MOLECULAR STUDY OF THERAPEUTIC TARGETS OF TYROSINE KINASE INHIBITORS IN ENDOMETRIAL STROMAL TUMORS

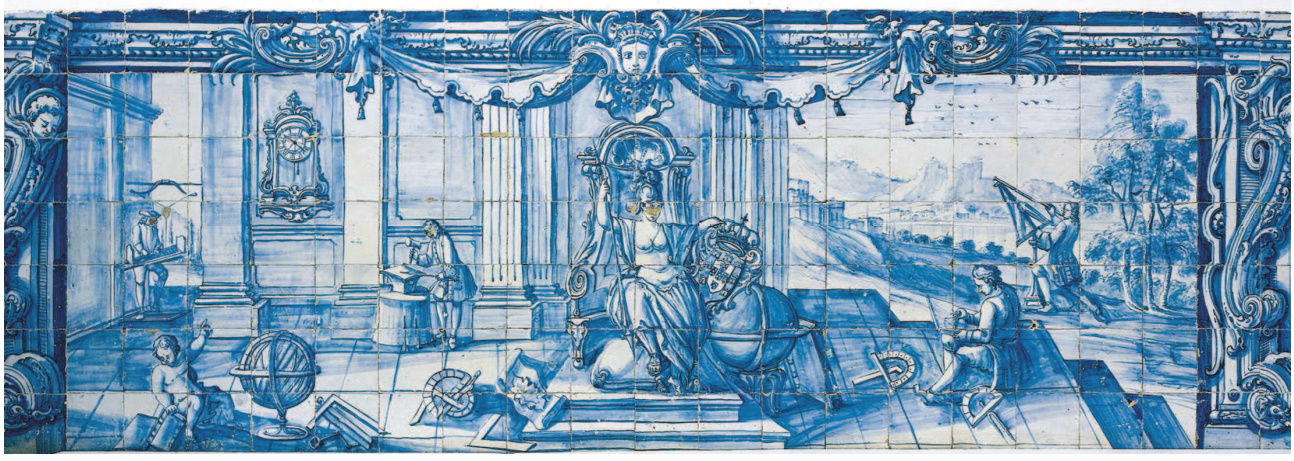
MOLECULAR AND PROTEIN EXPRESSION OF KIT, PDGFRA AND EGFR

Ruth Andreia Henriques Sardinha

Tese apresentada à Universidade de Évora
para obtenção do Grau de Doutor em Biologia

ORIENTADORES:
Professor Doutor Enrique de Álava
Professor Doutor Fernando Capela e Silva

ÉVORA, ABRIL 2014



INSTITUTO DE INVESTIGAÇÃO E FORMAÇÃO AVANÇADA

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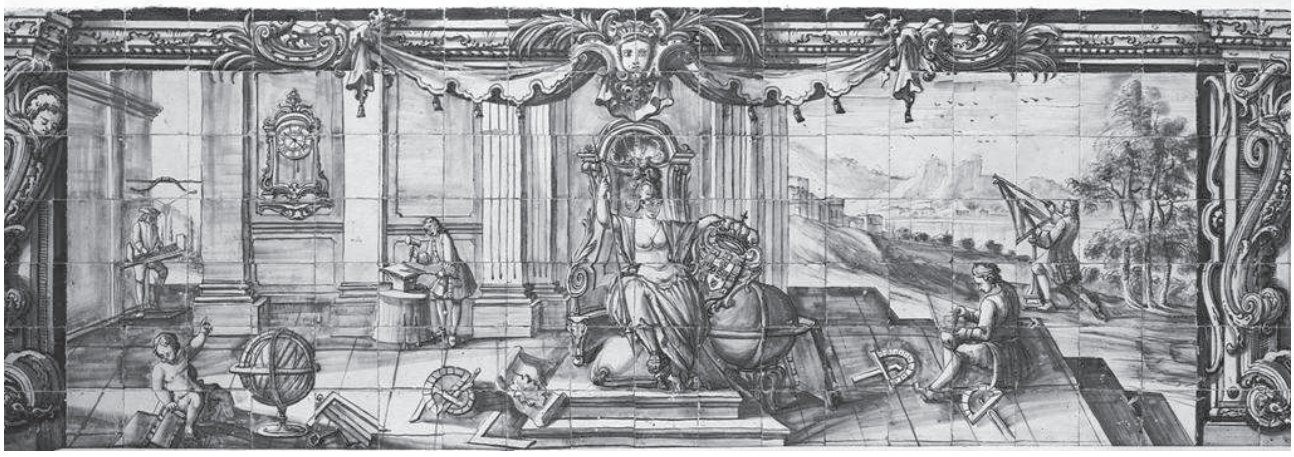

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Every day is new again
Every day is yours to win
And that's how heroes are made

Michael Stipe

À minha avó Dulce

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RESUMO

Estudo molecular dos alvos terapêuticos dos inibidores tirosina cinase em Tumores do Estroma Endometrial: Expressão Molecular e Proteica de KIT, PDGFRA e EGFR

Os tumores do estroma endometrial (EST) representam 15% dos sarcomas uterinos, e são caraterizados por recorrências tardias e metástases à distância. O tratamento sistémico destas neoplasias não está totalmente estabelecido e alguns estudos descrevem respostas objetivas ao inibidor tirosina cinase (TKI) imatinib, o que sugere uma nova estratégia terapêutica para estes tumores. Nesse sentido, o presente trabalho teve como objetivo efetuar uma análise retrospetiva dos possíveis alvos moleculares dos TKI em EST: KIT, PDGFRA e EGFR. Numa extensa série de EST que incluiu sarcomas do estroma endometrial de baixo grau (n=52) e sarcomas endometriais indiferenciados (n=13) foi efetuada a análise mutacional dos exões 9, 11, 13, e 17 do gene *KIT*, exões 12 e 18 do gene *PDGFRA* e exões 18, 19, 20 e 21 do gene *EGFR*. A expressão proteica de cada recetor foi avaliada por imunohistoquímica, e a técnica de hibridação *in situ* por fluorescência foi utilizada para avaliar o *status* do gene *EGFR*. A sobreexpressão proteica de KIT, PDGFRA e EGFR foi detetada em 2 (3%), 23 (35.4%), 7 (10.8%) dos casos, respetivamente. Não foram detetadas mutações ativadoras nos genes *KIT*, *PDGFRA* e *EGFR*, nem amplificação do gene *EGFR.* Em conclusão, a ausência de expressão significativa, amplificação e mutações ativadoras nestes recetores tirosina cinase sugere que é pouco provável que os EST possam beneficiar de terapias como TKI como tratamento sistémico.

Palavras-chave: Tumores do estroma endometrial, KIT, PDGFRA, EGFR, Inibidores tirosina cinase, Tratamento sistémico

ABSTRACT

Molecular study of therapeutic targets of tyrosine kinase inhibitors in Endometrial Stromal Tumors: Molecular and Protein Expression of KIT, PDGFRA and EGFR

Endometrial stromal tumors (EST) represent 15% of uterine sarcomas, and are characterized by late recurrences and distant metastasis. The systemic treatment of these malignancies is not well established and few reports describe objective responses to tyrosine kinase inhibitor (TKI) imatinib, which suggest a novel therapeutic strategy for these tumors. Due to these facts, the present work aimed to perform a retrospective analysis of possible molecular targets of TKIs in EST: KIT, PDGFRA and EGFR. In a large series of EST, which included endometrial stromal sarcomas (n=52) and undifferentiated endometrial sarcomas (n=13) the mutational analysis was performed for exons 9, 11, 13, and 17 of the *KIT* gene, exons 12 and 18 of the *PDGFRA* gene and exons 18, 19, 20 and 21 of the *EGFR* gene. Protein expression of each receptor was assessed by immunohistochemistry, and fluorescence *in situ* hybridization was used to evaluate *EGFR* gene status. Overexpression of KIT, PDGFRA, EGFR, was detected in 2 (3%), 23 (35.4%), 7 (10.8%) cases, respectively. Neither activating mutations in *KIT*, *PDGFRA* and *EGFR* genes nor amplification of EGFR gene was detected. In conclusion, absence of significant expression, amplification and activating mutations on these tyrosine kinase receptors suggest that it is unlikely that EST can benefit from therapies such as TKI on the systemic setting.

Keywords: Endometrial stromal tumors, KIT, PDGFRA, EGFR, Tyrosine kinase inhibitors, Systemic treatment

ABBREVIATIONS

AJCC – American Joint Committee of Cancer
APC – Adenomatous polyposis coli
AR – Antigen retrieval
ATC – Anaplastic thyroid cancer
ATP – Adenosine triphosphate
BSO – Bilateral salpingo-oophoferectomy
Chr – Chromosome
CIC – Centro de Investigación del Cáncer
CML – Chronic myeloid leukaemia
CMML – Chronic myelomonocytic leukaemia
DFS – Disease free-survival
DFSP – Dermatofibrosarcoma protuberans
EGF – Epidermal growth factor
EGFR – Epidermal growth factor receptor
ER – Estrogen receptor
ESMO – European Society for Medical Oncology
ESN – Endometrial stromal nodule
ESS – Low-grade endometrial stromal sarcoma
EST – Endometrial stromal tumors
ET – Endocrine therapy
FDA – U.S. Food and Drug Administration
FFPE – Formalin-fixed paraffin embedded
FIGO – International Federation of Gynaecology and Obstetrics
FISH – Fluorescence *in situ* hybridization
GAPDH – Glyceraldehyde-3-phosphate dehydrogenase
GBM – Glioblastoma multiforme
GCF – Giant cell fibroblastoma
GEIS – Grupo Español de Investigación en Sarcomas
GIST – Gastrointestinal stromal tumor
GnRH – Gonadotrophin-releasing hormone receptors
H&E – Hematoxylin and eosin
HIER – Heat-induced epitope retrieval
HNC – Head and neck cancer
HPF – High power field

IHC – Immunohistochemistry

IHES – Idiopathic hypereosinophilic syndrome

JAK/STAT – Janus kinase/signal transducers and activators of transcription

LEF – Lymphoid enhancing factor

LRC – Local regional control

MAPK – Mitogen-activated protein kinase

NBF – Neutral buffered formalin

NCCN – National Comprehensive Cancer Network

NSCLC – Non-small cell lung cancer

OS – Overall survival

PCR – Polymerase chain reaction

PDGF – Platelet-derived growth factor

PDGFRA – Platelet-derived growth factor receptor A

PDGFRB – Platelet-derived growth factor receptor B

PFS – Progression free-survival

PI3K – Phosphoinositol 3-kinase

PLC-γ – Phospholipase Cγ

PPLD – Pelvic and para-aortic lymphadenectomy

PR – Progesterone receptor

PTK – Protein tyrosine kinase

SCF – Stem cell factor

SCLC – Small cell lung cancer

SNP – Single nucleotide polymorphisms

STS – Soft tissue sarcoma

TCF – T-cell factor

TH – Total hysterectomy

TK – Tyrosine kinase

TKI – Tyrosine kinase inhibitor

TKR – Tyrosine kinase receptor

TMA – Tissue microarray

UES - P – Undifferentiated endometrial sarcoma with nuclear pleomorphism

UES - U – Undifferentiated endometrial sarcoma with nuclear uniformity

UES – Undifferentiated endometrial sarcoma

WHO – World Health Organization

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INTRODUCTION

Endometrial stromal tumors (EST)[1]: low-grade endometrial stromal sarcoma (ESS) and undifferentiated endometrial sarcomas (UES) are rare uterine sarcomas that present distinct histological, cytogenetic and molecular features.

ESS are characterized by a 5-year overall survival from 67% to 96% at 5-years, since it is usually diagnosed in early stages, whereas UES is an aggressive neoplasm that apparently shows poor prognosis, even in early stages, with 5-year survival rates ranging from 25-55%. Despite the indolent behaviour of ESS, both neoplasias are characterized by a high rate of late recurrences and distant metastasis. The standard surgical treatment includes total hysterectomy and bilateral oophorectomy, but an appropriate adjuvant and/or systemic treatment is not well established yet due to the disease rarity and the lack of randomized studies. Endocrine therapy is mainly applied to ESS based on hormonal receptors expression, and chemotherapy seems to have a limited efficacy on advanced disease, whereas radiotherapy shows good results on local-regional control rate.

Oncogene addiction is a common phenomenon in which a tumor becomes largely dependent on a single activated oncogene (Pecorino, 2008). An example of this phenomenon is the relationship between molecular alterations (gene amplification and mutation, autocrine/paracrine growth loops) that lead to constitutive activation of tyrosine kinase receptors (TKR) and pathogenesis of several solid tumors. Based on this, TKR are now considered the main drug targets for molecular therapies: monoclonal antibodies and tyrosine kinase inhibitors (TKI), which exploit the activated oncogene as the Achilles heel of the disease (Pierotti et al., 2010). Presence of *KIT* and *PDGFRA* mutations in gastrointestinal stromal tumor and presence of BCR-ABL fusion oncoprotein in chronic myeloid leukaemia are examples of this oncogene addiction and are the main predictors of response to imatinib mesylate. Another clear example is the association between activated EGFR (overexpression, gene amplification/mutations) and non-small cell lung cancer pathogenesis and response to treatment with anti-EGFR TKIs.

Some reports in uterine sarcomas and EST describe expression of TKR as PDGFRA/B, KIT and EGFR, which represent well-defined molecular targets of imatinib mesylate, gefitinib and erlotinib, and few reports described objective responses with imatinib in EST patients who express at least one TKI target. These findings point out that TKR expression in EST may represent a therapeutic target of TKI, as well as the need of an extensive evaluation of the

[1] Accordingly to the 2003 World Health Organization classification (Hendrickson and Tavassoli, 2003)

molecular targets of TKI on EST that may allow the identification of a novel therapeutic strategy for these malignancies.

The state of art and these evidences lead the Grupo Español de Investigación en Sarcomas (GEIS) to design the Project GEIS 18, which aim was to evaluate the gene status and protein expression of KIT, PDGFRA, and EGFR in a large series of EST, their distribution among the distinct EST subgroups and correlate the immunohistochemical expression with mutational status. The present Thesis is the result of Project GEIS 18, which main results were already published:

Original Publication: Sardinha, R., Hernandez, T., Fraile, S., Tresserra, F., Vidal, A., Gomez, M. C., et al. (2013). Endometrial stromal tumors: immunohistochemical and molecular analysis of potential targets of tyrosine kinase inhibitors. *Clinical Sarcoma Research* 2013, 3:3 doi: 10.1186/2045-3329-3-3. PMID: 23497641 [PubMed]. PMCID: PMC3599876 (Annex 1).

