a significantly higher risk of relapse in patients with deletions involving both codons. This more aggressive role of deletions simultaneously involving codons 557 and 558 can be explained by the critical autoinhibitory role on the tyrosine kinase activation process exerted by the regions of codons 557 and 558 which, when both are deleted, results in a considerable increase in phosphorylation spontaneous receptor and activation of the downstream pathway (99).

In particular, the outcome of intermediate-risk patients with Del-557/558 and high-risk patients without Del-557/558 was compared. Patients in the two groups hadidentically poorer prognoses in terms of RFS. According to the current research, subjects with GIST with intermediate-risk features and *KIT* exon 11 deletions or deletions/insertions in codons 557/558 may require adjuvant treatment with imatinib due to the high risk of relapse.

Subsequently, it was hypothesised how nVAF levels of pathogenic variants in *KIT* and *PDGFRA* may correlate with the prognosis of patients with localised, fully resected GIST.

In our cohort, patients with nVAF >50% showed less favourable RFS than patients in the group with nVAF ≤ 50%. The median nVAF was statistically higher in tumours with *KIT* mutation compared to *PDGFRA* . Furthermore, high levels of *KIT/PDGFRA*–nVAF mutant were associated with age of onset of GIST > 50 years, non-gastric site of primary tumours and primary tumour diameter > 5 cm.

We also observed that in KIT mutated intermediate-risk patients, higher nVAF was statistically associated with disease recurrence, compared to low nVAF.

These results suggest that the high allele frequency of the *KIT/PDGFRA* mutation in GIST could be one of the criteria for deciding whether to use adjuvant treatment.

Another aim of this project was to investigate the role that *YAP*, *TAZ* and other genes involved in this signalling pathway could play in GISTs, as predictors of biological tumour behaviour. Therefore, the gene expression of these genes in different molecular subtypes of GISTs was assessed.

Specifically, we selected a cohort of patients from which 18 had exon11 *KIT* mutations, 5 in *PDGFRA* and 4 WT.

Due to the small number of the cohort under investigation, statistical analysis did not reveal statistically significant data. However, it is interesting to observe that the most frequently expressed gene was *CTGF* (in 55.6% of the GISTs analysed) followed by *YAP* (48.1%) and *TAZ* (40.7%).

Patients showing this upregulation more frequently had GISTs with small bowel localisation and primary tumour diameter >5 cm.

Interestingly, all WT patients showed overexpression of *YAP* and 3 (75%) also overexpressed *TAZ* and *CTGF*.

According to the work of Chen et al. (85) KIT-independent and imatinib-resistant GISTs exhibit *YAP*/*TAZ*-induced overexpression of *CCDN1*. In our case, on the contrary, both WT and mutated GISTs presented an downregulation of this gene and also of *AXL*.

Of the 27 patients, 5 (18.5%) were metastatic at diagnosis and among these the most overexpressed gene was *CTGF* (3 patients, 60%).

According to another study, a gene that appears to be upregulated in GISTs is *LIX1*. It represents a marker of immaturity of the gastrointestinal mesenchyme and *LIX1* has been shown to induce, via Hippo's *YAP* effector, the proliferation and differentiation of mesenchymal progenitors and its overexpression is associated with poor prognosis (87). In our study, *LIX1* was overexpressed in only two cases, which however represented 66.7% (2/3) of the patients who showed tumour recurrence. These patients had tumours with a diameter >5cm and a mitotic index >5/50HPF, both carried pathogenic variants at *KIT* exon 11 and were classified as high risk.

Despite the small patient cohort, these preliminary reports may encourage future studies in a larger patient population.

Regarding the gene fusion analysis in WT patients, we did not identify any alterations. This result was however expected as the patients analyzed were few.

Although NTRK fusions are rare in wild-type GISTs, the unequivocal diagnosis can bring clinical benefits to patients.

In conclusion, the set of studies reported in this thesis lead to a deeper understanding of the molecular characterisation of GISTs, allowing patients to be stratified according to more detailed criteria and leading to an improvement in clinical management.

Indeed, in patients with localized GISTs completely resected we showed that *KIT* exon 11 deletions or deletion/insertion involving both codons 557 and 558 are genotypes indicative of more aggressive tumor behavior and higher risk of recurrence, supporting the importance of considering the PV type and codon location in routine risk stratification.

Furthermore, we demonstrated that higher levels of nVAF, in patients with localised GISTs presenting *KIT* or *PDGFRA* mutations, were independent predictors of prognosis and survival. These data could be relevant in the cohort of intermediate-risk patients to improve prognosis and the use of adjuvant imatinib.

Finally, gene expression analysis showed the potential role of Hippo and pathway-related genes as oncogenic mediators in GISTs, especially in WT patients and patients with

disease recurrence. Inhibitors of these pathways could expand in the future the therapetic landscape of these tumours.

CHAPTER 5

# Tables and Figures

**Table 1.** Prediction of the recurrence risk in GISTs based on the size, mitotic index, and anatomical site of the primary tumor (28).

| Size (cm) | Mitotic Index | Gastric | Duodenum | Jejunum/Ileum | Rectum |
|---|---|---|---|---|---|
| **<2** | <5 | None | None | None | None |
| **>2<5** | | Very low | Low | Low | Low |
| **>5<10** | | Low | Moderate | Insufficient data | Insufficient data |
| **>10** | | Moderate | High | High | High |
| **<2** | >5 | None | High | Insufficient data | High |
| **>2<5** | | Moderate | High | High | High |
| **>5<10** | | High | High | High | High |
| **>10** | | High | High | High | High |