1. REVIEW OF THE LITERATURE

Uterine sarcomas are rare neoplasms derived from or differentiating towards mesodermally derived tissues: endometrial stromal and myometrial smooth muscle cells, although differentiation towards heterologous tissues (skeletal muscle, cartilage) also may be seen (Hendrickson and Tavassoli, 2003; Nucci and Bradley, 2006). Uterine sarcomas represent 3-9% of uterine cancers (Brooks et al., 2004; Abeler et al., 2009) with an incidence of 0.4 to 1.7/100.000 women (Nordal and Thoresen, 1997; Koivisto-Korander et al., 2008). They present an aggressive clinical behaviour and a limited overall survival[2], and are classified into four histological subtypes: leiomyosarcomas, endometrial stromal tumors (EST), carcinosarcomas[3] and adenosarcomas (Hendrickson and Tavassoli, 2003). The pattern of recurrence include local recurrence and hematogenous spread with distant metastasis mainly in pelvis and lung (Sleijfer et al., 2007; Denschlag et al., 2007; Koivisto-Korander et al., 2008; Gadducci, 2011).

1.1 ENDOMETRIAL STROMAL TUMORS

In the 2003 World Health Organization (WHO) classification, EST are defined as mesenchymal neoplasms composed of cells that morphologically resemble non-neoplastic proliferative phase endometrial stroma (Hendrickson and Tavassoli, 2003; Nucci and Bradley, 2006).

1.1.1 EPIDEMIOLOGY

EST represents the second most frequent subtype of uterine sarcomas after leiomyosarcomas, accounting 15-20% of uterine sarcomas cases (Koivisto-Korander et al., 2008; Sampath et al., 2010). In Portugal, their actual incidence was estimated in 0.32/100.000 (Instituto Português de Oncologia, 2008; Instituto Nacional de Estatística, 2011), which is similar to the observed in Nordic countries (Koivisto-Korander et al., 2012).

[2] Overall survival: defined as the interval from the date of primary surgery to the date of death (Tanner et al., 2012a).
[3] Recently reclassified as a dedifferentiated or metaplastic form of endometrial carcinoma, is still included in most retrospective studies of uterine sarcomas (Prat, 2009).

1.1.2 CLASSIFICATION

Classification and terminology of malignant EST has undergone numerous changes as summarized in table 1. In accordance with the 2003 WHO classification they are classified into 3 categories: 1) benign endometrial stromal nodule (ESN), 2) low-grade endometrial stromal sarcoma (ESS) and 3) undifferentiated endometrial sarcoma (UES) (Hendrickson and Tavassoli, 2003). ESS is the most frequent malignant neoplasm corresponding to 60-80% of cases, whereas UES corresponds to approximately 20% of cases (Abeler et al., 2009; Kildal et al., 2009a).

Table 1 – Evolution of classification and terminology of Endometrial stromal tumors

Author	Criteria	Classification
Norris and Taylor (1966)	Tumor margins and mitotic index	Pushing margins - Stromal nodule
		Infiltrating margins: · MI<10 HPF - Endolymphatic stromal myosis · MI>10 HPF - Stromal sarcoma
Evans (1982)	Cellular pleomorphism and necrosis	· Endometrial stromal sarcoma · Poorly differentiated endometrial sarcoma
Chang et al. (1990)	Nuclear atypia	· Minimal nuclear atypia: ESS grade 1 · Significant nuclear atypia: ESS grade 2 or 3 · Pleomorphic undifferentiated sarcoma
AFIP Atlas (1992)	Nuclear atypia and resemblance with normal endometrium	· Low-grade endometrial stromal sarcoma · High-grade endometrial stromal sarcoma · Undifferentiated endometrial sarcoma
WHO (2003)	Resemblance to normal endometrium	· Low-grade endometrial stromal sarcoma · Undifferentiated endometrial sarcoma

Legend - AFIP: Armed Forces Institute of Pathology; MI: Mitotic index; HPF: High Power field. Adapted from Kurihara et al., 2008

1.1.3 CLINICAL FEATURES AND DIAGNOSIS

ESS is a clinically indolent neoplasm, more common in younger women (<50 years). 50-80% of patients are pre-menopausal (Li et al., 2005; Amant et al., 2007; Schick et al., 2012). Although diagnosed in early-stages, 15-25% of patients die of the disease (Abeler et al., 2009;

Cheng et al., 2011; Schick et al., 2012). UES represents an aggressive neoplasm usually diagnosed in advanced stages and frequent in postmenopausal women (Evans, 1982; Hendrickson and Tavassoli, 2003; Schick et al., 2012).

The main symptom is vaginal bleeding (menorrhagia, menometrorrhagia or post-menopausal bleeding) followed by abdominal pain. These tumors are diagnosed preoperatively by means of hysteroscopy and endometrial sampling, or postoperatively at the time of histological evaluation of a hysterectomy specimen (Gadducci et al., 2008; Bansal et al., 2008). Accurate preoperative diagnosis is difficult and many patients are often misdiagnosed as benign leiomyoma (Amant et al., 2003; Sagae et al., 2004; Cheng et al., 2011). Cytological examination has a limited value (Diesing et al., 2006; Li et al., 2008a), and radiological evaluation with computed tomography, magnetic resonance imaging and positron emission tomography with 18F-fluorodeoxyglucose have shown utility in the surgical management of disease (Koyama et al., 1999; Umesaki et al., 2001; Rha et al., 2003; Wu et al., 2011).

Hypertension and obesity are the most commonly related diseases (Salazar, 1978; Park et al., 2008), and some risk factors have been suggested, including pelvic irradiation, tamoxifen therapy and estrogen replacement therapy (Wickerham et al., 2002; Lin and Slomovitz; Chu et al., 2003).

Recurrences can be found to extend beyond the uterus in $^{1}/_{4}$ to ½ of ESS patients many years after the primary diagnosis or treatment (up to 25 years) (Chang et al., 1990; Abeler et al., 2009; Cheng et al., 2011; Schick et al., 2012), whereas $^{3}/_{4}$ of UES present extra-uterine disease at time of diagnosis and usually die 2-5 years after the diagnosis (Tanner et al., 2012a). Limited to the pelvis in ESS patients, some patients may develop distant metastases and the most commonly affected site is the lung with an incidence ranging between 7-28% (Berchuck et al., 1990; Goff et al., 1993; Aubry et al., 2002; Cheng et al., 2011). Distant metastasis of ESS to the brain, kidneys, bladder, bones, breast, and heart has also been reported. UES normally display a pattern of distant metastasis to unexpected locations (Gonzalez et al., 2010; Mentrikoski et al., 2012).

1.1.4 PATHOLOGICAL FEATURES

1.1.4.1 LOW-GRADE ENDOMETRIAL STROMAL SARCOMA

Defined as an invasive tumor composed of cells resembling the endometrial stromal cells in proliferative phase (Hendrickson and Tavassoli, 2003). Macroscopically, present as an irregular nodular growth or polypoid mass (mean size 7.7 cm) involving the endometrium,

myometrium or both and the sectioned surface appears yellow to tan with a soft consistency (figure 1.A). In some cases, cystic and myxoid degeneration and hemorrhagic foci without prominent necrosis can be observed (Evans, 1982; Norris and Taylor, 1966; Hendrickson and Tavassoli, 2003; Rosai, 2004; D'Angelo et al., 2009). The tumoral mass can display various degrees of permeation of the myometrium (figure 1.B) and intravenous worm-like plugs of tumor are often seen in the myometrium and parametrial and lymphatic veins (Evans, 1982; Chew and Oliva, 2010).

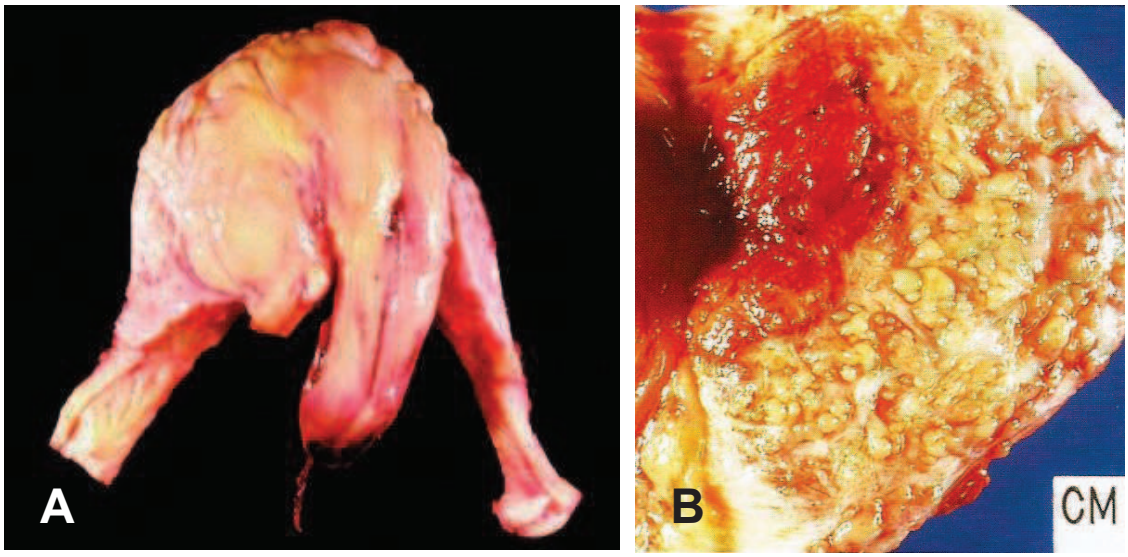

Figure 1 – Macroscopic features of low-grade Endometrial stromal sarcoma: A) Gross examination of low-grade endometrial stromal sarcoma. **B)** Low-grade endometrial stromal sarcoma with diffuse permeation of myometrium in the form of small nodules (images A and B scanned and adapted from Nucci and Bradley, 2006 and Rosai, 2004 respectively)

At the histological level these tumors are typically cellular and composed by uniform, small to medium and oval to spindle-shaped cells with eosinophilic cytoplasm that resemble the cell of normal endometrial stroma (figure 2.A and 2.B). These cells, having minimal cytological atypia and low mitotic activity (<5 MF/10HPF), are usually arranged in sheets and supported by a rich network of small arterioles resembling the spiral arterioles of normal endometrium (figure 2.A and 2.B) (Evans, 1982; Hendrickson and Tavassoli, 2003). Cells with foamy cytoplasm, perivascular hyalinization and cystic change associated with haemorrhage, infarction and necrosis may be seen (Evans, 1982; Norris and Taylor, 1966; Oliva et al., 2000). An extensive permeation of the myometrium by a characteristic tongue-like infiltrative growth (figure 2.C) with lymphovascular invasion can be observed in some cases (figure 2.D) (Oliva et al., 1999; Oliva et al., 2002a). The similarity with normal endometrium is also described at the ultrastructural (Akhtar et al., 1975; Gil-Benso et al., 1999) and cytological levels (Policarpio-Nicolas et al., 2007).

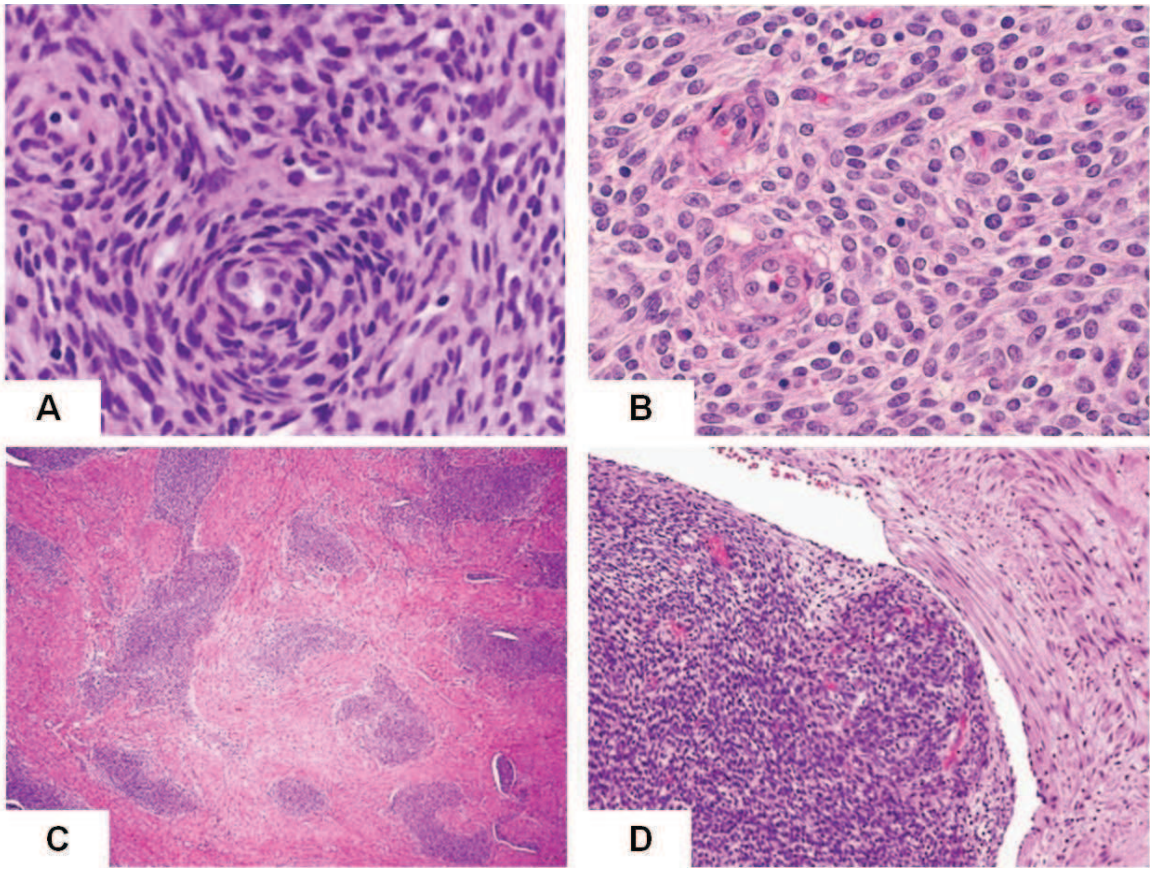

Figure 2 – Microscopic features of low-grade Endometrial stromal sarcoma: A and **B)** Typical histological appearance: high cellularity and small blood vessels; **C)** Extensive permeation of the myometrium by a characteristic tongue-like infiltrative growth; **D)** Vascular Invasion (image A and B scanned and adapted from Lee et al., 2012b and Kurihara, et al., 2008, images C and D scanned and adapted from Nucci and Bradley, 2006)

ESS may occasionally show unusual types of differentiation and these features include: smooth muscle differentiation (Oliva et al., 1998; McCluggage et al., 2001); fibrous and myxoid change (Oliva et al., 1999; Yilmaz et al., 2002; Kasashima et al., 2003); sex-cord like differentiation (Clement and Scully, 1976; Evans, 1982; McCluggage et al., 1996; Fukunaga et al., 1997); glandular differentiation (Norris and Taylor, 1966; Clement and Scully, 1992; McCluggage et al., 2009) and other unusual features such as epithelioid, clear and rhabdoid cells and fatty metaplasia (Lifschitz-Mercer et al., 1987; McCluggage et al., 1996; Kim et al., 1996; Oliva et al., 2002a; Baker et al., 2005).

1.1.4.2 UNDIFFERENTIATED ENDOMETRIAL SARCOMA

UES is by definition a high-grade endometrial sarcoma that lacks specific differentiation and bears no histological resemblance to endometrial stroma (Evans, 1982; Oliva et al., 2000; Hendrickson and Tavassoli, 2003). The endometrial stromal origin was associated to

topographic location, and the existence of tumors composed by low and high-grade component (Evans, 1982; Amant et al., 2006; Ohta et al., 2010).

Usually at gross examination UES are characterized by one or more polypoid fleshy, grey to yellow endometrial masses (mean size 14.15 cm), with prominent haemorrhage and necrosis (figure 3) (Evans, 1982; Hendrickson and Tavassoli, 2003; Lenhard et al., 2006D'Angelo et al., 2009).

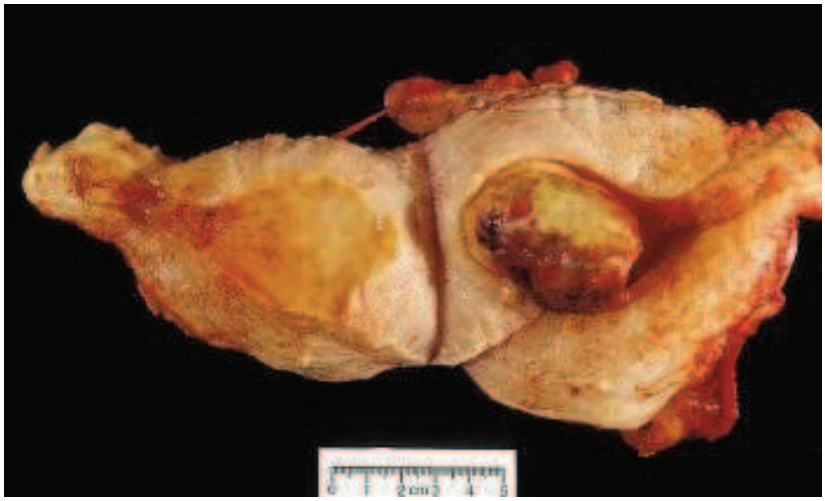

Figure 3 – Gross examination of Undifferentiated endometrial sarcoma
(image scanned from Nucci and Bradley, 2006)

Microscopically, they present high-grade spindle cells and polygonal cells arranged in sheets with marked nuclear hypercromatism and pleomorphism (figure 4.A), which are also seen at the ultrastructural level (Bocker and Stegner, 1975). These tumors lack the typical vasculature and growth pattern of ESS, and present high mitotic activity (>10 mitoses/10 HPF) with a destructive pattern of myometrial infiltration, accompanied by the presence of necrosis and vascular invasion (figure 4.B) (Hendrickson and Tavassoli, 2003; Abeler et al., 2009). In some cases rhabdomyosarcomatous and osteosarcomatous differentiation is evident, as well as the presence of osteoclastic giant cells (Evans, 1982; Bardarov et al., 2011).

Due to the existence of tumors with high and low-grade component (Amant et al., 2006; Ohta et al., 2010), a new classification system based on cellular pleomorphism and on the relationship with endometrial stroma was suggested: UES with nuclear uniformity (UES-U) with low-grade (spindle-shaped cells) and high-grade component (round cells) with minor nuclear pleomorphism and the permeative pattern of myometrial invasion seen in ESS (figure 4.C), and UES with nuclear pleomorphism (UES-P) that present a high degree of nuclear pleomorphism and no resemblance of endometrial stroma (figure 4.D) (Kurihara et al., 2008).

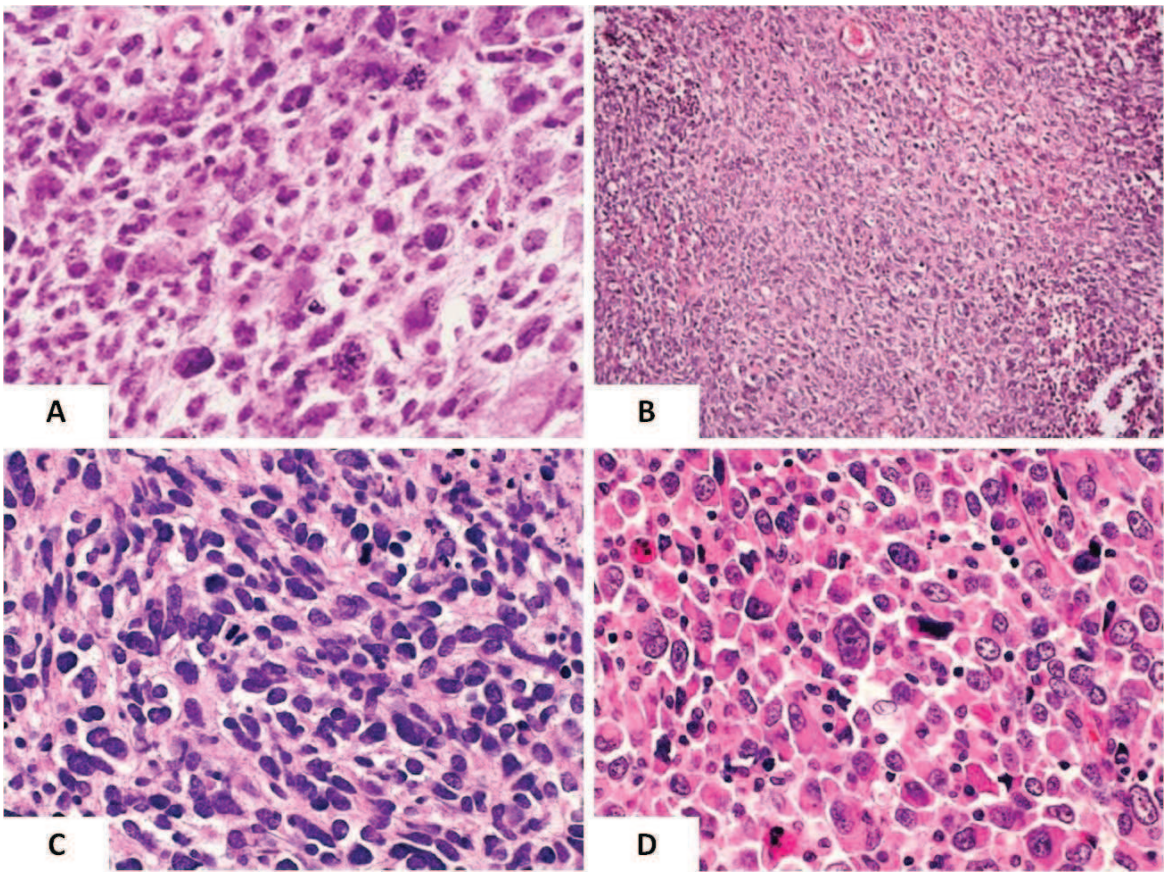

Figure 4 – Microscopic features of Undifferentiated endometrial sarcoma: A) Classical pattern with prominent mitotic figures; **B)** Mixture of spindle and polygonal cells associated with necrosis; **C)** Undifferentiated endometrial sarcoma with nuclear uniformity; **D)** Undifferentiated endometrial sarcoma with nuclear pleomorphism (images A and B scanned and adapted from Nucci and Bradley, 2006 and Chew and Oliva, 2010 respectively, images C and D scanned and adapted from Kurihara, et al., 2008)

1.1.4.3 EXTRAUTERINE ENDOMETRIAL STROMAL TUMORS

The occurrence of extrauterine EST is a unusual event, which can be explained by two alternative hypotheses i) malignant transformation of endometriosis, and ii) *de novo* tumor development from mesothelial pluripotent müllerian cells (Kang et al., 2008). Most cases are ESS, and a minor proportion corresponds to UES (<8%) (Kusaka et al., 2006; Alcazar et al., 2012). Extrauterine locations include mainly ovary, and pelvic and abdominal cavity (Chang et al., 1993; Alcazar et al., 2012; Lan et al., 2012), with a similar histologic pattern seen in uterine EST (Bosincu et al., 2001; Levine et al., 2001; Kim et al., 2008a). The behaviour of these neoplasms is more reminiscent of high grade uterine EST (Chang et al., 1993).

1.1.5 IMMUNOPHENOTYPE

EST immunoprofile is quite variable and as mesenchymal tumors they normally express vimentin with a lower prevalence of myogenic markers and cytokeratins (table 2). Absence or low expression (<5%) is seen for endothelial markers, oxytocin, S100, EMA, HMB45, CD99 and CD68 (Oliva et al., 2002b; Loddenkemper et al., 2003; Abeler and Nenodovic, 2011), whereas expression for Wilms tumor protein was described mainly in ESS cases (Agoff et al., 2001; Sumathi et al., 2004; Coosemans et al., 2007).

CD10, also known as common acute lymphocytic leukaemia antigen, is expressed in several normal and neoplastic tissues, including endometrial stromal cells. It is the most sensitive marker for EST: in 70-100% of ESS and in approximately 51% of UES (table 2) (Chu et al., 2001; Toki et al., 2002; Jakate et al., 2013), but it may also be expressed in other mesenchymal uterine tumors as leiomyosarcomas and carcinosarcomas (Baker and Oliva, 2007).

ESS is considered a hormone-sensitive tumor since it presents a consistent expression of estrogen (ER) and progesterone (PR) receptors (Reich et al., 2000; Chu et al., 2003), aromatase (Reich and Regauer, 2004) and gonadotrophin-releasing hormone receptors (GnRH) (Reich et al., 2005a). ER and PR expression in ESS is about 85-100%, while in UES-U cases varies between 30-60% and are usually absent in UES-P cases (Kurihara et al., 2008; Lee et al., 2012a).

Ki-67 expression is low (<10%) in ESS, but normally >20% in UES cases (D'Angelo et al., 2009; Jakate et al., 2013; Lee et al., 2012a). Nuclear accumulation of p53 protein is found in 50% of UES-P cases, and is absent or focally expressed in ESS and UES-U cases (Nordal et al., 1998; Blom et al., 1999; Lee et al., 2012a; Jakate et al., 2013).

As described in breast cancer (Rowlands et al., 2004) and in some soft tissue sarcomas (STS) (Saito et al., 2006), abnormal nuclear β-catenin expression and cyclin D1 overexpression were also described in EST. Nuclear β-catenin is expressed in approximately 50-60% of ESS, 85% of UES-U and 33% of UES-P (Ng et al., 2005; Kurihara et al., 2008; Jung et al., 2008; Kildal et al., 2009b). On the other hand, cyclin D1 expression in ESS and UES-P tumors is absent or focal (≤5%), while diffuse nuclear staining is found in the high grade component of UES-U (25-85%), which normally presents β-catenin and cyclin D1 co-expression (Kurihara et al., 2010).

Table 2 – Immunoprofile of Endometrial stromal tumors subtypes

	Vimentin	Pan-Cytokeratin	Calponin	α-muscle actin	Desmin	H-caldesmon	CD10	ER	PR	β-catenin	Cyclin D1	Ki-67	p53
ESS	-/+++	-/+++	-/++	-/+++**	-/++**	-/+++**	++/+++	+/+++	-/+++	+++	-/+	<10%	-/+
UES – U	-/+++	-/+++	-/++	-/+	-/++	-/+	-/++*	+/+++	++/+++	+++	++/+++	>20%	-/+
UES – P								-	-	+++	-/+		++/+++

Legend: **-** : negative; **-/+**: focal and/or weak; **++**: moderate, local or diffuse; **+++**: intense and diffuse; **-/++**: focal and/or weak to moderate, local or diffuse; **-/+++**: focal and/or weak to intense and diffuse; **++/+++**: moderate, local or diffuse to intense and diffuse; *mainly in areas of spindle cell component; **mainly in areas of smooth muscle differentiation

1.1.6 MOLECULAR AND CYTOGENETIC FEATURES

ESS usually displays a diploid status, whereas UES are frequently aneuploid tumors (Hitchcock and Norris, 1992; Blom et al., 1999; Kildal et al., 2009a). Microsatellite stability was observed in all EST (Amant et al., 2001; Moinfar et al., 2004) and loss of heterozygosity with at least one polymorphic DNA marker is a common event in these tumors and in surrounding non-neoplastic endometrial and myometrial tissues, suggesting that loss of function of tumor suppressor genes and no mismatch repair deficiency may play a key role in EST pathogenesis (Moinfar et al., 2004). Loss of heterozygosity of *PTEN* gene was observed in 37% of cases, but no mutations in *PTEN* gene were described (Amant et al., 2002; Moinfar et al., 2004). Other tumor suppressor genes may appear altered such as the *FHIT*, *P53* or *WT1* (Moinfar et al., 2004), and mutations on *P53* gene were only described in 3 UES-P cases (Kurihara et al., 2008).

In a recent gene expression analysis that included 7 ESS, overexpression of genes involved in intracellular and transcriptional regulation of proliferation, invasion and metastasis was described (Davidson et al., 2013). Despite the abnormal accumulation of β-catenin observed in EST cases, no mutations were found in both modulators of Wnt pathway: *CTNNB1* (β-catenin) and adenomatous polyposis coli (*APC*) genes (Jung et al., 2008; Kurihara et al., 2010). However, microarray and quantitative real-time reverse-transcription polymerase chain reaction analyses showed that secreted frizzled-related proteins SFRP1 and SFRP4, known as putative modulators of the Wnt-signalling pathway, to be significantly downregulated in EST: in relation to normal endometrium, the expression of SFRP4 was decreased in both ESS and UES, being lower in the latter more aggressive form (Hrzenjak et al., 2004).

Cytogenetically, EST are characterized by the presence of several chromosomal translocations and rearrangements: in ESS they normally involve chromosome (Chr) 7 (7p), 6 (6p), 17 (17q) and 10 (10q) (Chiang and Oliva, 2011), while several chromosomal gains (2q, 4q, 6q, 7p, 9q and 20q) and losses (3q, 10p and 14q) were observed in UES tumors which present a complex karyotype (Gil-Benso et al., 1999; Halbwedl et al., 2005).

ESS are characterized by the recurrent chromosomal translocation *t*(7:17)(p15;q21) that results in the fusion of two *zinc finger* genes *JAZF1* and *JJAZ1* (also named *SUZ12*) located on Chr 7p15 and Chr 17q21 respectively (Koontz et al., 2001). The specific function of this *JAZF1*/*JJAZ1* gene fusion is unclear, although it was described to confer resistance to apoptosis and increased proliferative capacity (Li et al., 2008b). Expression of these two genes *JAZF1* and *JJAZ1* in normal endometrium (Huang et al., 2004) and the presence of gene fusion in ESS and in their benign counterpart - ESN, suggest a common molecular

pathway and may be an early event in ESS tumorigenesis (Huang et al., 2004; Li et al., 2008b). In subsequent larger series, reverse-transcription polymerase chain reaction and fluorescence *in situ* hybridization (FISH) assays demonstrated that these rearrangements were found in approximately 50% of ESS tumors (classical and variants) (Huang et al., 2004; Hrzenjak et al., 2005; Nucci et al., 2007; Amador-Ortiz et al., 2011), rarely in UES cases (Kurihara et al., 2008; Kurihara et al., 2010), and absent in any other mesenchymal uterine tumors (Hrzenjak et al., 2005; Nucci et al., 2007; Chiang et al., 2011).