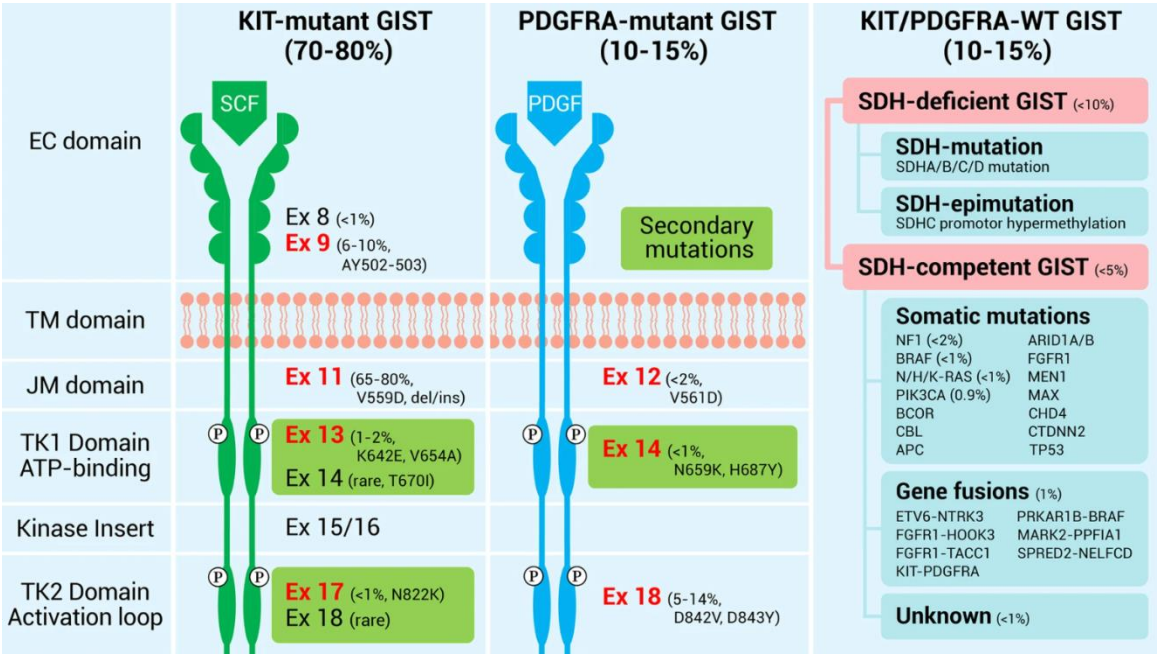


**Figure 1.** The genomic profiles of GISTs (100).

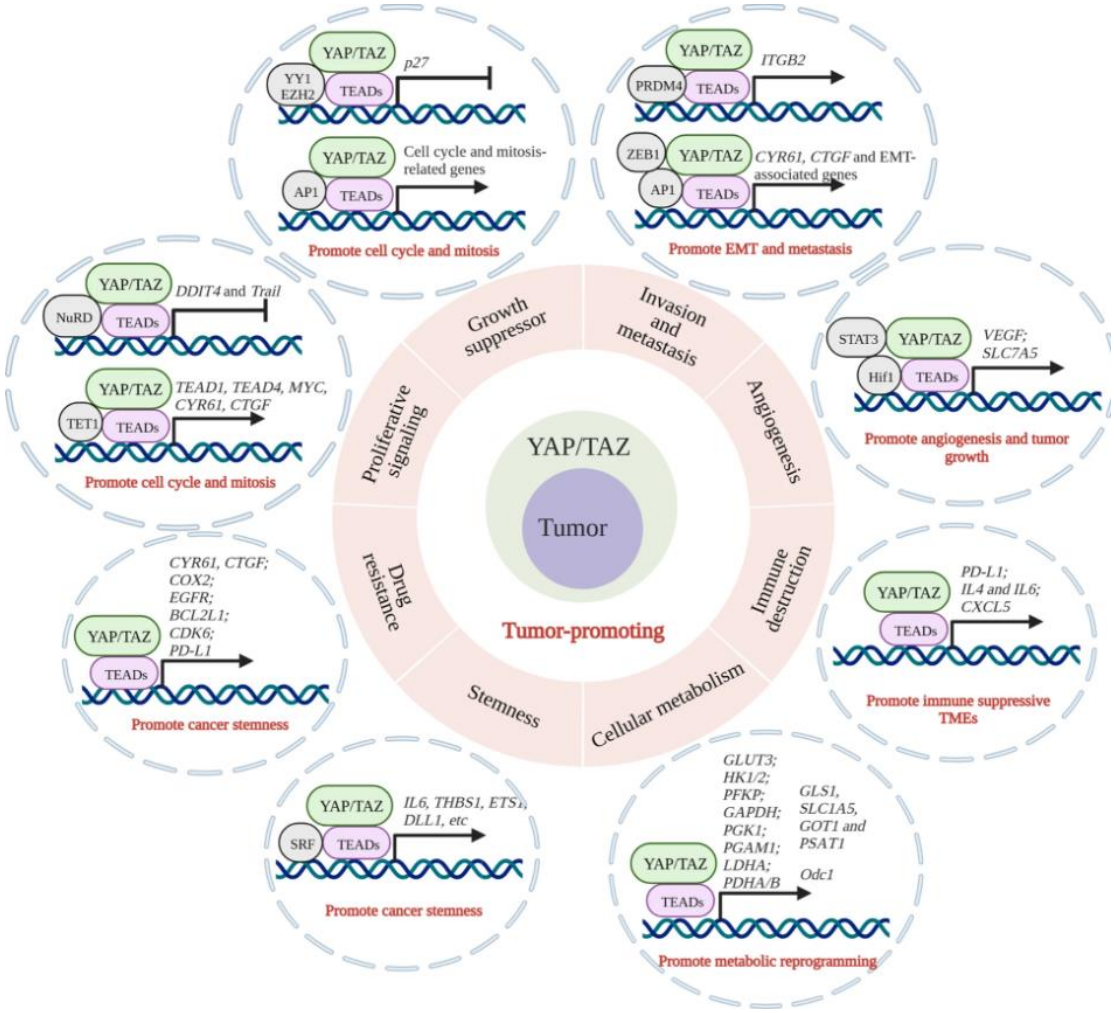


**Figure 2.** Potential tumour-promoting mechanisms of YAP/TAZ in human tumours (101).

**Table2:** Oligonucleotide sequences (Fw and Rv) designed for amplification of exons 9-11-13-17 of the *KIT* gene and exons 12-14-18 of the *PDGFRA* gene.

| **Primers** | | |
|---|---|---|
| **Gene** | **Forward Sequence** (5'- 3') | **Reverse Sequence** (5'- 3') |
| ***KIT* ex 9** | ACC TCT AAC TTT GTT TTA AAA GTA TGC | GGA ATG AAC TTA AAA TCA TGA CTG |
| ***KIT* ex 11** | GTT CTC TCT CCA GAG TGC TCT AA | GTT CCT TAA AGT CAC TGT TAT GTG TAC |
| ***KIT* ex 13** | GTA TGG TAC TGC ATG CGC TT | CAT TGC CAA AAT CAT ATT AAA ATG |
| ***KIT* ex 17** | CTG AAT ACT TTA AAA CAA AAG TAT TGG | TTA TGA AAA TCA CAG GAA ACA ATT T |
| ***PDGFRα* ex 12** | TGG AGT GAA CGT TGT TGG A | AGA TCT CTA TTC TGC CAA GGC |
| ***PDGFRα* ex 14** | CAG GAT TAG TCA TAT TCT TGG TTT TT | TTC TAT TCC CTG CCA TGT GT |
| ***PDGFRα* ex 18** | CAG TCT TGC AGG GGT GAT G | GCA CCG AAT CTC TAG AAG CA |

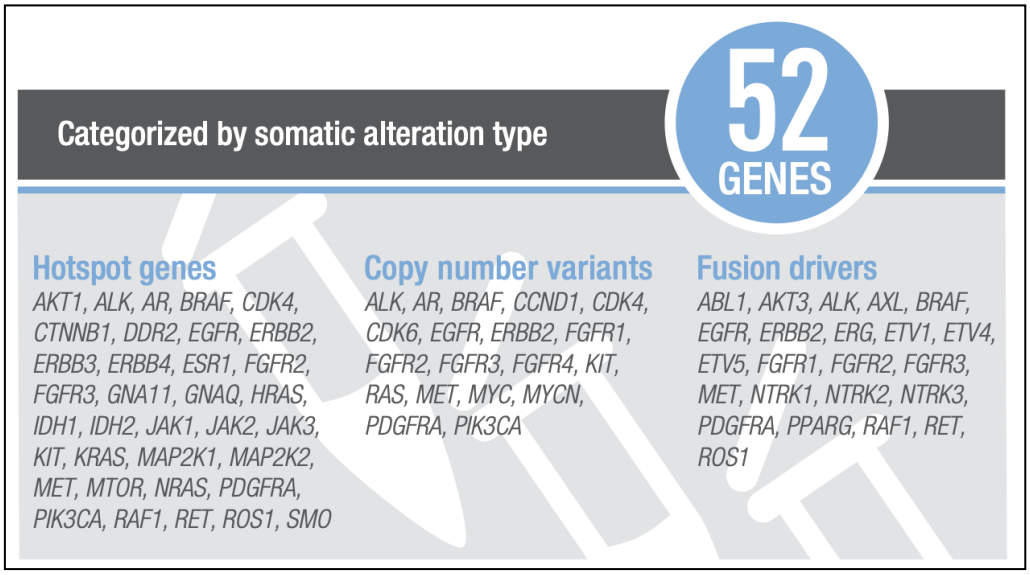


**Figure 3**: Oncomine Focus Panel Assay

**Table 3** . Preparation of the reaction mix

| Component | Volumeper Reaction,µl | FinalConcentration |
|---|---|---|
| **Supermix** | 5 | 1x |
| **Reversetranscriptase** | 2 | 20U/µl |
| **300 mMDTT** | 1 | 15mM |
| **Targetprimers/probe*** | 1.1 | 900nM/250nM |
| **RNase-/DNase-freewater** | Variable | — |
| **TotalRNA** | Variable | 20 ng per reaction |
| **Total volume** | **22** µl | **—** |

**Table 4.** Thermal cycling conditions

| CyclingStep | Temperature, °C | Time | NumberofCycles |
|---|---|---|---|
| **Reversetranscription** | 50 | 60min | 1 |
| **Enzymeactivation** | 95 | 10min | 1 |
| **Denaturation** | 95 | 30sec | 45 |
| **Annealing/extension** | 55 | 1 min* | |
| **Enzymedeactivation** | 98 | 10min | 1 |
| **Hold** | 4 | 30min | 1 |

*Check/adjust ramp rate settings to~2°C/sec.

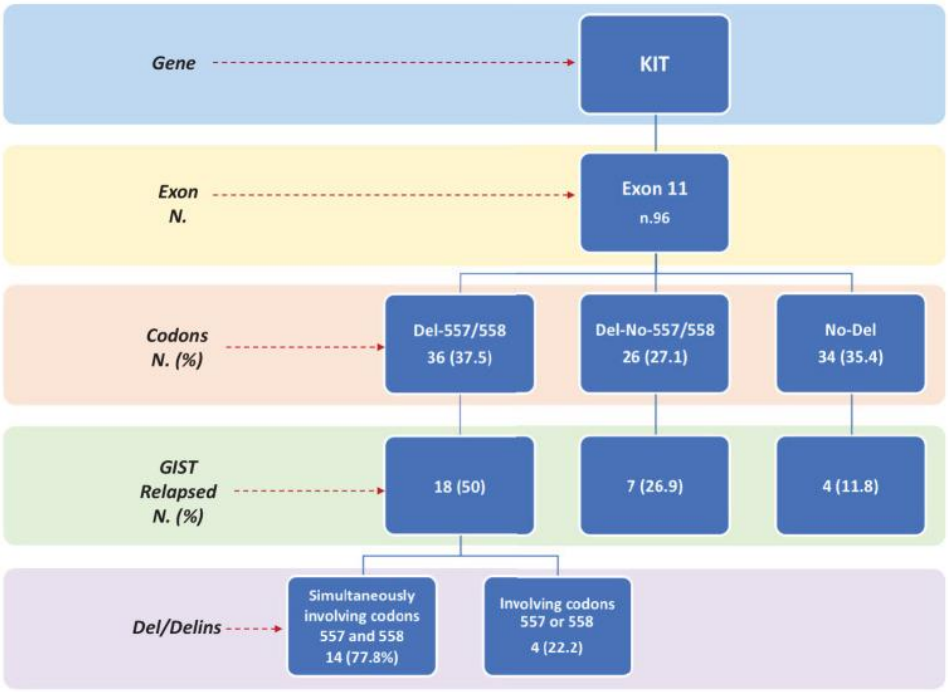


**Figure 4**. Diagram of mutational status and outcomes of the patients with *KIT* exon 11 GISTs included in the study. Abbreviations: Del, deletions; Delins, deletion/insertions; GIST, gastrointestinalstromaltumor; *KIT*, KIT proto-oncogene receptor tyrosinekinase.

**Table 5.** Clinico-pathological characteristics of patients with GISTs according to *KIT* exon 11 critical mutations

*Comparison Del-557/558 *versus* Del-No-557/558 *versus* No-Del.

Del, deletion; Del-557/558, *KIT* exon 11 deletion or deletion/insertions involving 557 and/or 558 codons; Del-No-557/558, *KIT* exon 11 deletion or deletion/insertions in codons other than 557 and/or 558; Delin, deletion/insertion; GIST, gastrointestinal stromal tumor; HPF, high-power field; *KIT*, KIT proto-oncogene receptor tyrosine kinase; M–L, Miettinen–Lasota; No-Del, *KIT* exon 11 duplication, insertion, or single nucleotide variant; NS, not significant.

| | **Total *KIT* exon** | ***KIT* exon 11 subgroups** | | | ***p* value*** |
|---|---|---|---|---|---|
| | | **Del-557/558 *n* (%)** | **Del-No-557/558 *n* (%)** | **No-Del *n* (%)** | **–** |
| *N*.patients | 96 | 36 (37.5) | 26 (27.1) | 34 (35.4) | – |
| Gender | | | | | |
| Male | 56 (58.3) | 24 (60.6) | 13 (50) | 19 (55.9) | NS |
| Female | 40 (41.7) | 12 (31.4) | 13 (50) | 15 (44.1) | |
| Age at diagnosis (years) | | | | | |
| Median | 60 | 59 | 56.5 | 63.5 | NS |
| Mean | 59 | 58 | 53.9 | 61.1 | |
| Range | 33–82 | 33–76 | 33–67 | 39–82 | |
| Site of origin | | | | | |
| Gastric | 58 (60.4) | 27 (75) | 12 (46.1) | 19 (55.9) | 0.03 |
| Small bowel/other | 38 (39.6) | 9 (25) | 14 (53.9) | 15 (44.1) | |
| Baseline diameter | | | | | |
| ⩽5 cm | 41 (42.7) | 10 (27.8) | 16 (61.5) | 15 (44.1) | 0.02 |
| >5 cm | 55 (57.3) | 26 (72.2) | 10 (38.5) | 19 (55.9) | |
| Baseline mitosis | | | | | |
| ⩽5/50 HPF | 51 (53.1) | 13 (36.1) | 15 (57.7) | 23 (67.6) | 0.02 |
| >5/50 HPF | 45 (46.9) | 23 (63.9) | 11 (42.3) | 11 (32.4) | |
| M–L risk categories | | | | | |
| Very low/low | 22 (22.9) | 3 (8.3) | 10 (38.5) | 9 (26.5) | 0.008 |
| Intermediate | 35 (36.5) | 13 (36.2) | 12 (46.1) | 10 (29.4) | |
| High | 39 (40.6) | 20 (55.5) | 4 (15.4) | 15 (44.1) | |

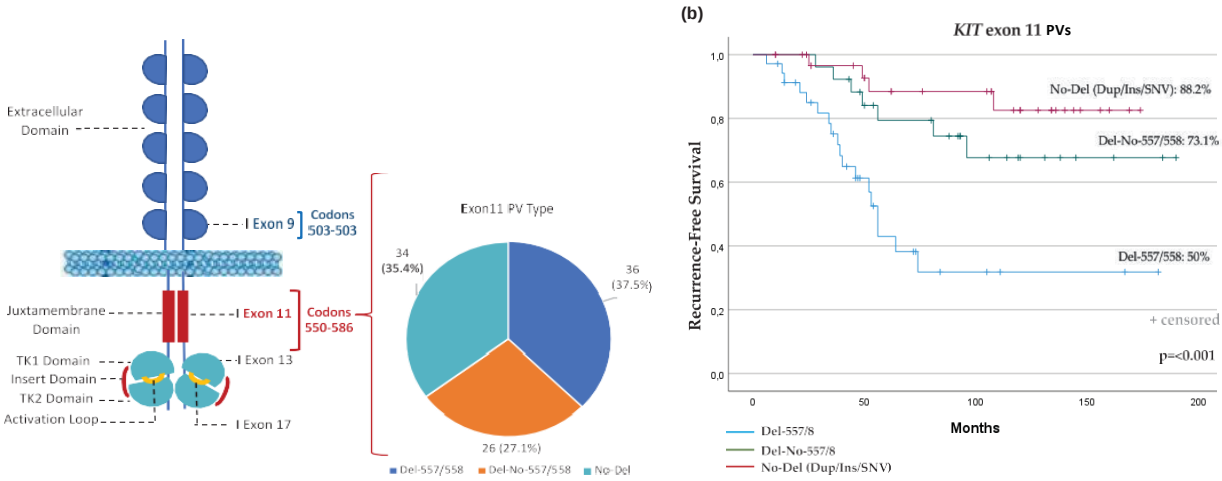


**Figure 5 .** (a) Schematic KIT receptor structure and locations of activating mutational hotspots. The KIT functional domains include five immunoglobulin-like domains (extracellular domain), juxtamembrane domain, TK1 domain, insert domain, TK2 domain, and activation loop. Number of patients in the three groups of *KIT* exon 11 PVs: (i) deletion or deletion/insertion in codons 557 and/or 558 ('Del-557/558'); (ii) deletion or deletion/ insertion in codons other than 557 and/or 558 ('Del-No-557/558'); (iii) duplication, insertion, or SNV ('No-Del'). (b)RFS in *KIT* exon 11 patients according to PV type and location.

KIT, KIT proto-oncogene receptor tyrosine kinase; PV, pathogenic variant; RFS, recurrence-free survival; SNV, single nucleotide variant; TKI, tyrosine kinase 1; TK2, tyrosine kinase 2.

**Table 6.** Univariate and multivariate analysis of prognosticfactors for RFS in *KIT* exon 11-mutated patients

| RFS | Univariate Cox regression | | Multivariable Cox regression | |
|---|---|---|---|---|
| | HR (95% CI) | *p* value | HR (95% CI) | *p* value |
| Gender (female *versus* male) | 0.65 (0.31–1.39) | NS | | |
| Age (>50 years *versus* ⩽50 years) | 0.75 (0.33–1.69) | NS | | |
| Primitive tumor diameter (⩽5 cm *versus* >5 cm) | 4.72 (1.91–11.66) | < 0.001 | 2.76 (1.01–7.57) | 0.04 |
| Mitosis (⩽5/50 HPF *versus* >5/50 HPF) | 3.34 (1.57–7.10) | 0.001 | 1.50 (0.60–3.73) | NS |
| Gastric site of origin (yes *versus* no) | 2.13 (1.03–4.43) | 0.03 | 2.49 (1.13–5.48) | 0.02 |
| M–L risk categories (very low/low *versus* intermediate/high) | 4.67 (1.89–11.54) | 0.001 | 2.34 (0.77–7.18) | NS |
| Exon 11 Del or Delins 557 and/or 558 no *versus* yes) | 0.19 (0.08–0.42) | < 0.001 | 0.23 (0.1–0.53) | 0.001 |

CI, confidence interval; Del, deletion; Delin, deletion/insertion; HPF, high-power field; HR, hazard ratio; *KIT*, KIT proto-oncogene receptor tyrosine kinase; M–L, Miettinen–Lasota; NS, not significant; RFS, recurrence-free survival.

**Table 7**. Metastatic sites in patients with relapse after curative surgery with *KIT* exon 11 mutations. Patients were classified as GIST carriers with deletion or deletion/insertion involving codons 557/558 or other PVs of *KIT* exon 11

| Metastatic sites | Total patients *n* (%) | Deletion or deletion/insertion codons 557/558* *n*. (%) | Other exon 11 PVs* *n* (%) |
|---|---|---|---|
| Liver metastasis | 5 (17.2) | 3 (16.7) | 2 (18.2) |
| Peritoneal metastasis | 19 (65.6) | 13 (72.2) | 6 (54.5) |
| Liver and peritoneal metastasis | 5 (17.2) | 2 (11.1) | 3 (27.3) |

*Pearson's chi-square test: comparison deletion or deletion/insertion codons 557/558 *versus* other exon 11 PVs; $p = 0.5$.