Rearrangements between PHD finger protein-1 gene (*PHF1*) at 6p21 with *JAZF1* and the enhancer of polycomb gene (*EPC1*) at 10p11 (Micci et al., 2006; Panagopoulos et al., 2008), as well with MYST/Esa1-associated factor 6 (*MEAF6*) from 1p34 (Panagopoulos et al., 2012) were also described in uterine and extrauterine ESS, and may play a pivotal role in ESS tumorigenesis as an alternative biology pathway distinct from the *JAZF1/JJAZ1* fusion.

The recurrent non-random translocation *t*(10;17)(q22;p13) was described in ESS cases that do not harbour *JAZF1* rearrangements (Leunen et al., 2003; Regauer et al., 2008; Amant et al., 2011). Recent studies showed that leads to a gene fusion between *YWHAE* on Chr 17 and either *FAM22A* or *FAM22B* on Chr 10 and results in a protein fusion 14-3-3ε with oncogenic properties (Lee et al., 2012b) and that it is specific of a subset of ESS with high-grade features and aggressive clinical behaviour compared to *JAZF1* ESS (Lee et al., 2012b; Isphording et al., 2012).

Additionally, comparison of gene expression profile between 4 *JAZF1/JJAZ1* ESS and 3 *YWHAE/FAM22* ESS showed that *CCND1* gene that encodes for cyclin D1 was the most upregulated gene in the *YWHAE/FAM22* ESS group (Lee et al., 2012b), which is consistent with cyclin D1 expression in the high-grade round cells component of these tumors (≥70%) (Lee et al., 2012c; Croce et al., 2013) and support the evidence that cyclin D1 was the most sensitive marker for EST with *YWHAE* rearrangement (Croce et al., 2013). In fact, a new diagnostic algorithm was suggested for EST based not only on morphologic features, but also on cytogenetic and immunophenotypic features (figure 5) (Lee et al., 2012c).

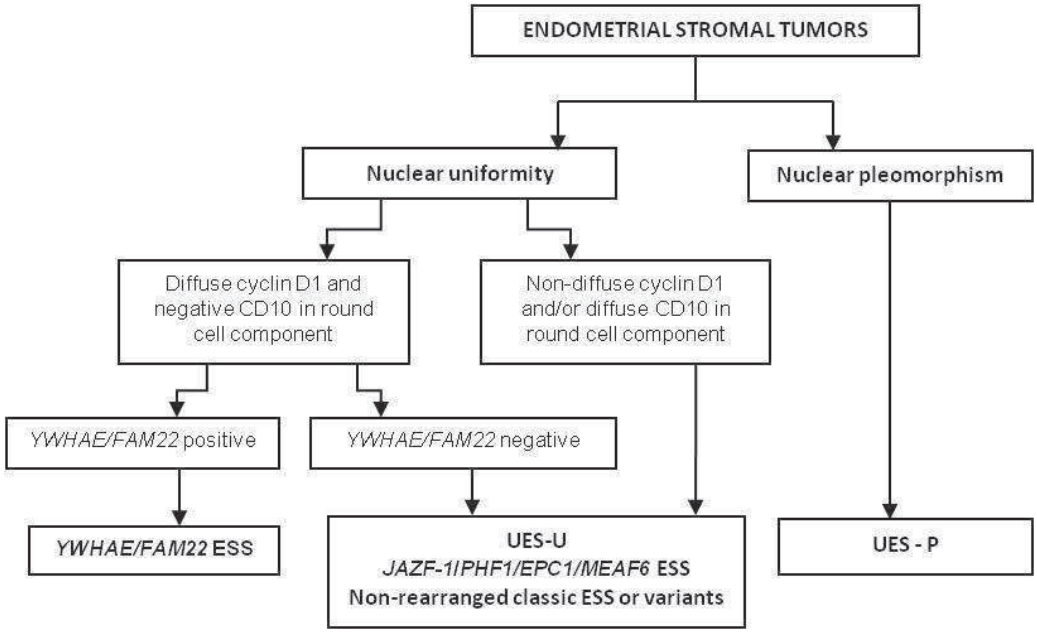

Figure 5 – Proposed diagnostic algorithm for Endometrial stromal tumors (adapted from Lee et al., 2012a)

1.1.7 STAGING AND PROGNOSIS

Endometrial stromal tumors (EST) staging was recently updated with the new surgical staging system of International Federation of Gynaecology and Obstetrics (FIGO) and the classification of American Joint Committee on Cancer (AJCC) system, in order to reflect the distinct pathological and clinical behaviour of each tumor and allowing an adequate management (table 3) (Prat, 2009; AJCC, 2010; Garg et al., 2010).

Numerous clinical and pathological prognostic factors have been described for these tumors (Chew and Oliva, 2010), but FIGO stage is considered as the most important prognostic factor in EST, since it is described as an important independent predictor of survival, influencing not only overall survival (OS) but also disease-specific survival[4] (table 4) (Denschlag et al., 2007; Koivisto-Korander et al., 2008; Chan et al., 2008; Abeler et al., 2009). Tumor grade was considered an independent predictor for worse survival (Nordal et al., 1996; Chauveinc et al., 1999; Denschlag et al., 2007; Chan et al., 2008; Schick et al., 2012), and clearly supports the fact that ESS and UES have a distinct pathological and clinical behaviour (Bartosch et al., 2010).

[4] **Disease-specific survival:** defined as the percentage of people in a study who have survived a particular disease since diagnosis or treatment (Kaplan and Malmgren 2006).

Other pathological factors which are associated to OS include mitotic index (Abeler et al., 2009; Feng et al., 2013) and nuclear atypia (Nordal et al., 1996; Abeler et al., 2009) which is in agreement with the recently proposed classification of EST (Kurihara et al., 2008; Lee et al., 2012a). Lymphovascular invasion seems to have significant prognostic influence in UES, with a 5-year OS of 83% and 17% when vascular invasion was absent or present (Denschlag et al., 2007; Abeler et al., 2009).

Table 3 – Staging System for Endometrial stromal tumors

TNM Categories	FIGO Stages	Definition
TX		Primary tumor cannot be assessed
T0		No evidence of primary tumor
T1	I	Tumor limited to the uterus
T1a	IA	Tumor 5 cm or less in greatest dimension
T1b	IB	Tumor more than 5 cm
T2	II	Tumor extends beyond the uterus, within the pelvis
T2a	IIA	Tumor involves adnexa
T2b	IIB	Tumor involves other pelvic tissues
T3	III	Tumor infiltrates abdominal tissues (not just protruding into the abdomen)
T3a	IIIA	One site
T3b	IIIB	More than one site
NX		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1	IIIC	Lymph node metastasis
T4	IVA	Tumor invades bladder or rectum
M0		No distant metastasis
M1	IVB	Distant metastasis (excluding adnexa, abdominal and pelvic tissues)

Adapted from Prat, 2009 and AJCC, 2010

Table 4 – Influence of FIGO stage on overall survival and disease-specific survival

Overall Survival				
Study	**N**	**Stage**	**ESS**	**UES**
Abeler et al. (2009)	ESS - 83 UES - 25	**I**	84% 5-years 77% 10-years	57% 5-years
		II	62% 5-years 49% 10-years	0% died within 5 years
		III	40% 5-years 40% 10 years	
		IV	0% died within 2 years	
Disease-Specific Survival				
	N	**Stage**	**EST**	
Chan et al. (2008)	EST - 831	**I-II**	89.3%	
		III-IV	50.3%	

1.2 CLINICAL MANAGEMENT OF ENDOMETRIAL STROMAL TUMORS

1.2.1 SURGICAL TREATMENT

Surgery is the cornerstone of the treatment of EST both in disease limited to uterus and known or suspected extrauterine disease (National Comprehensive Cancer Network [NCCN], 2013), and in general includes total hysterectomy (TH) and bilateral salpingo-oophoferectomy (BSO). The complete surgical staging that includes pelvic and para-aortic lymphadenectomy (PPLD) and the role of BSO in ESS is not well established (Gadducci et al., 2008; Amant et al., 2009).

Due to the consistently high expression of hormonal receptors in ESS (Reich et al., 2000), BSO has been recommended even in premenopausal women with early stage disease based in increased risk of recurrence in women who retained the ovaries (Berchuck et al., 1990; Reich et al., 2000; Li et al., 2008a; Beck et al., 2012; Feng et al., 2013). In the same way, other studies pointed that ovarian preservation did not affect the outcome, especially in early-stages of disease (Chu et al., 2003; Li et al., 2005; Amant et al., 2007; Chan et al., 2008; Shah et al., 2008; Kim et al., 2008b), which suggests that surgical procedure should be

discussed on an individual basis with the patient (Amant et al., 2007; Shah et al., 2008; Malouf et al., 2010).

The role of PPLD in patients with EST is not conclusive, since node involvement seems to be more frequent in advanced stage ESS and there is no evidence of the real benefit of PPLD in UES (Signorelli et al., 2010; Malouf et al., 2013). The estimate incidence is about 0-9%, and despite the evidence of higher incidence in some cases (10-45%) (Riopel et al., 2005; Reich et al., 2005b; Cheng et al., 2011; Schick et al., 2012), its impact on management and outcome is minimal (Chan et al., 2008; Barney et al., 2009; Garg et al., 2010; Schick et al., 2012).

The role of other staging procedures such as peritoneal cytology, omentectomy, peritoneal biopsies is unclear, and in cases of EST with extrauterine disease complete cytoreductive surgery/debulking is advocated (Berchuck et al., 1990; Leath et al., 2007; Gadducci et al., 2008; Amant et al., 2009; Casali and Blay, 2010).

1.2.2 ADJUVANT AND SYSTEMIC TREATMENT

Adjuvant therapy options following surgery include radiotherapy, chemotherapy, endocrine therapy (ET) and observation, either alone or in combination (NCCN, 2013). Lack of specific prospective trials of adjuvant therapies, the limited number of studies and cases reported and the long disease free interval observed in ESS, are reasons for the absence of a specific adjuvant and systemic treatment for EST. Due to the hormonal-sensitive status of ESS, ET is normally advised in both adjuvant and systemic settings whereas adjuvant radiotherapy for local control is recommended for advanced stages (NCCN, 2013). Adjuvant therapy for UES is controversial, and parallels with uterine leiomyosarcoma treatment. In stage I disease treatment includes observation and adjuvant chemotherapy and radiotherapy (Tanner et al., 2012a; NCCN, 2013), whereas in higher stages due to increased risk of systemic relapse, adjuvant chemotherapy and radiotherapy are advocated (NCCN, 2013).

1.2.2.1 ENDOCRINE THERAPY

Early ESS treatment with tamoxifen was discontinued due the evidences of association with development of EST (Eddy and Mazur, 1997; Sesti et al., 2005; Christie et al., 2008; Engin, 2008; Kashiyama et al., 2013) and disease progression (Chu et al., 2003; Spano et al., 2003; Pink et al., 2006; Brechot et al., 2007).

Nowadays ET strategy is mainly based on blocking the estrogen receptor (ER) synthesis by two main mechanisms: progestins that exert an anti-estrogen effect by reducing receptor content and the ability of the endometrium to synthesize new receptors, and aromatase inhibitors that inhibit estrogen biosynthesis in peripheral tissues by the aromatase enzyme complex (Reich and Regauer, 2005; Chumsri et al., 2011).

Based on its well established ER and progesterone receptor (PR) expression (Reich et al., 2000), treatment with progestins have long been explored as a therapeutic strategy in ESS, namely medroxyprogesterone acetate and megestrol acetate (Garrett and Quinn, 2008). The efficacy of this treatment is influenced by cell-specific expression of different levels of steroid receptors and their isoforms and is associated to light side-effects (Chu et al., 2003; Garrett and Quinn, 2008; Africander et al., 2011). In the adjuvant setting it seems to have an impact on relapse rate: Chu et al. (2003) described a recurrence rate of 67% and 31% in ESS patients (early and advanced stages) who did not receive and receive progestin therapy, respectively, which is in line with observed by Amant et al. (2007). Few cases report an overall response rate of 60% in recurrent disease (Amant et al., 2009; Ioffe et al., 2009; Mizuno et al., 2012), and benefits on regression or stabilization of metastatic disease (Pink et al., 2006; Garavaglia et al., 2010; Mizuno et al., 2011). In UES cases it has a limited role, although partial response to progestins was observed in an UES patient with measurable disease that showed ER expression (Tanner et al., 2012a).

ESS presents a higher expression of aromatase in advanced stages (Reich and Regauer, 2004), and inhibition of this particular enzyme via aromatase inhibitors, whose were developed as an effective treatment for advanced breast cancer (Maluf et al., 2001; Chumsri et al., 2011), represents an alternative treatment modality for recurrent ESS (Reich and Regauer, 2004). Used in recurrent setting as 1st line treatment (Krauss et al., 2007; Ioffe et al., 2009; Sylvestre and Dunton, 2010), and also as 2nd line after progression on progestin therapy (Dahhan et al., 2009; Ioffe et al., 2009; Shoji et al., 2011), present an overall response rate of 67% (Altman et al., 2012). Additionally, shrinkage of tumor relapses was observed alone or in combination with progestin therapy, which means an adjuvant role in the surgical management of the relapses (Leunen et al., 2004; Leiser et al., 2004; Shoji et al., 2011).

Other approaches in hormonal treatment of ESS include use of Gn-RH agonists mainly in management of relapses (Burke and Hickey, 2004; Alkasi et al., 2009; Dupont and Disaia, 2010). In a phase II trial, stable disease was observed in one of the two ESS recurrent patients treated with mifepristone, a selective PR modulator (Ramondetta et al., 2009).

1.2.2.2 CHEMOTHERAPY

Adjuvant chemotherapy has a limited benefit in ESS (Klaritsch et al., 2006; Li et al., 2008a), and few studies pointed as systemic treatment especially in unresectable and recurrent disease (Sutton et al., 1996; Yokoyama et al., 2004) and in cases who progressed after hormonal treatment (Gadducci et al., 2008; Amant et al., 2009). Adjuvant chemotherapy was successful used in surgical management of a patient that present an ESS with high-grade features and the *t*(10;17)(q22;p13) (Leunen et al., 2003).