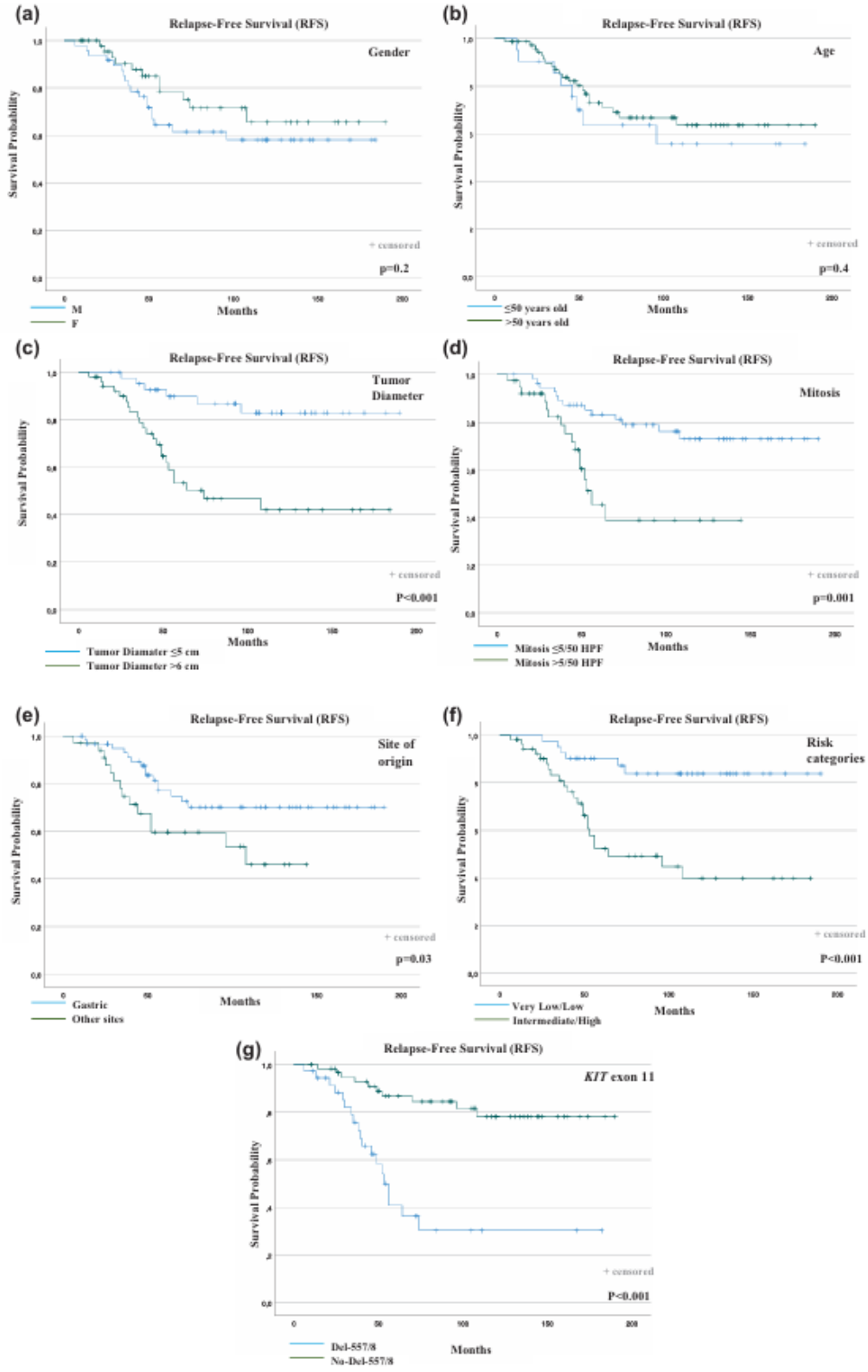


**Figure 6** . Outcome analysis according to clinical and biological factors. (a) RFS according to gender. (b) RFS according to age. (c) RFS according to tumor diameter. (d) RFS according to mitosis. (e) RFS according to the site of origin. (f) RFS according to risk categories. (g) RFS according to *KIT* exon 11 PV type. Abbreviations: Del-557/558, *KIT* exon 11 deletion or deletion/insertion involving 557 and/or 558 codons; Del-No-557/558, *KIT* exon11 deletion or deletion/insertion in codons other than 557 and/or 558; HPF,high-power field;*KIT*, KIT proto-oncogene receptor tyrosine kinase; PV, pathogenic variant; RFS, recurrence-free survival.

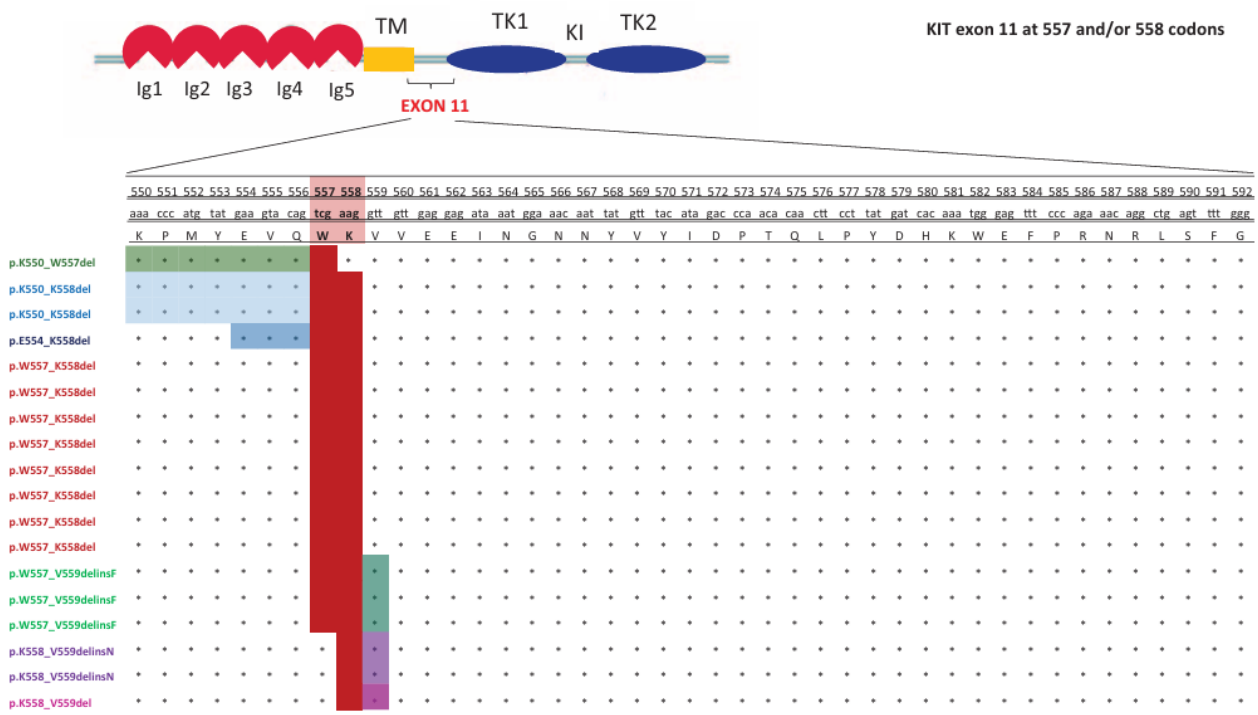


**Figure 7.** The exact *KIT* exon 11 PV classification of relapsed patients with GISTs with a deletioninvolvingonly one or both 557 and 558 codons (in red). Abbreviations: GIST, gastrointestinal stromal tumor; Ig, immunoglobulin; *KIT*, KIT proto-oncogene receptor tyrosine kinase; PV, pathogenic variant; TKI, tyrosine kinase 1; TK2, tyrosine kinase 2.

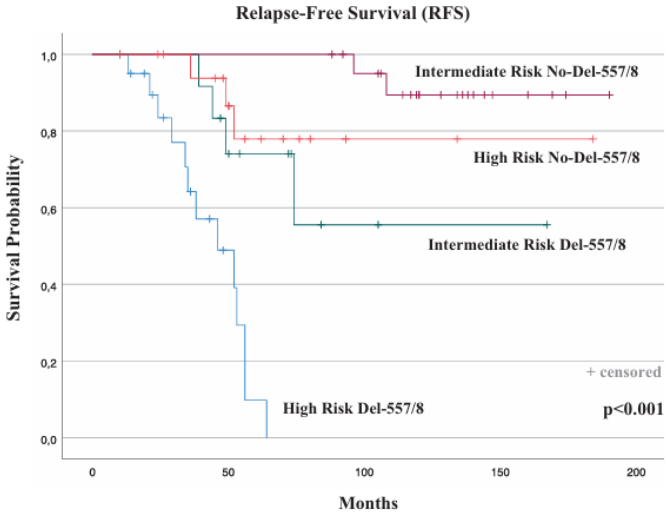


**Figure 8**. Outcome analysis in the intermediate-risk *versus* high-risk *KIT* exon 11-mutated patients according to the presence/absence of deletion or deletion/insertion of the codons 557/558. *KIT*, KIT proto-oncogene receptor tyrosine kinase.

**Table 8**. Patient and disease characteristics of patients with GIST localized.
*Del-557/8: deletions (del) or deletion/insertion (delins) involving 557 and/or 558 codons of *KIT* exon 11.
**Others: del or delins in other codons than *KIT* exon 11 557 and/or 558 codons, or *KIT* exon 11 duplications, insertions or SNVs. Abbreviations: IQR, interquartile range; nVAF, normalized variant allele frequency.

| Characteristic | No. of patients (%) | nVAF ≤ 50% | nVAF > 50% | *P*-value |
|---|---|---|---|---|
| No of patients | 200 | 79 | 121 | — |
| Gender | | | | .08 |
| Male | 111 (55.5) | 50 (63.3) | 61 (50.4) | |
| Female | 89 (44.5) | 29 (36.7) | 60 (49.6) | |
| Age groups (years) | | | | .03 |
| ≤50 | 45 (22.5) | 24 (30.4) | 21 (17.4) | |
| >50 | 153 (76.5) | 54 (68.3) | 99 (81.8) | |
| Missing | 2 (1) | 1 (1.3) | 1 (0.8) | |
| Site of origin | | | | .04 |
| Gastric | 104 (52) | 48 (60.7) | 56 (46.3) | |
| Others | 95 (47.5) | 30 (38) | 65 (53.7) | |
| Missing | 1 (0.5) | 1 (1.3) | 0 | |
| Baseline diameter | | | | .02 |
| ≤5 cm | 89 (44.5) | 44 (55.7) | 45 (37.2) | |
| >5 cm | 102 (51) | 34 (43) | 68 (56.2) | |
| Missing | 9 (4.5) | 1 (1.3) | 8 (6.6) | |
| Baseline mitosis | | | | .4 |
| ≤5/mmq | 111 (55.5) | 42 (53.2) | 69 (57) | |
| >5/mmq | 73 (36.5) | 32 (40.5) | 41 (33.9) | |
| Missing | 16 (8) | 5 (6.3) | 11 (9.1) | |
| Histology | | | | 1 |
| Spindle cell | 111 (55.5) | 43 (54.5) | 68 (56.2) | |
| Epithelioid/mixed | 71 (35.5) | 28 (35.4) | 43 (35.5) | |
| Missing | 18 (9) | 8 (10.1) | 10 (8.3) | |
| Mutated *KIT* gene | | | | .04 |
| Yes | 159 (79.5) | 57 (72.2) | 102 (84.3) | |
| No | 41 (20.5) | 22 (27.8) | 19 (15.7) | |
| *KIT* exons | | | | .5 |
| Exon 11 | 130 (81.8) | 45 (78.9) | 85 (83.4) | |
| Other exons | 28 (17.6) | 12 (21.1) | 16 (15.7) | |
| Missing | 1 (0.6) | 0 | 1 (0.9) | |
| *KIT* exon 11 | | | | .2 |
| Del-557/8* | 41 (31.5) | 11 (24.4) | 30 (35.3) | |
| Others** | 89 (68.5) | 34 (75.6) | 55 (64.7) | |

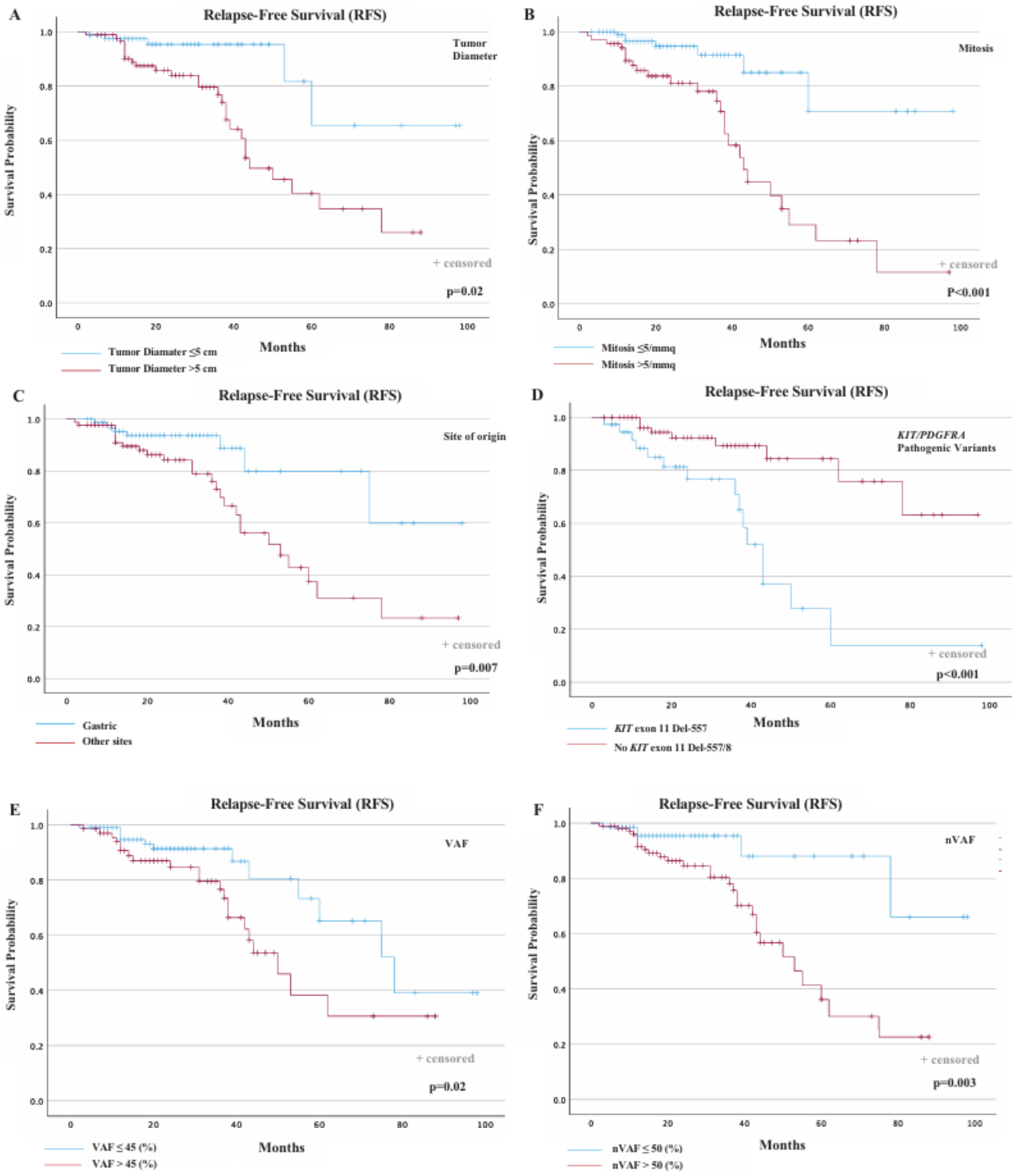


**Figure 9**. RFS according to prognostic factors. RFS according to (**A**) diameter of the primary tumor; (**B**) baseline mitosis; (**C**) site of origin; (**D**) *KIT* or *PDGFRA* PVs; (**E**) VAF; (**F**) nVAF. RFS, relapse-free survival; VAF, variant allele frequency; nVAF, normalized VAF.

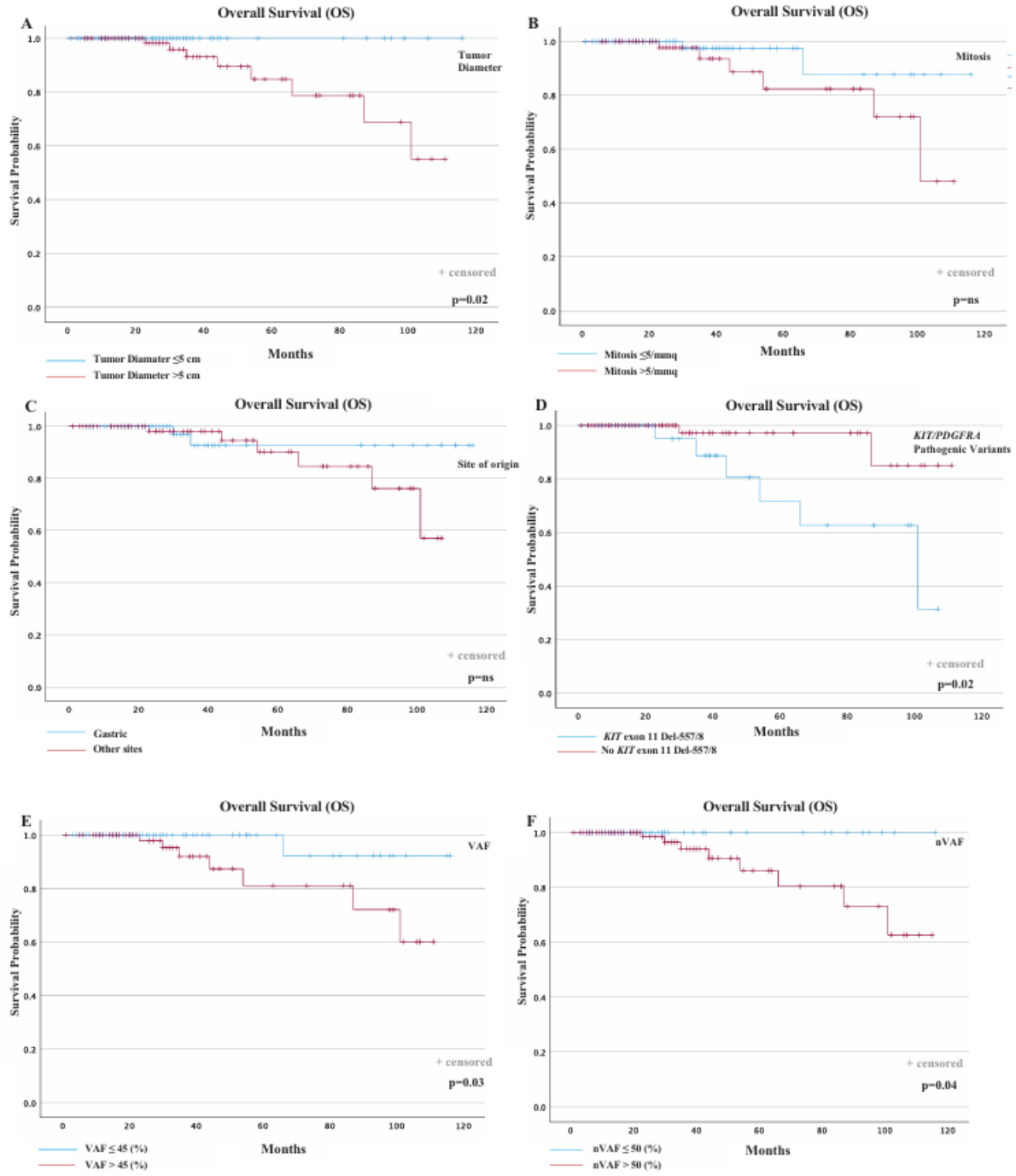


**Figure 10.** OS according to prognostic factors. OS according to (**A**) diameter of primary tumor; (**B**) baseline mitosis; (**C**) site of origin; (**D**) *KIT* or *PDGFRA* PVs; (**E**) VAF; (**F**) nVAF. OS, overall survival; VAF, variant allele frequency; nVAF, normalized VAF.