Some objectives responses in metastatic disease have been described (Lehrner et al., 1979; Berchuck et al., 1990), although only two prospective phase II trials showed more consistent results: in phase II trial of Gynaecologic Oncology Group an overall response rate of 33.3% was observed in metastatic or recurrent ESS treated with ifosfamide (Sutton et al., 1996), whereas in SAKK phase II trial which include only 5 ESS patients, a combination of ifosfamide plus doxorubicin was evaluated in advanced or metastatic gynaecologic sarcomas and a response rate of 40% was observed in the ESS group (Leyvraz et al., 2006). Nevertheless in a recent review of outcome of 74 ESS patients, none complete or partial response was observed in patients with recurrent ESS treated with anthracyclines and mitotic-inhibitors based chemotherapy: 40% of patients showed stable disease and 60% disease progression (Cheng et al., 2011).

In the case of UES no prospective trials have been conducted specifically for advanced stages and few case reports have been published on responses to doxorubicin and ifosfamide-based treatment (Berchuck et al., 1990; Yamawaki et al., 1997). Li et al. (2008) described an improvement in recurrence control in early stage UES treated with adjuvant chemotherapeutic agents. A partial response to carboplatin and paclitaxel was described in one UES patient (Szlosarek et al., 2000) and other reports showed good surgical management of UES tumors with ifosfamide and cisplatin-based adjuvant chemotherapy (Yamawaki et al., 1997; Numa et al., 2003).

In a retrospective study including only 21 UES patients, a patient with early stage UES showed no evidence of disease and recurrence with an OS of 109 months after treatment with gemcitabine/docetaxel combination. In the group of patients with measurable disease a response rate of 62% was observed with gemcitabine/docetaxel and doxorubicin-based regimens in 1st line, whereas in 2nd line or additional chemotherapy for progressive disease, the response rate was around 19% (Tanner et al., 2012a).

1.2.2.3 RADIOTHERAPY

In the literature, radiotherapy role in OS and local regional control (LRC) of EST is unclear due to the low frequency of EST, absence of randomized data and the few reports which do not differentiate between ESS and UES (Seddon and Davda, 2011).

A multicentric retrospective study of impact of postoperative radiotherapy showed an improvement of radiotherapy on LRC, disease free interval and OS on uterine sarcomas, including ESS patients (Ferrer et al., 1999). Gadduci et al. (1996) described a benefit on LRC only in early stages ESS: none of patient relapses after adjuvant radiotherapy Gadducci et al., 1996, and a complete response was observed in one ESS patient after postoperative radiotherapy (Ishiko et al., 2000). Non-randomized studies showed an improvement of overall local control rate in both categories (Weitmann et al., 2001; Weitmann et al., 2002; Valduvieco et al., 2010), which was also observed in a retrospective analysis that include 361 women with EST treated with radiotherapy (Sampath et al., 2010; Sampath and Gaffney, 2011).

Nevertheless, adjuvant radiotherapy seems to have a low impact on survival improvement (Li et al., 2008a; Barney et al., 2009; Schick et al., 2012). More recently, the retrospective analysis of the Rare Cancer Network, which includes 59 women diagnosed with EST, showed that adjuvant radiotherapy had a significant effect on disease free-survival (DFS), LRC and OS in both ESS and UES (all stages) and was an independent prognostic factor for OS and DFS (Schick et al., 2012).

Similar findings were described in a recent series of 30 UES cases (Malouf et al., 2013). In fact, the significant effect on LRC rate which are approximately 94% and 50% with and without radiotherapy respectively, and the improvement of LRC in locally advanced or recurrent disease (Barney et al., 2012) support the use of this therapeutic option for EST and/or in palliative setting (Gadducci et al., 1996; Weitmann et al., 2001; Schick et al., 2012).

1.3 TYROSINE KINASE RECEPTORS

The tyrosine kinase receptors (TKR) belong to the protein tyrosine kinases family, which are main mediators of several signalling pathways that have a role in cellular differentiation and proliferation (Kolibaba and Druker, 1997; Hubbard and Till, 2000), and are considered “star players” in the story of carcinogenesis (Pecorino, 2008).

Distributed in approximately 20 receptor families according to their sequence similarity, structural characteristics and distinct motifs (Kolibaba and Druker, 1997; Robinson et al., 2000), the TKR share a common structural organization: (1) a glycosylated extracellular ligand-biding domain (Ig-like loops, cysteine-rich domains, fibronectin domains), (2) a single transmembrane α helix, and (3) an intracellular domain with catalytic tyrosine kinase domain with well conserved additional regulator domains (juxtamembrane domain and a carboxyl-terminal tail) (figure 6) (Kolibaba and Druker, 1997; Bennasroune et al., 2004).

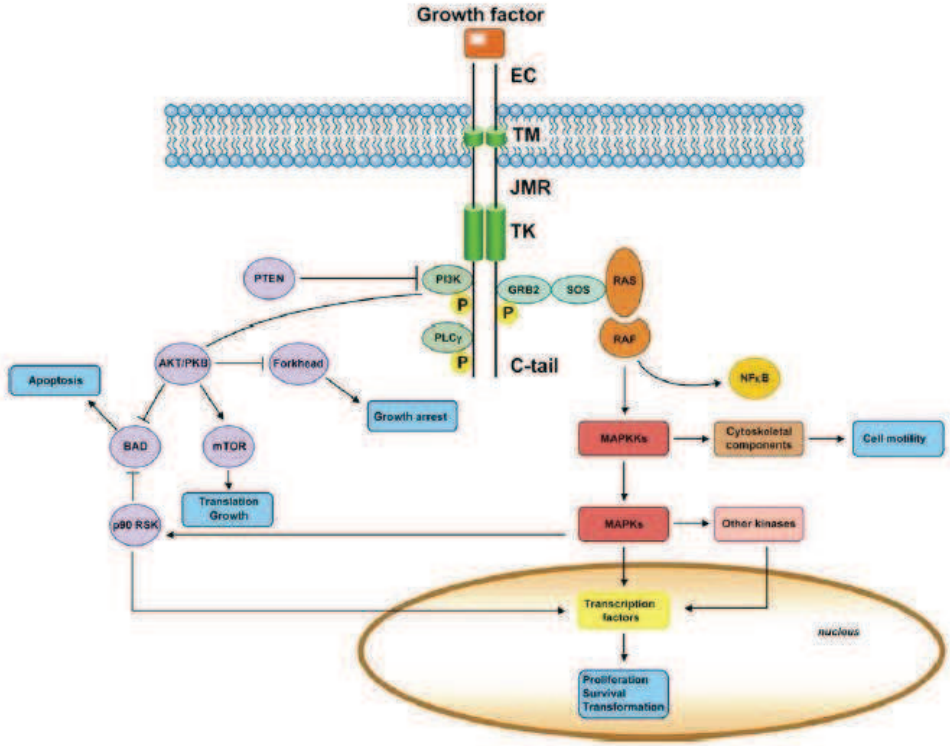

Figure 6 – Overview of the structure and signalling mechanism of tyrosine kinase receptor: schematic representation of TKR structure (extracellular domain [EC], transmembrane domain [TM], and intracellular domain with juxtamembrane domain [JMR] and a carboxyl-terminal tail [C-tail]) and its related signalling pathways (image adapted from Castellone et al., 2008)

In normal cells, TKR activity is normally tightly controlled and regulated (Bache et al., 2004): (i) the TKR is activated by its specific ligands which induce receptor dimerization, (ii) subsequent activation of receptor kinase activity, and leads to (iii) *trans*-phosphorylation of specific tyrosine residues in the catalytic domain, that provides docking sites for downstream molecules containing SH2 domains which activates the signal transduction pathways within the cells responsible for cell proliferation, differentiation, migration or metabolic changes (figure 6) (Blume-Jensen and Hunter, 2001; Andrae et al., 2008).

Dysregulation or constitutive activation of TKR can occur by several processes that includes autocrine/paracrine growth loops, gene rearrangements, and oncogenic mutations, and it has been shown to correlate with the development and progression of numerous human cancers (Robertson et al., 2000; Zwick et al., 2001; Pierotti et al., 2010; Takeuchi and Ito, 2011), which turned them into selective anti-cancer targets (Bennasroune et al., 2004).

1.3.1 KIT RECEPTOR AND PLATELET-DERIVED GROWTH FACTOR RECEPTOR A

KIT receptor and platelet-derived growth factor receptor A (PDGFRA) are members of the type III TKR family which includes also platelet-derived growth factor receptor B (PDGFRB), FMS-related tyrosine kinase 3 (FLT-3) and colony-stimulating factor-1 receptor (CSF-1R). These TKR are characterized by an extracellular domain with five Ig-like domains and an intracellular tyrosine kinase (TK) domain split by 100 amino acids composed of TK1, kinase insert, and TK2 (figure 7.A) (Zwick et al., 2001).

KIT gene is located at Chr 4q12 and encodes the transmembrane KIT protein with 145 kD expressed in diverse cells types, including interstitial Cajal cells, mast cells and hematopoietic stem cells, germ cells and melanocytes (Arber et al., 1998; Lammie et al., 1994; Elmore et al., 2001; Miettinen and Lasota, 2005). Its natural ligand is the Stem cell factor (SCF), produced in mast cells and fibroblasts (Yarden et al., 1987; Williams et al., 1990).

PDGFRA presents a molecular size of 170 kD and is a product of *PDGFRA* gene located adjacently to *KIT* gene in Chr 4q12, with high homology to *PDGFRB* gene located on Chr 5 (Matsui et al., 1989), and is normally found in connective tissue and glia but lacking in most epithelia (Andrae et al., 2008). The platelet-derived growth factor (PDGF) family includes at least five dimeric forms (PDGF AA, PDGF AB, PDGF BB, PDGF CC, and PDGF DD), and are well known ligands and can be produced by different cell types (Alvarez et al., 2006). The PDGF dimers activate PDGFRA and PDGFRB, thereby stimulating the proliferation and migration of mesenchymal cells (Ostman and Heldin, 2007). PDGFRA and its ligands (AA,

AB and CC) are essential during the embryonal development of several organs and systems (Alvarez et al., 2006; Andrae et al., 2008).

1.3.1.1 ONCOGENIC ACTIVATION OF KIT AND PDGFRA

Constitutive activation of KIT and PDGFRA on tumor cells has been described to occur by three mechanisms: (1) autocrine and/or paracrine stimulation of the receptor by its ligand, (2) cross activation by other kinases and/or loss of regulatory phosphatase activity, and (3) acquisition of activating mutations (Burger et al., 2005). The main activated pathways in response of constitutive activation include mitogen-activated protein kinase (MAPK), phosphoinositol 3-kinase (PI3K), phospholipase Cγ (PLC-γ) and Janus kinase/signal transducers and activators of transcription (JAK/STAT) (Duensing et al., 2004; Kang et al., 2005), which contributes to tumor development, and regulate the cell cycle and cell differentiation through activation of transcription factors (figure 7.B) (Miettinen et al., 2002).

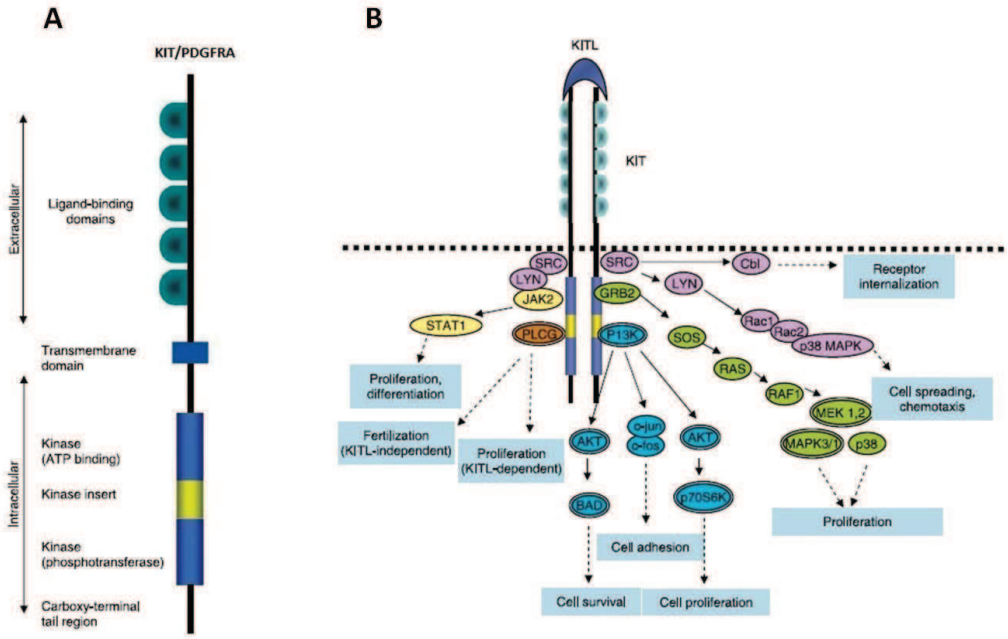

Figure 7 – KIT and PDGFRA structure and signalling: A) Schematic representation of KIT and PDGFRA structure**; B)** Signalling pathways and dowstream signalling cellular functions activated by KIT receptor (adapted from Mithraprabhu and Loveland, 2009)

1.3.1.1.1 Autocrine/Paracrine growth-loops

KIT autocrine stimulation by its ligand SCF was observed in tumor cell lines (Turner et al., 1992; Rygaard et al., 1993; Krystal et al., 1996; Hines et al., 1999) and coexpression of KIT and SCF in both normal and corresponding malignant tissues (Matsuda et al., 1993), suggests an autocrine/paracrine stimulation in growth and development of several human malignancies including small cell lung cancer (SCLC), colorectal cancer (Bellone et al., 2006), breast cancer, gynaecologic malignancies (Inoue et al., 1994) and soft tissue sarcomas (STS) (Landuzzi et al., 2000; Negri et al., 2009). KIT overexpression is found in a variety of human tumors as acute myeloid leukaemia (Heinrich et al., 2002), mastocytosis, melanomas (Arber et al., 1998; Went et al., 2004), SCLC (Sihto et al., 2005) and limited in some STS subtypes (Hornick and Fletcher, 2002). In gastrointestinal stromal tumors (GIST), its expression is observed in 95-100% of cases (Went et al., 2004; Miettinen and Lasota, 2005;; Sihto et al., 2005).

PDGFRA expression and its ligands has been reported in malignant gliomas (Hermanson et al., 1992; Martinho et al., 2009), Leydig tumor cells (Gnessi et al., 2000), hepatocellular cancer (Stock et al., 2007), cervical cancer (Taja-Chayeb et al., 2006), GISTs (Negri et al., 2009) and in malignant peripheral nerve sheath tumors (Holtkamp et al., 2006), suggesting an involvement of autocrine PDGFRA signalling on these tumors.