**Table 9.** Univariable and multivariable analysis of prognostic factorsfor RFS and OS in localized patients with GIST. Abbreviations: Del, deletions; Delins, deletions/insertions; HR, hazard ratio; OS, overall survival; RFS, relapse-free survival; VAF, variant allele frequency; nVAF, norm

| RFS | Univariable Cox regression | | Multivariable Cox regression | |
|---|---|---|---|---|
| | HR (95%CI) | *P*-value | HR (95%CI) | *P*-value |
| Gender (F vs. M) | 0.58 (0.29-1.19) | Ns | | |
| Primitive tumor diameter (≤5 cm vs. >5 cm) | 3.94 (1.58-10.26) | .005 | 1.08 (0.31-3.77) | ns |
| Mitosis (≤ 5/mmq vs. >5/mmq) | 4.59 (1.98-10.63) | <.001 | 4.26 (1.36-13.38) | .01 |
| Gastric site of origin (No vs. yes) | 2.83 (1.28-6.27) | .01 | 1.50 (0.52-4.31) | ns |
| Exon 11 Del or Delins 557 and/or 558 (No vs. yes) | 0.21 (0.09-0.49) | <.001 | 0.22 (0.08-0.63) | .005 |
| nVAF (≤ 50% vs. > 50%) | 3.83 (1.47-9.93) | .006 | 4.97 (1.29-19.11) | .01 |

| OS | Univariable Cox regression | | Multivariable Cox regression | |
|---|---|---|---|---|
| | HR (95%CI) | *P*-value | HR (95%CI) | *P*-value |
| Gender (F vs. M) | 0.84 (0.19-3.61) | ns | | |
| Primitive tumor diameter (≤ 5 cm vs. >5 cm) | 1.19 (0.48-2.98) | ns | | |
| Mitosis (≤ 5/mmq vs. >5/mmq) | 3.37 (0.68-16.79) | ns | | |
| Gastric site of origin (No vs. yes) | 2.15 (0.43-10.85) | ns | | |
| Exon 11 Del or Delins 557 and/or 558 (No vs. yes) | 0.18 (0.03-0.91) | .03 | | |
| nVAF (≤ 50% vs. > 50%) | 6.92 (0.85-56.62) | ns | | |

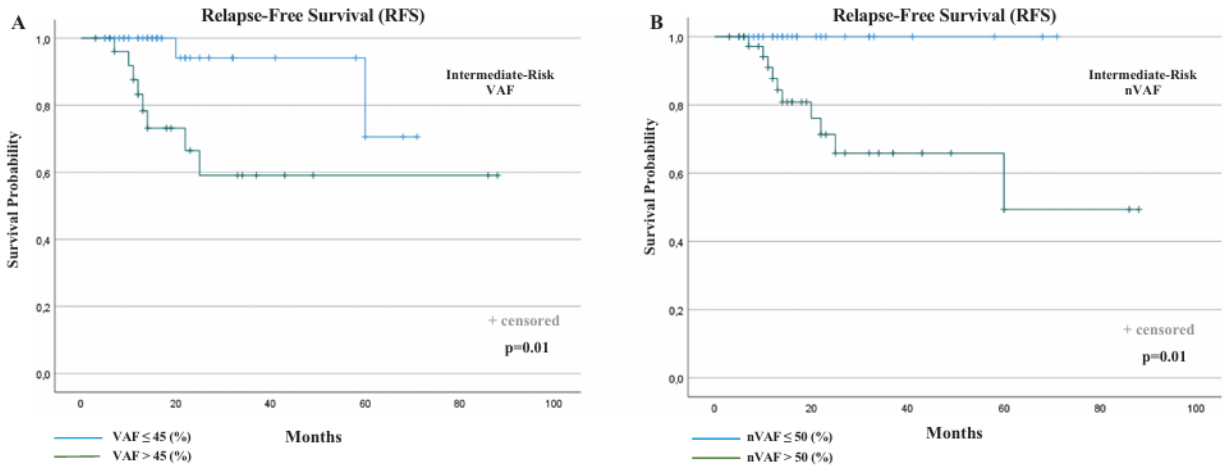


**Figure 11 .** RFS according to VAF (**A**) and nVAF (**B**) in the sub-population of intermediate-risk, *KIT* mutated, GIST patients. RFS, relapse-free survival; VAF, variant allele frequency; nVAF, normalized VAF.

**Table 10**. Pathogenetic variants in patients with GISTs. Abbreviations: SNV, Single Nucleotide Variant, Del/Ins, Deletion and Insertion.

| Gene | Exon | Type of variant | HGVS Nomenclature | Proteinchange | No. patients |
|---|---|---|---|---|---|
| ***KIT*** | 11 | Duplication | c.1716_1717insGAC | p.Asp572dup | 1 |
| | | Duplication | c.1731_1754dup | p.Tyr578_Pro585dup | 1 |
| | | Duplication | c.1717_1761dup | p.Pro573_Asn587dup | 1 |
| | | Deletion | c.1649_1654delAACCCA | p.Lys550_Pro551del | 1 |
| | | Deletion | c.1686_1724del | p.Ile563_Gln575del | 1 |
| | | Deletion | c.1658_1669del | p.Tyr553_Gln556del | 1 |
| | | Deletion | c.1735_1737delGAT | p.Asp579del | 1 |
| | | Deletion | c.1665_1676delACAGTGGAAGGT | p.Gln556_Val559del | 1 |
| | | Deletion | c.1687_1716del | p.Ile563_Asp572del | 1 |
| | | Deletion | c.1669_1674delTGGAAG | p.Trp557_Lys558del | 4 |
| | | Deletion | c.1658_1669delATGAAGTACAGT | p.Tyr553_Gln556del | 1 |
| | | Deletion | c.1661_1675delAAGTACAGTGGAAGG | p.Glu554_Lys558del | 2 |
| | | Deletion | c.1669_1683delTGGAAGGTTGTTGAG | p. Trp557_Glu561del | 2 |
| | | Deletion | c.1672_1674delAAG | p.Lys558del | 1 |
| | | Deletion | c.1690_1734del | p.Asn564_Tyr578 | 1 |
| | | Deletion | c.1648-6_1665del | p.Lys550_Val555del | 1 |
| | | Deletion | c.1679_1681delTTG | p.Val560del | 1 |
| | | Deletion | c.1648_1674del | p.Lys550_Lys558del | 2 |
| | | Deletion | c.1655_1672del | p.Met552_Trp557del | 1 |
| | | Deletion | c.1735_1737delGAT | p.Asp579del | 1 |
| | | Del/Ins | c.1679_1680delTTinsAG | p.Val560Glu | 2 |
| | | Del/Ins | c.1671_1676delGAAGGT | p.W557_V559delinsCys | 1 |
| | | Del/Ins | c.1670_1678delGGAAGGTTG | p.Trp557_Val560delinsPhe | 1 |
| | | Del/Ins | c.1703_1727delins | p.Tyr568_Leu576delinsCysCys | 1 |
| | | Del/Ins | c.1673_1674insTCC | p.Lys558delinsAsnPro | 1 |
| | | Del/Ins | c.1670_1675del | p.Trp557_Val559delinsPhe | 1 |
| | | SNV | c.1669T>C | p.Trp557Arg | 1 |
| | | SNV | c.1669T>A | p.Trp557Arg | 2 |
| | | SNV | c.1676T>A | p.Val559Asp | 5 |
| | | SNV | c.1676T>G | p.Val559Gly | 5 |
| | | SNV | c.1679T>A | p.Val560Asp | 2 |
| | | SNV | c.1727T>C | p.Leu576Pro | 3 |
| | 9 | Duplication | c.1509_1510insGCCTAT | p.Ala502_Tyr503dup | 6 |
| | 13 | SNV | c.1964A>G | p.Asn655Ser | 1 |
| | | SNV | c.1924A>G | p.Lys642Glu | 1 |
| ***PDGFRA*** | 12 | Del/Ins | c.1698_1712del | p.Ser566_Glu571delinsArg | 1 |
| | 18 | Deletion | c.2526_2537del | p.Ile843_Asp846del | 1 |
| | | SNV | c.2525A>T | p.Asp842Val | 5 |
| ***BRAF*** | 15 | SNV | c.1799T>A | p.Val600Glu | 1 |
| ***MAP2K1*** | 2 | SNV | c.166C>A | p.Gln56Lys | 1 |

**Table 11**. Clinical and pathological characteristics of patients with GIST based on the gene expression value of *YAP*, *TAZ* and *CTGF*. *Abbreviations:* Del, deletion; Del-557/558, *KIT* exon 11 deletion or deletion/insertions involving 557 and/or 558 codons; Del-No-557/558, *KIT* exon 11 deletion or deletion/insertions in codons other than 557 and/or 558; HPF, high-power field; No-Del, *KIT* exon 11 duplication, insertion, or single nucleotide variant; NA, not availabe.