1.3.1.1.2 Gene amplification and somatic mutations

KIT and *PDGFRA* gene amplification was described in malignant peripheral nerve sheath tumors (Holtkamp et al., 2006) and *PDGFRA* gene amplification is a common event in malignant gliomas (Hermanson et al., 1996; Martinho et al., 2009), and seems to be the genetic alteration that precedes PDGFRA overexpression. An involvement of PDGFRA signalling on disease through gene fusions was described in idiopathic hypereosinophilic syndrome (IHES) that is characterized by the presence of *FIP1L1-PDGFRA* gene fusion (Cools et al., 2003; Score et al., 2006;), and in chronic myeloid leukaemia (CML) (Baxter et al., 2002; Trempat et al., 2003).

Gain-of-function mutations of *KIT* and *PDGFRA* (figure 8) confer constitutional phosphorylation of respective TKR and activation of downstream signalling pathways independent of ligand binding (Hirota et al., 1998; Hirota et al., 2003; Pierotti et al., 2010).

KIT mutations are present in 60-80% of GISTs (Hirota et al., 1998; Heinrich et al., 2003b; Kern et al., 2011), where the presence of activating mutations correlates with KIT and

PDGFRA expression (Kang et al., 2005; Negri et al., 2009) and with less frequency in seminonas (Kemmer et al., 2004), adult mastocytosis (Buttner et al., 1998), melanoma (Curtin et al., 2006) and acute myeloid leukaemia (Beghini et al., 2004). *PDGFRA* mutations are present in 10-20% of GISTs and are mutually exclusive in relation to *KIT* mutations (Heinrich et al., 2003a; Lasota and Miettinen, 2006; Kern et al., 2011). Involved on GIST pathogenesis (Hirota et al., 1998; Kitamura et al., 2001; Hirota et al., 2003) with a specific clinicopathologic connotation, are associated with response to tyrosine kinase inhibitor (TKI) imatinib mesylate (Corless et al., 2004).

The pattern of mutations includes point mutations, in frame deletions, and internal tandem duplications (table 5) (Heinrich et al., 2003b; Corless et al., 2004; Debiec-Rychter et al., 2004; Debiec-Rychter et al., 2006) and are more common in intracellular juxtamembrane domain of the KIT receptor encoded by exon 11 (57–70%), followed by the extracellular domain encoded by exon 9 (5–18%) (figure 8) (Lasota and Miettinen, 2008). In *PDGFRA* approximately 90% of mutations are found in exon 18 (Corless et al., 2005; Hoeben et al., 2008) and with less frequency at exon 12 and rarely in exon 14 (figure 8) (Pierotti et al., 2010).

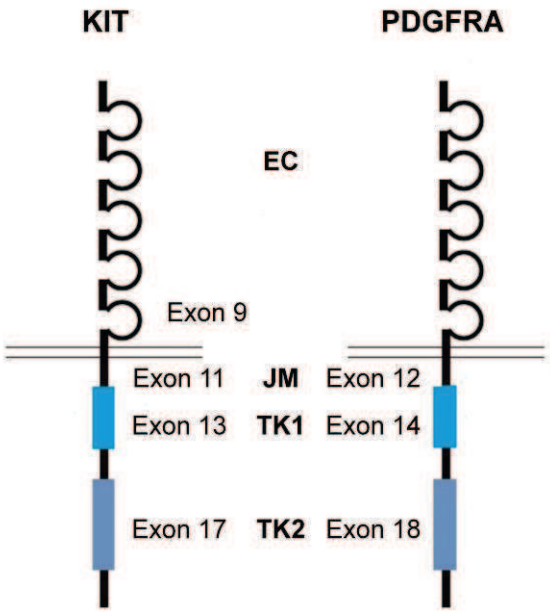

Figure 8 – Overview of most common KIT and PDGFRA hotspots. Legend: EC - extracellular domain; JM – juxtamembrane domain; TK – tyrosine kinase domain (adapted from Lasota and Miettinen, 2006)

Table 5 – Types and hotspots of activating mutations of *KIT* and *PDGFRA* in GISTs

Gene	Type of mutation	Hotspots (codons)
KIT		
Exon 9	Duplications and substitutions	502-503: **Ala502_Tyr503dup**
		506-508
Exon 11	Substitutions	557, 559, 560, 576
	In-frame deletions-insertions (5´)	550-561: **Trp557_Lys558del**
	In-frame deletions (3´)	**Asp579del**
	Duplications	570
Exon 13	Substitutions	642: **Lys642Glu**
		635: **Glu635Lys**
		641, 643, 647, 651, 655
Exon 17	Substitutions	822: **Asn822Lys**
		816,820
PDGFRA		
Exon 12	Substitutions	561: **Val561Asp**
		556, 563
	Deletions-insertions	559-572
Exon 14	Substitutions	659: **Asn659Tyr**
Exon 18	Substitutions	842: **Asp842Val**
		846, 849
	Deletions	840-848
	Deletions-insertions	840-849

Legend: in bold the most common mutations of *KIT* and *PDGFRA* genes. Adapted from Lasota and Miettinen, 2008.

1.3.1.2 KIT AND PDGFRA IN UTERINE SARCOMAS AND ENDOMETRIAL STROMAL TUMORS

KIT expression in uterine leiomyosarcomas and carcinosarcomas was widely studied in several studies and reports (Winter et al., 2003; Wang et al., 2003; Caudell et al., 2005; Menczer et al., 2005; Serrano et al., 2005; Nakayama et al., 2006; Zafrakas et al., 2008), after the suggestion that this receptor might be a potential alternative therapeutic approach for these high-grade tumors (Raspollini et al., 2003; Raspollini et al., 2004; Raspollini et al., 2005). Nevertheless KIT expression in uterine sarcomas remains controversial, varying from 0% to 100%, in part due to the number of cases selected, antibody´s choice and scoring system (Miettinen and Lasota, 2005; Menczer et al., 2005; Serrano et al., 2005), whereas consistent PDGFRA expression was observed in one study that included 13 uterine leiomyosarcomas and 18 carcinosarcomas (Adams et al., 2007).

In the literature, several case reports and small series that include less than 15 EST cases showed that the variability of KIT expression was the same observed in uterine sarcomas (Appendix 1). In a series of 37 ESS, all cases were negative for KIT receptor (Liegl et al., 2006), and similar results were reported in recent series of 23 ESS and 5 UES: KIT expression was observed only in a UES case with weak expression in 10% of cells (Cossu-Rocca et al., 2012a). Discordant results were reported by Park et al. (2013) which reported KIT expression in 82.1% of 39 ESS cases (Park et al., 2013). PDGFRA expression was observed in 33-100% of evaluated ESS cases (Appendix 1), and in 80% (4/5) of UES cases (Cossu-Rocca et al., 2012a).

Gene mutation analysis of *KIT* exons 9, 11, 13 and 17 and *PDGFRA* exons 12 and 18 was performed in cases that express KIT and/or PDGFRA (Liegl et al., 2006; Salvatierra et al., 2006; Mitsuhashi et al., 2007; Trojan et al., 2009), and in a series of 28 EST cases (Cossu-Rocca et al., 2012a), but no activating mutations were identified in any of the investigated exons. Gene expression analysis of *PDGFRA* gene in 14 EST showed overexpression levels in one ESS (3.8 fold) that presented PDGFRA protein overexpression at the immunohistochemical level (Cossu-Rocca et al., 2012a).

1.3.2 EPIDERMAL GROWTH FACTOR RECEPTOR

The epidermal growth factor receptor (EGFR) belongs to the ErbB/HER family of ligand activated TKR, which include also HER-2/ErbB2, HER-3/ErbB-3 and HER4/ErbB-4 (Ranson, 2004). EGFR (ERBB, ERBB1) was the first TKR to be described and is a transmembrane protein of 170 kDa encoded by the *EGFR* gene located on the short arm of the Chr 7 (*7p11.2*) (Davies et al., 1980). The extracellular domain of EGFR consists in four subdomains designated I, II, III and IV forming the ligand-binding pocket (figure 9.A) (Ogiso et al., 2002). The natural ligands of this receptor include transcriptional growth factor alpha and epidermal growth factor (EGF), among others (Normanno et al., 2006).

The binding of EGF or other ligands to EGFR results in the formation of homodimers or heterodimers with others members of EGFR family, triggering a cascade of signalling pathways as MAPK, PI3K, JAK/STAT, PLC-γ and SRC tyrosine kinases (figure 9.B) (Wells, 1999; Schlessinger, 2002; Buettner et al., 2002; Normanno et al., 2006; Zandi et al., 2007).

EGFR and its ligands are present in epithelial and stromal cells, in glial and smooth muscle cells (Wells, 1999; Normanno et al., 2006), being responsible for the mediation of many tumorigenic processes, including cell survival, cell cycle progression, angiogenesis, tumor cell invasion, and metastatic spread (Laskin and Sandler, 2004).

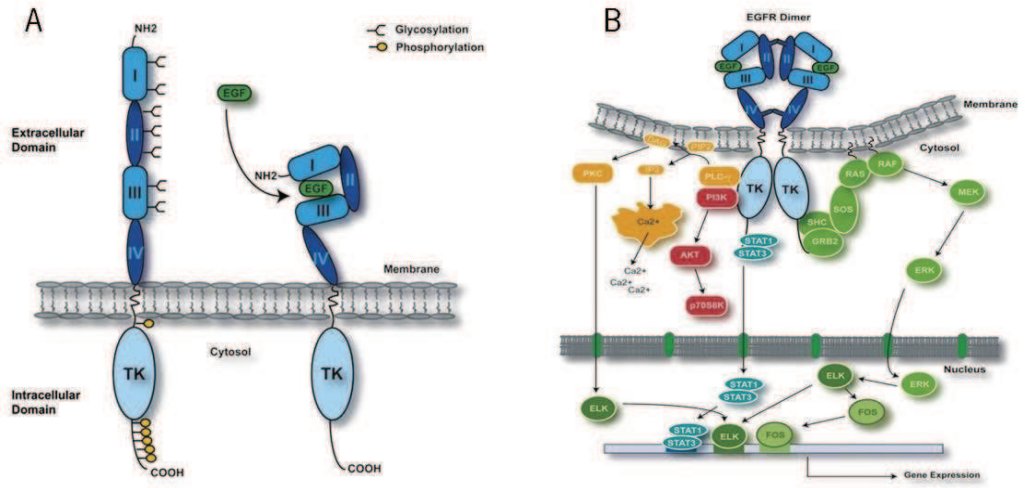

Figure 9 – EGFR structure and signalling: A) Schematic representation of EGFR structure: extracellular (I-IV domains, transmembrane and intracellular domain with the tyrosine kinase domain (TK); **B)** Activation of EGFR and related signalling pathways after ligand binding (adapted from Zandi et al., 2007)

1.3.2.1 EGFR DYSREGULATION IN CANCER

Dysregulated EGFR has been frequently associated to various forms of human cancer development (Prenzel et al., 2001; Arteaga, 2002). There are many mechanisms that lead to EGFR dysregulation, which include increased levels of EGFR protein, autocrine ligand production, and *EGFR* mutations, among others (figure 10) (Zandi et al., 2007).

Overexpression and/or amplification of the *EGFR* gene, as well as activating mutations are prevalent in many cancers types, and due its role in tumor promotion these mechanisms has been intensely studied as therapeutic targets (Zandi et al., 2007).

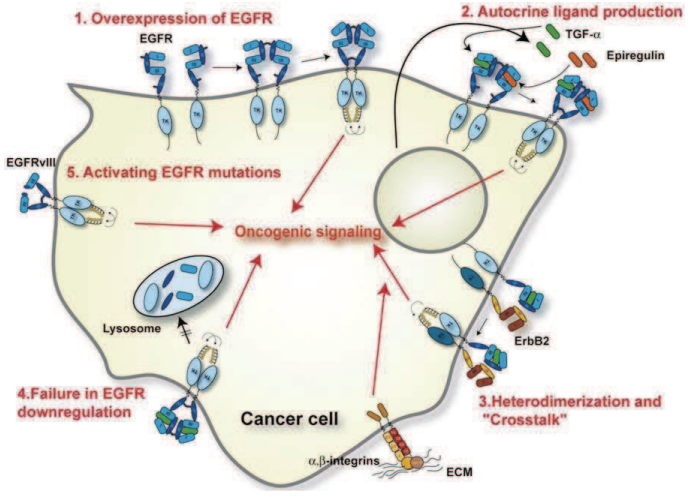

Figure 10 – Mechanisms leading to EGFR oncogenic signaling (adapted from Zandi et al., 2007)

1.3.2.1.1 EGFR overexpression and gene amplification

EGFR is expressed in more than one third of solid tumors (Laskin and Sandler, 2004), and overexpression and constitutive activation of downstream signalling pathways can occur mainly due to three mechanisms: (1) autocrine/paracrine growth factor loops, where EGFR is overexpressed in the presence of its cognate ligands or when overexpression of the ligand occurs in the presence of its associated receptor (Zwick et al., 2001; Zandi et al., 2007), (2) *EGFR* gene amplification (Zandi et al., 2007) and (3) increased activity of the EGFR promoter or deregulation at the translational and post-translational levels (Zandi et al., 2007).

In preclinical studies, coexpression of EGFR and its cognate ligands are shown to induce a malignant phenotype in cultured cells (Di Fiore et al., 1987; Di Marco et al., 1989) and rat xenografts (Kawamata et al., 1994). The coexpression of both in several solid tumors such as head and neck (HNC), oesophageal, cervical, ovarian, pancreatic and bladder cancers (Nicholson et al., 2001) supported an autocrine growth loop role on tumorigenesis (Salomon et al., 1995; Kolibaba and Druker, 1997; Grandis et al., 1998; Nicholson et al., 2001; Arteaga, 2001). EGFR has been found to be overexpressed in several solid tumors, including HNC, colorectal cancer, glioblastoma multiforme (GBM) and non-small cell lung cancer (NSCLC).

Gene amplification is another molecular mechanism to increase EGFR levels that usually, but not always, results in EGFR protein overexpression (Laskin and Sandler, 2004). Normally found in GBM (37-58%) (Libermann et al., 1985; Wong et al., 1987; Wikstrand et al., 1998) and in NSCLC (30-40%), was also described in breast cancer (Bhargava et al., 2005) and ovarian cancer (Lassus et al., 2006; Vermeij et al., 2008; Stadlmann et al., 2006), among others. In contrast to other solid tumors, in NSCLC and GBM protein expression is almost always associated to gene amplification (Hirsch et al., 2003; Cappuzzo et al., 2005a; Nicholas et al., 2006; Hirsch et al., 2006).

EGFR protein expression was found to be related with reduced recurrence-free and OS in HNC, ovarian, cervical, bladder and oesophageal cancers (Nicholson et al., 2001; Lassus et al., 2006). In NSCLC was associated to high metastatic rate, poor tumor differentiation, and increased rate of tumour proliferation (Pavelic et al., 1993), while the impact of EGFR expression and gene amplification on survival is a controversial issue (Hirsch et al., 2003).