| **Characteristics** | **N. of Patients (%)** | ***YAP*** | | ***TAZ*** | | ***CTGF*** | |
|---|---|---|---|---|---|---|---|
| | | **<1** | **≥1** | **<1** | **≥1** | **<1** | **≥1** |
| **N. of patients** | 27 | 14 (51.9%) | 13 (48.1%) | 16 (59.3%) | 11 (40.7%) | 12 (44.4%) | 15 (55.6%) |
| **Gender** | | | | | | | |
| Male | 18 (66.7%) | 11(78.6%) | 7(53.8%) | 10(62,5%) | 8(72.8%) | 8(66.7%) | 10(66.7%) |
| Female | 9 (33.3%) | 3 (21.4%) | 6(46.2%) | 6(37.5%) | 3(27.2%)) | 4(33.3%) | 5(3.33%) |
| **Age at diagnosis** | | | | | | | |
| Media | 68 | 64 | 63 | 68 | 56 | 69 | 59 |
| Mediana | 63.7 | 68 | 72 | 71 | 59 | 71 | 59 |
| Range | 23-86 | 28-86 | 23-86 | 47-86 | 23-86 | 41-86 | 23-86 |
| **Age groups** | | | | | | | |
| ≤50 | 6 (22.2%) | 2 (14.3%) | 4(30.8%) | 1(6.2%) | 5(45.4%) | 1(8.3%) | 5(33.3%) |
| >50 | 21(77.8%) | 12(85.7%) | 9(69.2%) | 15(93.8%) | 6(54.6%) | 11(91.7%) | 10(66.7%) |
| **Site of origin** | | | | | | | |
| Gastric | 18(66.7%) | 11 (78.6%) | 7(53.8%) | 12(75%) | 6(54.6%) | 9(81.8%) | 9(60%) |
| Small bowel | 9(33.3%) | 3 (21.4%) | 6(46.2%) | 4(25%) | 5(45.4%) | 3(27.3%) | 6(40%) |
| **Baseline diameter** | | | | | | | |
| ≤5 cm | 7(25.9%) | 5(35.7%) | 2(15.4%) | 5(31.2%) | 2(18.2%) | 3(27.3%) | 4(26.7%) |
| >5 cm | 18(66.7%) | 8(57.1%) | 10(76.9%) | 10(62.5%) | 8(72.8%) | 8(66.7%) | 10(66.7%) |
| N.A. | 2 | 1 | 1 | 1 | 1 | 1 | 1 |
| **Baseline Mitosis** | | | | | | | |
| ≤5/50 HPF | 12(44.4%) | 6(42.9%) | 6(46.1%) | 6(37.5%) | 6(54.6%) | 5(41.7%) | 7(46.7%) |
| >5/50 HPF | 10(37%) | 5(35.7%) | 5(38.5%) | 5(31.2%) | 5(45.4%) | 3(25%) | 7(46.7%) |
| N.A. | 5 | 3 | 2 | 5 | / | 4 | 1 |
| **Histology** | | | | | | | |
| spindle cell | 15(55.6%) | 7(50%) | 8(61.5%) | 10(62.5%) | 5(45.4%) | 7(58.3%) | 8(53.3%) |
| epithelioid | 2(7.4%) | 2(14.3%) | 0 | 1(6.2%) | 1(9.1%) | 1(8.3%) | 1(6.7%) |
| mixed | 8(29.6%) | 4(28.6%) | 4(30.8%) | 4(25%) | 4(36.4%) | 3(25%) | 5(33.3%) |
| N.A. | 2 | 1 | 1 | 1 | 1 | 1 | 1 |
| **Risk class** | | | | | | | |
| None | 3(11.1%) | 2(14.3%) | 1(7.7%) | 3(18.7%) | / | 1(8.3%) | 2(13.3%) |
| Very Low/Low | 6(22.2%) | 3(21.4%) | 3(23.1%) | 3(18.7%) | 3(27.3%) | 3(25%) | 3(20%) |
| Moderate | 1(3.7%) | 1(7.1%) | / | 1(6.2%) | / | 1(8.3%) | / |
| High | 11(40.7%) | 3(21.4%) | 8(61.5%) | 3(18.7%) | 8(72.7%) | 1(8.3%) | 10(66.7) |
| N.A. | 6 | 5 | 1 | 5 | 1 | 6 | / |
| **Mutated Gene** | | | | | | | |
| *KIT* ex11 | 18(66.7%) | 11(78.6%) | 7(53.8%) | 13(81.3%) | 5(45.4%) | 10(83.4%) | 8(53.3%) |
| *PDGFRA* | 5(18.5%) | 3(21.4%) | 2(15.4%) | 2(12.5%) | 3(27.3%) | 1(8.3%) | 4(26.7%) |
| WT | 4(14.8%) | / | 4(30.8%) | 1(6.2%) | 3(27.3%) | 1(8.3%) | 3(20%) |
| ***KIT* Exon 11** | | | | | | | |
| Del-557/8 | 7(38.9%) | 6(54.5%) | 1(14.3%) | 6(46.1%) | 1(20%) | 5(50%) | 2(25%) |
| Del-No-557/8 | 2(11.1%) | 2(18.2%) | / | 2(15.4%) | / | 1(10%) | 1(12.5%) |
| No-Del | 9(50%) | 3(27.3%) | 6(85.7%) | 5(38.5%) | 4(80%) | 4(40%) | 5(62.5%) |
| ***PDGFRA*** | | | | | | | |
| D842V | 4(80%) | 2(66.7%) | 2(100%) | 2(100%) | 2(66.7%) | 1 | 3(75%) |
| Others | 1(20%) | 1(33.3%) | / | / | 1(33.3%) | / | 1(25%) |
| **VAF(%)** | | | | | | | |
| <50% | 17(73.9%) | 9(64.3%) | 8(88.9%) | 11(73.3%) | 6(75%) | 7(63.6%) | 10(83.3%) |
| ≥50% | 6(26.1%) | 5(35.7%) | 1(11.1%) | 4(26.7%) | 2(25%) | 4(36.3%) | 2(16.7%) |
| **Metastatic GIST atdiagnosis** | 5(18.5%) | 4(80%) | 1(20%) | 4(80%) | 1(20%) | 2(40%) | 3(60%) |
| **Localized GIST** | 22(81.5%) | 12(54.5%) | 10(45.5%) | 10(45.5%) | 12(54.5%) | 12(54.5%) | 10(45.5%) |
| **Metastatic Sites** | | | | | | | |
| Liver | 4 (80%) | 4(100%) | / | 3(75%) | 1(25%) | 2(50%) | 2(50%) |
| Peritonuem | / | / | / | / | / | / | / |
| Both | 1(20%) | / | 1(100%) | 1(100%) | / | / | 1(100%) |
| **Recurrence** | | | | | | | |
| Yes | 3(13.6%) | / | 3(100%) | / | 3(100%) | 1(33.3%) | 2(66.7%) |
| No | 19(86.4%) | 12(63.1%) | 7(36.8%) | 10(52.6%) | 9(47.4%) | 11(57.9%) | 8(42.1%) |
| **Imatinib I line** | | | | | | | |
| Yes | 5(100%) | 4(80%) | 1(20%) | 4(80%) | 1(20%) | 2(40%) | 3(60%) |
| No | / | / | / | / | / | / | / |

**Table 12**. Clinical and pathological characteristics of patients with GIST based on the gene expression value of *CCDN1, LIX1, NFKB1 e AXL*. *Abbreviations:* Del, deletion; Del-557/558, *KIT* exon 11 deletion or deletion/insertions involving 557 and/or 558 codons; Del-No-557/558, *KIT* exon 11 deletion or deletion/insertions in codons other than 557 and/or 558; HPF, high-power field; No-Del, *KIT* exon 11 duplication, insertion, or single nucleotide variant; NA,not availabe.