1.3.2.1.2 *EGFR* mutations

EGFR activating mutations comprise three subtypes: (1) large deletions or duplications in extracellular and (2) intracellular domains that are responsible by EGFR mutated variants (I-

V), and (3) small mutations at intracellular TK domain (Normanno et al., 2006). The well-known mutant form EGFRvIII characterized by deletion of exons 2-7 of the *EGFR* gene, is frequently detected in association to *EGFR* amplification in GBM (Pedersen et al., 2001; Kuan et al., 2001), and may play a pivotal role in breast cancer progression (Tang et al., 2000).

The last group of *EGFR* activating mutations are small mutations that lead to changes in the intracellular TK domain and are restricted to the first four exons (18, 19, 20 and 21) that encode the TK domain (Lynch et al., 2004; Paez et al., 2004). *In vitro* studies showed that *EGFR* mutations induce constitutive TK activation, prolong the activity of ligand-activated receptors (Lynch et al., 2004; Sordella et al., 2004), have a higher sensitivity to EGFR inhibitors (Lynch et al., 2004; Paez et al., 2004; Chen et al., 2006), and includes: 1) small deletions around 6-7 codons in exon 19 (amino acid residues 746-753), 2) missense mutations in exons 18 and 21 (e.g. G719A/C and L858R respectively, among others) and 3) small duplications or insertions in exon 20 (Zandi et al., 2007; Zhang and Chang, 2007).

Mutations on exon 19 and 21 are the most frequent (>80%) (Zhang and Chang, 2007), and although its prevalence varies by ethnicity, they are associated to a specific group of NSCLC patients (Lynch et al., 2004; Paez et al., 2004; Normanno et al., 2006) and correlated with clinical responsiveness to treatment with EGFR tyrosine kinase inhibitors (TKI) (Lynch et al., 2004; Paez et al., 2004; Tsao et al., 2005; Takano et al., 2005).

Described in approximately 5-17% of NSCLC (Paez et al., 2004; Shigematsu et al., 2005; Marchetti et al., 2005; Eberhard et al., 2005; Cappuzzo et al., 2005b; Rosell et al., 2009; Boch et al., 2013) and 7% of HNC (Lee et al., 2005), *EGFR* mutations were not found in primary tumors of the breast, colon, kidney, pancreas, brain (Lynch et al., 2004) and uterine sarcomas (Murray et al., 2010) and are rare in ovary cancer (Schilder et al., 2005; Lassus et al., 2006; Vermeij et al., 2008).

1.3.2.2 EGFR IN UTERINE SARCOMAS AND ENDOMETRIAL STROMAL TUMORS

EGFR expression in uterine sarcomas was described in few studies: in carcinosarcomas was observed in 45-82% of cases, mainly in sarcomatous component (Swisher et al., 1996; Sawada et al., 2003; Livasy et al., 2006; Cimbaluk et al., 2007) and in 30% of adenosarcomas (Swisher et al., 1996). Sato et al. (2005) describe expression in 8 of 11 uterine leiomyosarcomas cases (Sato et al., 2005).

In a large study for evaluation of EGFR expression that include 36 cases of uterine leiomyosarcomas, a variable expression between 10-33% was observed in this group depending of the antibody and scoring system used (Kersting et al., 2006). Gene amplification was observed in one patient with uterine leiomyosarcoma (Kersting et al., 2006).

In EST cases, EGFR expression was described for the first time by Moinfar et al. (2005): in 23 cases of EST (20 ESS and 3 UES), EGFR expression was observed in 70% and 100% of ESS and UES cases, respectively. No expression of other members of EGFR family was observed (Moinfar et al., 2005). However, subsequent reports of expression of EGFR in EST are controversial (Appendix 1): absence of EGFR in ESS cases was observed in two studies (Cheng et al., 2011; Park et al., 2013), whereas in a recent series of EST, EGFR expression was significant in 14 of 28 tumors (50%), namely 43% of ESS and 80% of UES, with the staining intensity ranging from 1+ to 3+, and percentages of positive cells varying from 20% to 80% (Cossu-Rocca et al., 2012a).

EGFR amplification was described in one patient with UES (Mitsuhashi et al., 2007), but in a small series of 10 EST (7 ESS and 3 UES), FISH analysis did not show gene amplification in any of the tumors studied (Capobianco et al., 2012). In both studies that assessed the hotspots of *EGFR* gene (exons 18 to 21) no activating mutations were described in EST cases (Murray et al., 2010; Cossu-Rocca et al., 2012a).

1.4 TYROSINE KINASE INHIBITORS

The good knowledge of crystal structure of protein tyrosine kinases (PTK) and its crucial role on regulation and dysregulation of several signalling pathways that controls cellular differentiation and proliferation, pointed the PTK as a major drug target group (Cohen, 2002; Bennasroune et al., 2004). Tyrosine kinase inhibitors (TKI) are a type of available targeted therapy agents developed to interfere with these dysregulated molecular targets. These small molecules compete with adenosine triphosphate (ATP) for binding to the TK domain, blocking the catalytic activity and abnormal signalling (Levitzki, 1999; Broekman et al., 2011), and includes imatinib mesylate that inhibit selectively BCR-ABL and PDGFR family members, and EGFR inhibitors: gefitinib and erlotinib, among others.

1.4.1 IMATINIB MESYLATE

Imatinib Mesylate (STI571, Glivec®, Novartis, Basel, Switzerland) belongs to the group of 2-phenylamino-pyrimidine small molecules, and acts as a competitive inhibitor of ATP binding to the kinase domain (Heinrich et al., 2000; Hartmann et al., 2009). A potent inhibitor of the *ABL* tyrosine kinase gene (Carroll et al., 1997), the first target of this drug was the oncogene *BCR-ABL* fusion gene. The activity of imatinib was extended to other TKR, namely type III TKR PDGFRA/B and KIT (Buchdunger et al., 1996; Carroll et al., 1997; Pardanani and Tefferi, 2004) and a putative role on β-catenin, member of Wnt pathway, was suggested (Zhou et al., 2003; Rao et al., 2006).

1.4.1.1 *BCR-ABL* FUSION GENE

The *BCR-ABL* fusion gene, the cytogenetic hallmark of chronic myeloid leukaemia (CML), results from a fusion between the *BCR* gene located on Chr 22 and the *ABL* gene located on Chr 9 that lead to *t*(9;22) translocation or Philadelphia chromosome (Ph), (Heisterkamp et al., 1983; Faderl et al., 1999). Based on the specific breakpoints of the rearrangement, several different isoforms of the BCR-ABL fusion protein can be generated (p190, p210 and p230 isoforms), which correlate with different leukaemia phenotypes (Arora and Scholar, 2005).

Approximately 95% of CML express the BCR-ABL gene fusion protein (Druker et al., 1996), which is also present in 5% to 10% of patients with acute lymphoblastic leukaemia (Carroll et al., 1997; Buchdunger et al., 1996). Activation of ABL tyrosine kinase and consequent tyrosine phosphorylation that is required for the transforming function of BCR-ABL proteins is induced by sequences from the first exon of *BCR* gene (Carroll et al., 1997). This activation lead to subsequent dysregulation of intracellular signalling with enhanced proliferative capability and resistance to apoptosis of hematopoietic stem or progenitor cells, and to a massive increase in myeloid cell numbers (Arora and Scholar, 2005). Other genes can substitute *BCR* in activating the transforming ability and tyrosine kinase activity of ABL (e.g. *TEL* gene) (Carroll et al., 1997).

Inhibitory effect of Imatinib in the growth of BCR-ABL positive cells was observed *in vivo* and *in vitro* (Buchdunger et al., 1996; Druker et al., 1996), as well as the reduction BCR-ABL positive colonies (Druker et al., 1996). In the treatment of Ph^+ CML patients in whom treatment with interferon alpha had failed, imatinib induced major responses (Druker et al., 2001a), and are also effective in BCR-ABL positive adult lymphoblastic leukaemia (Druker et

al., 2001b). In 2001, it was approved for treatment of CML (FDA, 2001), and later for adult patients with relapsed or refractory Ph^+ acute lymphoblastic leukaemia.

1.4.1.2 KIT ONCOPROTEIN

Activation of the KIT tyrosine kinase by somatic mutations has been documented in a number of human malignancies, including GIST, seminoma, acute myeloid leukaemia, and mastocytosis. In addition, paracrine or autocrine activation of this kinase has been postulated in numerous other malignancies, including SCLC and ovarian cancer (Longley et al., 2001; Heinrich et al., 2002; Burger et al., 2005).

Over 70% of SCLC express the KIT receptor (Wang et al., 2000) and imatinib inhibitory growth effect was also evaluated in SCLC cell lines that express KIT and its ligand stem cell factor (SCF) (Wang et al., 2000; Krystal et al., 2000), but in phase II clinical trials no response to imatinib was observed in patients with progressive SCLC with KIT expression (Dy et al., 2005; Krug et al., 2005).

Using biochemical and cell-based assays, treatment with imatinib showed a decrease of growth of SCF-dependent cells and block of anti-apoptotic activity of SCF at a concentration similar to the required for inhibition of *BCR-ABL* (IC_{50} of 100 nmol/L) (Heinrich et al., 2000; Buchdunger et al., 2000). Imatinib inhibit the SCF-dependent activation of KIT, MAP kinase activation and AKT kinase activation, without altering total protein levels of KIT, MAP kinase, or AKT (Heinrich et al., 2000; Buchdunger et al., 2000).

In a human mast cell leukaemia cell line (HMC-1) which expresses juxtamembrane mutant KIT polypeptide, the inhibitory effect of imatinib on the kinase activity was superior than on ligand-dependent activation of the wild-type receptor which suggested that monotherapy with KIT TKIs will be effective only against tumors for which growth is driven almost entirely by KIT activity (Heinrich et al., 2000).

In GISTs cell lines that express *KIT* activating mutations (K558NP, K642E), an inhibitory effect of imatinib effect was observed through decreased proliferation, inhibition of foci formation, and the induction of apoptosis (Tuveson et al., 2001), whereas it failed to do in cell lines that carry the typical codon 816 mutation (D816V) seen in human mastocytosis (Ma et al., 2002; Zermati et al., 2003; Heinrich et al., 2003a; Akin et al., 2003).

Treatment of a patient with metastatic GIST with imatinib showed a complete metabolic response and a decrease in tumor volume (Joensuu et al., 2001). Subsequently, imatinib efficacy and safety in GIST patients was evaluated in phase I and II trials: pathological

response to imatinib was observed in more than half of the patients with advanced unresectable or metastatic GISTs-KIT positive (van Oosterom et al., 2001; Blanke et al., 2001; Demetri et al., 2002), and presence of *KIT* mutations was associated to higher chance to response (p=0.0117) (Blanke et al., 2001).

The relationship between mutation of *KIT* and clinical response to imatinib was evaluated in *in vitro* studies and phase II trial with GIST patients: in the patients that harbour exon 11 *KIT* mutations, the partial response rate was 83.5%, whereas patients with tumors containing an exon 9 *KIT* mutation (extracellular domain) or no detectable mutation had partial response rates of 47.8% (p=0.0006) and 0.0% (p<0.0001), respectively (Heinrich et al., 2003a). Furthermore, patients whose tumors contained exon 11 *KIT* mutations had a longer event-free and overall survival (OS) than those tumors that express either exon 9 *KIT* mutations or had no detectable kinase mutation (Heinrich et al., 2003a).

Similar results were observed in phase I/II studies of the European Organization for Research and Treatment of Cancer (EORTC) - Soft Tissue and Bone Sarcoma Group and the US-Finnish group (Debiec-Rychter et al., 2004; Blanke et al., 2008) and in a phase III studies in patients with advanced GIST (Debiec-Rychter et al., 2006; Heinrich et al., 2008a), and support the evidence that the type and location of mutation serves as an independent predictor for disease free-survival and the likelihood of achieving response to imatinib (Singer et al., 2002; Debiec-Rychter et al., 2006; Debiec-Rychter et al., 2004).

Actually it is well established that in patients with advanced GIST, 90% of patients respond to imatinib when their tumors have a *KIT* exon 11 mutation, 50% if the tumor harbour a *KIT* exon 9 mutation, whereas in absence of mutation only a subset of patients respond to imatinib (European Society for Medical Oncology [ESMO], 2012**;** NCCN, 2013). Imatinib is approved worldwide as systemic and adjuvant treatment for malignant GISTs (Dagher et al., 2002; Cohen et al., 2010; ESMO, 2012; NCCN, 2013)

1.4.1.3 PLATELET-DERIVED GROWTH FACTOR RECEPTOR A

PDGFRA dysregulation through activating mutations and autocrine growth loops (overexpression and gene rearrangements) were well described in GIST (Hirota et al., 2003), Leydig cell tumors (Gnessi et al., 2000), malignant gliomas (Hermanson et al., 1992; Martinho et al., 2009), haematological disorders among others.

Malignant gliomas commonly co-expressed PDGFRA and it ligand (Hermanson et al., 1996), and since PDGFRA overexpression occur in both malignant and in low-grade astrocytomas,

it may be an early event in progression pathway of malignant gliomas, and activation of PDGFRA through an autocrine loop may be a pervasive feature of malignant gliomas (Hermanson et al., 1992; Vassbotn et al., 1994; Guha et al., 1995). PDGFRA has been shown to be overexpressed, amplified, mutated, or truncated (*KDR-PDGFRA* and *PDGFRA*$^{\Delta 8,9}$ gene fusions) in these tumors, although the incidence of these alterations are rare (Raymond et al., 2008; Alentorn et al., 2012).

In cell cultures studies imatinib selectively induced the inhibition of growth of cells that express PDGFRA autophosphorylation loops, as well the inhibition of growth on PDGFRA transformed phenotype cells *in vivo* and *in vitro* (Kilic et al., 2000; Hagerstrand et al., 2006). Oral administration of imatinib prolonged the survival in a mice inoculated with PDGFRA transformed phenotype glioblastoma cells (Kilic et al., 2000).

KDR-PDGFRA fusion transcript (*KP* fusion) and *PDGFRA*$^{\Delta 8,9}$ gene fusion transforming oncogenes that induce the ligand-independent receptor activation, are present in GBM that present *PDGFRA* amplification and PDGFRA overexpression (Ozawa et al., 2010). An inhibitory effect of imatinib was observed in both gene fusions through the reduction of the level of phosphotyrosine together with the active downstream signalling in a concentration dependent manner, and inhibition of the gene fusion-induced transformed phenotype (Ozawa et al., 2010). In the phase II trials with patients with recurrent malignant gliomas, a few patients presented a partial response to the treatment and the 6-month progression free-survival (PFS) was only observed in 3%-10% patients, which suggest that imatinib has minimal single-agent activity in malignant gliomas (Wen et al., 2006; Raymond et al., 2008).