| Characteristics | *CCND1* | | *LIX1* | | *NFKB1* | | *AXL* | |
|---|---|---|---|---|---|---|---|---|
| | <1 | ≥1 | <1 | ≥1 | <1 | ≥1 | <1 | ≥1 |
| **N. of patients** | 27 (100%) | 0 | 25 (92.6%) | 2 (7.4%) | 22 (81.5%) | 5 (18.5%) | 27 (100%) | 0 |
| **Gender** | | | | | | | | |
| Male | 18(66.7%) | | 16(64%) | 2(100%) | 13(59.1%) | 5(100%) | 18(66.7%) | |
| Female | 9 (33.3%) | | 9(36%) | / | 9(40.9%) | / | 9 (33.3%) | |
| **Age at diagnosis** | | | | | | | | |
| Media | 68 | | 63 | 65 | 64 | 60 | 68 | |
| Mediana | 63.7 | | 68 | 65 | 69 | 59 | 63.7 | |
| Range | 23-86 | | 23-86 | 59-72 | 23-86 | 46-86 | 23-86 | |
| **Age groups** | | | | | | | | |
| ≤50 | 6 (22.2%) | | 6(24%) | / | 4(18.2%) | 2(40%) | 6 (22.2%) | |
| >50 | 21(77.8%) | | 19(76%) | 2(100%) | 18(81.8%) | 3(60%) | 21(77.8%) | |
| **Site of origin** | | | | | | | | |
| Gastric | 18(66.7%) | | 17(68%) | 1(50%) | 16(72.7%) | 2(40%) | 18(66.7%) | |
| Small bowel | 9(33.3%) | | 8(32%) | 1(50%) | 6(27.3%) | 3(60%) | 9(33.3%) | |
| **Baseline diameter** | | | | | | | | |
| ≤5 cm | 7(25.9%) | | 7(28%) | / | 6(27.3%) | 1(20%) | 7(25.9%) | |
| >5 cm | 18(66.7%) | | 16(64%) | 2(100%) | 15(68.2%) | 3(60%) | 18(66.7%) | |
| N.D. | 2 | | 2 | / | 1 | 1 | 2 | |
| **Baseline Mitosis** | | | | | | | | |
| ≤5/50 HPF | 12(44.4%) | | 12(58%) | / | 10(45.4%) | 2 (40%) | 12(44.4%) | |
| >5/50 HPF | 10(37%) | | 8(32%) | 2(100%) | 7(31.8%) | 3(60%) | 10(37%) | |
| N.D. | 5 | | 5 | / | 5 | / | 5 | |
| **Histology** | | | | | | | | |
| spindle cell | 15(55.6%) | | 14(56%) | 1(50%) | 13(59.1%) | 2(40%) | 15(55.6%) | |
| epithelioid | 2(7.4%) | | 2(8%) | / | 2(9.1%) | / | 2(7.4%) | |
| mixed | 8(29.6%) | | 7(28%) | 1(50%) | 6(27.3%) | 2(40%) | 8(29.6%) | |
| N.D. | 2 | | 2 | / | 1 | 1 | 2 | |
| **Risk class** | | | | | | | | |
| None | 3(11.1%) | | 3(12%) | / | 3(13.6%) | / | 3(11.1%) | |
| Very Low/Low | 6(22.2%) | | 6(24%) | / | 6(27.3%) | / | 6(22.2%) | |
| Moderate | 1(3.7%) | | 1(4%) | / | 1(4.5%) | / | 1(3.7%) | |
| High | 11(40.7%) | | 9(36%) | 2(100%) | 6(27.3%) | 5(100%) | 11(40.7%) | |
| N.D. | 6 | | 6 | / | 6 | / | 6 | |
| **Mutated Gene** | | | | | | | | |
| *KIT* ex11 | 18(66.7%) | | 16(64%) | 2(100%) | 15(68.2%) | 3(60%) | 18(66.7%) | |
| *PDGFRA* | 5(18.5%) | | 5(20%) | / | 4(18.2%) | 1(20%) | 5(18.5%) | |
| WT | 4(14.8%) | | 4(16%) | / | 3(13.6%) | 1(20%) | 4(14.8%) | |
| ***KIT* Exon 11** | | | | | | | | |
| Del-557/8 | 7(38.9%) | | 7(43.7%) | / | 7(46.7%) | / | 7(38.9%) | |
| Del-No-557/8 | 2(11.1%) | | 2(12.6%) | / | 2(13.3%) | / | 2(11.1%) | |
| No-Del | 9(50%) | | 7(43.7%) | 2 | 6(40%) | 3(100%) | 9(50%) | |
| ***PDGFRA*** | | | | | | | | |
| D842V | 4(80%) | | 4(80%) | / | 3(75%) | 1(100%) | 4(80%) | |
| Others | 1(20%) | | 1(20%) | / | 1(25%) | / | 1(20%) | |
| **VAF(%)** | | | | | | | | |
| <50% | 17(73.9%) | | 15(71.4%) | 2(100%) | 13(68.4%) | 4(100%) | 17(73.9%) | |
| ≥50% | 6(26.1%) | | 6(28.6%) | / | 6(31.6%) | / | 6(26.1%) | |
| **Metastatic GIST atdiagnosis** | 5(100%) | | 5(100%) | / | 5(100%) | / | 5(100%) | |
| **Localized GIST** | 22(100%) | | 20(91%) | 2(9%) | 17(77.3%) | 5(22.7%) | 22(100%) | |
| **Metastatic Sites** | | | | | | | | |
| Liver | 4(100%) | | 4(100%) | | 4(100%) | | 4(100%) | |
| Peritonuem | / | | / | | / | | / | |
| Both | 1(100%) | | 1(100%) | | 1(100%) | | 1(100%) | |
| **Recurrence** | | | | | | | | |
| Yes | 3(100%) | | 1(33.3%) | 2(66.7%) | / | 3(100%) | 3(100%) | |
| No | 19(100%) | | 19(100%) | / | 17(89.5%) | 2(10.5%) | 19(100%) | |
| **Imatinib I line** | | | | | | | | |
| Yes | 5(100%) | | 5(100%) | | 5(100%) | | 5(100%) | |
| No | / | | / | | / | | / | |

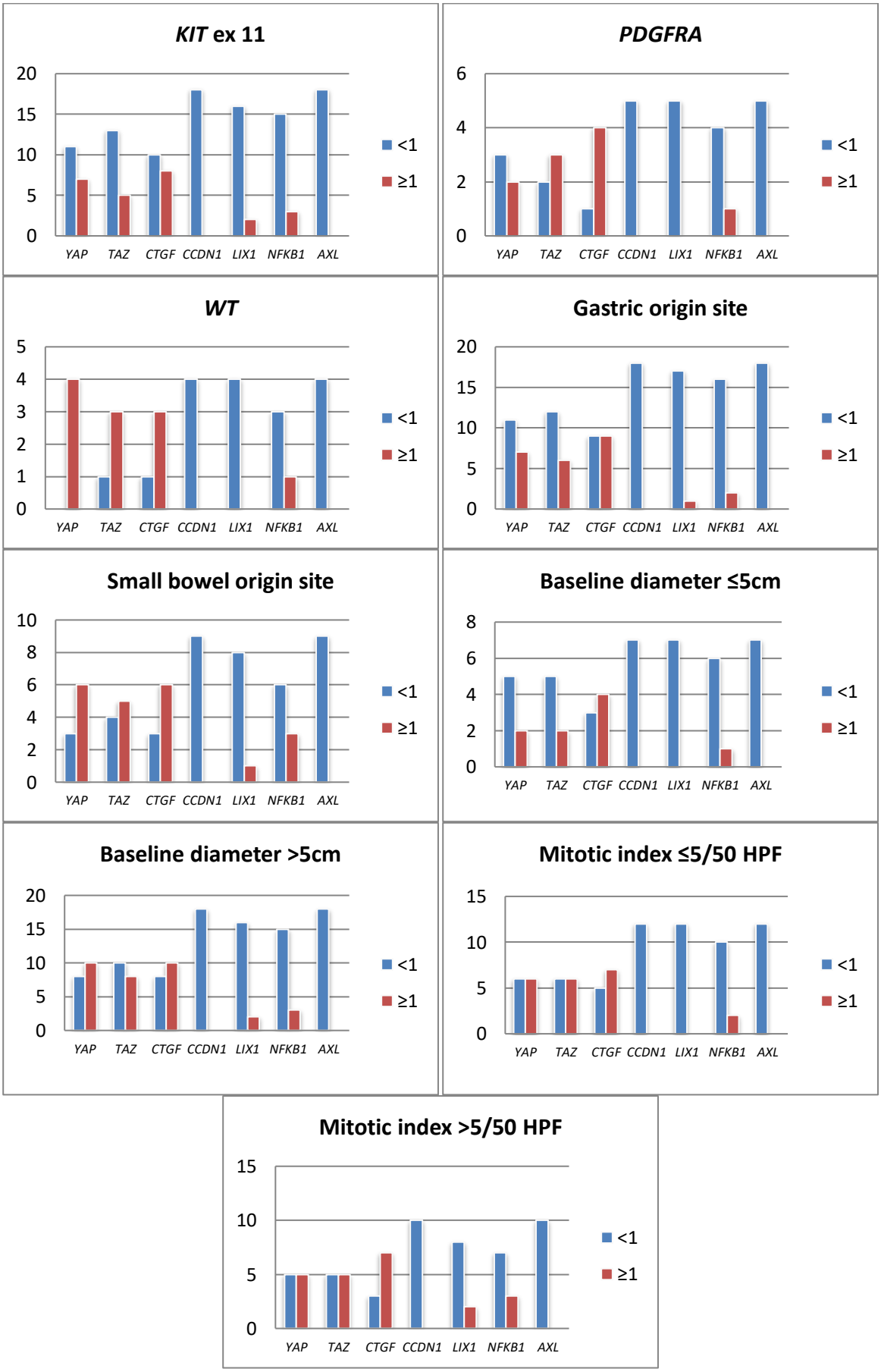


**Figure 12**. Gene expression values compared to mutational status and known prognostic factors.

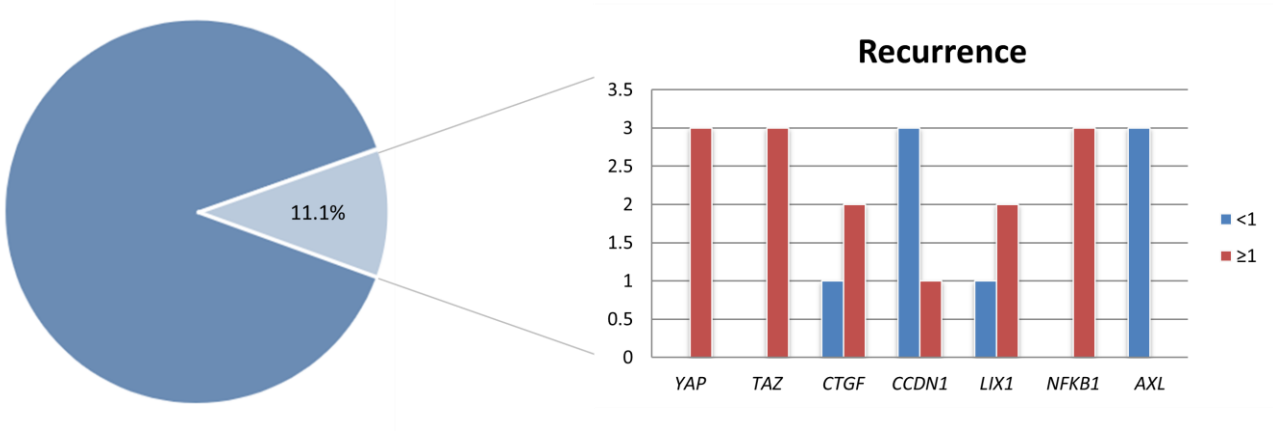


**Figure 13**. Gene expression in GIST patients with tumor recurrence.

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# Scientific Products

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**ABSTRACTS**

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