An involvement of PDGFRA signalling in idiopathic hypereosinophilic syndrome (IHES), characterized by persistent hypereosinophilia and other organ damage associated symptoms (Ostman and Heldin, 2007), has been established by the demonstration of presence of the *PDGFRA* gene rearrangements (Cools et al., 2003; Score et al., 2006). The most well characterized is the fusion of *FIP1-Like-1* gene (*FIP1L1)* to *PDGFRA* gene caused by an interstitial deletion on chromosome 4 (4q12) (Cools et al., 2003). Present in approximately 12% of IHES cases (Score et al., 2006), was also described in other eosinophilic and myeloproliferative disorders (Trempat et al., 2003; Klion et al., 2004; Pardanani et al., 2004; Walz et al., 2006).

In the experiments of Cools et al. (2003), the expression of FIP1L1-PDGFRA fusion transformed the murine hematopoietic cell line Ba/F3 to interleukin-3–independent growth and was constitutively tyrosine-phosphorylated in these cells. Deletion of the FIP1L1 moiety (amino acids 4 to 233) abrogated this type of growth, indicating that this part of the fusion protein was essential for the activation of the chimeric kinase (Cools et al., 2003).

Ba/F3 cells expressing FIP1L1-PDGFRA were efficiently inhibited by lower concentrations of imatinib than Ba/F3 cells expressing BCR-ABL. In the same way, imatinib inhibited tyrosine phosphorylation of FIP1L1-PDGFRA and its downstream target STAT5 (Cools et al., 2003).

Imatinib treatment of patients with IHES induced hematologic remission during more than 3 months in 81% of patients, which 56% (5/9) presented the *FIP1L1-PDGFRA* fusion gene (Cools et al., 2003), and responses have also been observed in patients with other *PDGFRA* gene rearrangements (Trempat et al., 2003; Score et al., 2006; Walz et al., 2006).

In a prospective multicenter study of idiopathic or primary HES, all the patients that carried the *FIP1L1-PDGFRA* rearrangement achieved a complete hematologic and molecular remission, whereas in 36 patients that did not carry the rearrangement, only 14% achieved a complete hematologic response (Baccarani et al., 2007). Similar results were observed in other studies (Klion et al., 2004; Heinrich et al., 2008b; Arefi et al., 2012; Legrand et al., 2013). Imatinib is described as the treatment of choice of these disorders, although optimal dosing, duration, and possibility of discontinuation are still a matter of debate (Legrand et al., 2013).

A subset of GISTs lacking *KIT* mutations carries intragenic activation mutations in *PGDFRA* (Heinrich et al., 2003c). Presence of *PDGFRA* exon 12 and 18 mutations was associated to increased baseline tyrosine phosphorylation of PDGFRA in absence of ligand stimulation in GIST cell lines and tumors, and lead to activation of different signalling pathways (AKT, MAPK and STAT) (Heinrich et al., 2003c). Expression of *PDGFRA* mutant isoforms in cultured cells revealed imatinib sensitivity of exon 12 mutations and of exon 14 substitution (N659K), whereas the isoforms that involve the codon D842 exon 18 (D842V, RD841-842KI, DI842-843IM) were resistant to the imatinib (Heinrich et al., 2003a; Hirota et al., 2003; Corless et al., 2005). D842Y activating mutation seems to be sensitive, as well other exon 18 mutations (Corless et al., 2005). Inhibitory effect of Imatinib on survival was also observed in proliferation studies with BA/F3 cell lines stably expressing selected *PDGFRA* mutant isoforms: survival of the V561D, D846Y and Y849C lines was inhibited by imatinib at clinically relevant concentrations, while the D842V clone was completely resistant to the drug (Corless et al., 2005).

Absence of response was observed in metastatic GIST patients with the imatinib-resistant *PDGFRA* D842V mutation (Heinrich et al., 2003a; Debiec-Rychter et al., 2004), whereas two of three patients with imatinib-sensitive PDGFRA oncoproteins achieved a partial response with imatinib therapy (Heinrich et al., 2003a). *PDGFRA* mutations are mutually exclusive in relation to *KIT* mutations (Heinrich et al., 2003c) and due to sensitivity to imatinib, evaluation

of *PDGFRA* mutations is advised in patients with advanced GIST which can be candidate to imatinib treatment therapy (NCCN, 2013).

1.4.1.4 PLATELET-DERIVED GROWTH FACTOR RECEPTOR B

The *PDGFRB* gene located on Chr 5 (*5q32)* encodes the platelet-derived growth factor receptor B (PDGFRB), a cell surface tyrosine kinase receptor, member of the PDGFR family, and its natural ligands include PDGF–B and D isoforms (Ostman and Heldin, 2007).

PDGFRB is a well characterized plasma membrane receptor with endogenous tyrosine kinase activity, which is autophosphorylated in response to binding of PDGF ligands (Carroll et al., 1996). This PDGF binding ligands induces diverse physiological effect depending on the cell in which PDGFRB is expressed and what biological responses are measured (Carroll et al., 1996). In normal conditions, PDGFRB autophosphorylation is accompanied by binding of a variety of proteins that mediate downstream signal transduction events, and lead to activation of several signalling pathways like PLCγ, PI3K, Src, RAS, etc., as well as activation of STAT 1 and 3 (Carroll et al., 1996; Ostman and Heldin, 2007) . On the other hand, PDGF isoforms stimulate the proliferation, survival, and motility of connective tissue cells (Heldin and Westermark, 1999), and has an important role on embryonal development (Pietras et al., 2003).

The PDGFB/PDGFRB autocrine system, based on the production of the factor, has been proposed as the pathogenic mechanism in a growing list of human tumours (Greco et al., 1998; Ostman and Heldin, 2007), including soft tissue tumours (Wang et al., 1994), leukaemia (Golub et al., 1994), malignant gliomas (Hermanson et al., 1992), dermatofibrosarcoma protuberans (DFSP) and giant-cell fibroblastoma (GCF) (Simon et al., 1997).

First experiments described imatinib *in vitro* inhibition of ligand-stimulated phosphorylation in cultured cells that express PDGFRB, although overall levels of expressed PDGFRB protein did not change after exposure to imatinib (Buchdunger et al., 1996). In BALB/c3T3 cells that require PDGFR autocrine loop stimulation, imatinib also induced growth inhibition (Buchdunger et al., 1996).

1.4.1.4.1 *ETV6-PDGFRB* fusion gene

The fusion transcript in which the tyrosine kinase domain of the *PDGFRB* on Chr 5 is coupled to a novel *ETS*-like gene (*ETV6*) on Chr 12, lead to *t*(5;12)(q33;p13) and results in *ETV6/PDGFRB* fusion gene (Golub et al., 1994; Tomasson et al., 1999). Other rearrangements involving *PDGFRB* gene has been described in BCR-ABL negative chronic myeloid leukemias, and include the same breakpoint in the *PDGFRB* gene which leads to activation of the PDFGRB tyrosine kinase domain (Ostman and Heldin, 2007). The partner genes include *Huntingtin interacting protein 1* (*HIP-1*) (Ross et al., 1998), *Rabaptin-5* (Magnusson et al., 2001), *CEV14* (Abe et al., 1997) and *H4/D10S170* (Kulkarni et al., 2000).

The *ETV6/PDGFRB* fusion gene leads to receptor homodimerization and activation of PDGFRB tyrosine kinase activity and is characteristic of a subset of patients with chronic myelomonocytic leukaemia (CMML) (Golub et al., 1994; Carroll et al., 1996; Tomasson et al., 1999). The transforming potential of this fusion gene in primary lymphoid lineage cells was evidenced by the development of B and T lymphoblastic lymphomas in different transgenic lines of mice (Tomasson et al., 1999).

Studies of Carroll et al. (1997) and Tomasson et al. (1999) described imatinib inhibition in cells lines that express *ETV6/PDGFRB* (Carroll et al., 1997; Tomasson et al., 1999). Imatinib treatment of transgenic mice induced a significant prolongation of survival compared with the group control (figure 11.A). Latency was also prolonged during the imatinib treatment, which suggest that drug may have inhibited growth of these cells or prevent tumor progression, without eradicate *ETV6/PDGFRB* cells.

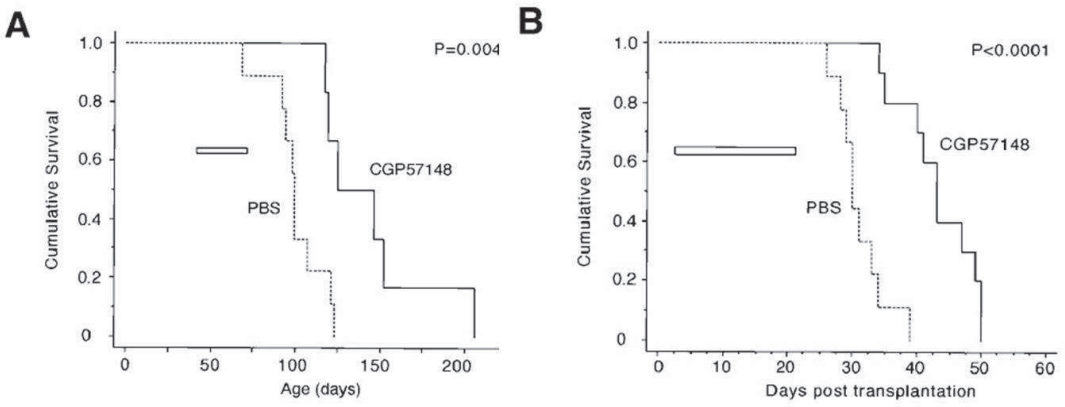

Figure 11 – Effect of imatinib on survival of *ETV6*/*PDGFRB* transgenic mice: (A) Premalignant mice model and (B) Tumor transplant model (images A and B adapted from Tomasson et al., 1999)

In a transplant model where tumor cells derived from affected lymph nodes of transgenic animals were injected intravenously, animals treated with imatinib had a statistically significant inhibition of development of lymphoblastic lymphoma and survived longer than control animals (figure 11.B) (Tomasson et al., 1999). Furthermore tumor cells were apparently not killed by the drug treatment; imatinib seemed to inhibit the proliferation of cells mediated by *ETV6/PDGFRB*, but after cessation of therapy tumor cells were again able to proliferate (Tomasson et al., 1999).

Imatinib effectively inhibited all growth of the *RAB5EP-PDGFBR*-transformed Ba/F3 cell line, with an inhibitory concentration lower (0.03 μM) than *BCR-ABL* (0.3 μM). In western blotting assays, an inhibitory effect of imatinib was also observed on phosphorylated STAT1, indicating blockade of downstream effectors (Magnusson et al., 2002). In the same experiment, a patient with CMML carrying the *RAB5EP-PDGFBR* fusion gene, which presents progressive and persistent disease, was treated with imatinib. Four weeks after initiation of imatinib therapy, molecular testing revealed marked reduction of *RAB5EP-PDGFBR* fusion transcript in peripheral blood cells, and a molecular remission (Magnusson et al., 2002).

In a phase II study (Novartis STIB2225) imatinib treatment was evaluated in 4 patients who had chronic myeloproliferative diseases. The *t*(5;12)(q33;p13) and subsequent *ETV6/PDGFRB* fusion gene were detected in only 3 patients, whereas in the fourth patient an unknown gene at 12q13 is presumably fused to *PDGFRB* gene (Apperley et al., 2002). Mononuclear cells with the *t*(5;12)(q33;p13) showed *in vitro* sensitivity to treatment with imatinib, and in all patients with chromosomal rearrangements involving *PDGFRB* was observed normalization of the blood count, disappearance of eosinophilia and other side effects. Cytogenetic remission was achieved with imatinib given at 400 mg/day (Apperley et al., 2002).

1.4.1.4.2 *COL1A1/PDGFB* fusion gene

Dermatofibrosarcoma protuberans (DFSP) and giant cell fibroblastoma (GCF) are recurrent, infiltrative skin tumors. Standard management of DFSP includes surgical resection, but presents a high recurrence rate (Sjoblom et al., 2001; Rubin et al., 2002; McArthur et al., 2005). These tumors are characterized by the reciprocal translocation *t*(17;22)(q22;q13), that results in a fusion between *Collagen type 1 alpha 1* (*COL1A1*) gene and the *Platelet-derived Growth Factor* (*PDGFB*) gene whose product is the mature PDGFB, which exerts its

pathogenic effect through autocrine or paracrine interaction with PDGFRB present on the cell surface of the DFSP (Simon et al., 1997; Greco et al., 1998; O'Brien et al., 1998).

In the work of Greco et al. (1998) and Shimizu et al. (1999) transfection of NIH3T3 mouse fibroblasts cells with DNA of 4 DFSP that carry the *t*(17;22) and express PDGFRB, resulted in tumor formation (Greco et al., 1998; Shimizu et al., 1999). Greco et al. (1998) showed through PCR analysis of NIH3T3 transformants DNA the presence of both exon 1 and 4 of *COL1A1* and *PDGFB* genes respectively, and the presence of *PDGFB* gene which indicates that gene fusion results in a constitutive expression of human PDGFB (Greco et al., 1998) as suggested previously (Simon et al., 1997).

The putative role of PDGFB as the mitogenic factor with autocrine and paracrine properties of DFSP cells was also confirmed in the studies of Shimizu et al. (1999), where in phosphotyrosine immunoblotting experiments, the B5/5 (*COL1A1/PDGFB* transfectant) extracts display a strong signal, indicating autocrine stimulation of PDGFRB by the *COL1A1/PDGFB* gene fusion (Shimizu et al., 1999). Shimizu et al. (1999) also showed in their experiments that addition of imatinib dramatically alters the morphology of B5/5 cells that harbour the *COL1A1/PDGFB* gene fusion to a phenotype that is indistinguishable from that of the control cells, which indicated that the altered morphology occurred as a consequence of PDGF receptor activation (Shimizu et al., 1999).

Greco et al. (2001) demonstrated that unlike BCR-ABL positive cells and similar to observed in *ETV6-PDGFRB* positive cells (Tomasson et al., 1999), treatment of NIH-3T3 cells transformed by the *COL1A1/PDGFB* rearrangement (5A cell line) with imatinib at different doses induced a morphological reversion of the transformed phenotype (Greco et al., 2001). The effect of imatinib was also assayed on 12 different DFSP-transformed NIH3T3 cell lines derived from the transfection DFSP tumor-DNA, and in all of them treatment with 1 μM imatinib induced reversion of the transformed phenotype (Greco et al., 2001). Under both conditions, addition of imatinib produced a significant dose reduction (>60%) of cell proliferation (figure 12.A), although inactivation of PDGFRB did not trigger apoptosis (Greco et al., 2001), which is in line with the observed in cells carrying the oncogenic *ETV6-PDGFRB* rearrangement (Tomasson et al., 1999), but contrasts with results observed in KIT positive SCLC lines and BCR-ABL leukaemia cells (Carroll et al., 1997; Krystal et al., 2000).

A reduction of tyrosine phosphorylation of PDGFRB was also observed after imatinib treatment (figure 12.B), as well an inhibitory growth effect (Greco et al., 2001), which was also previously described by Shimizu et al. (1999).

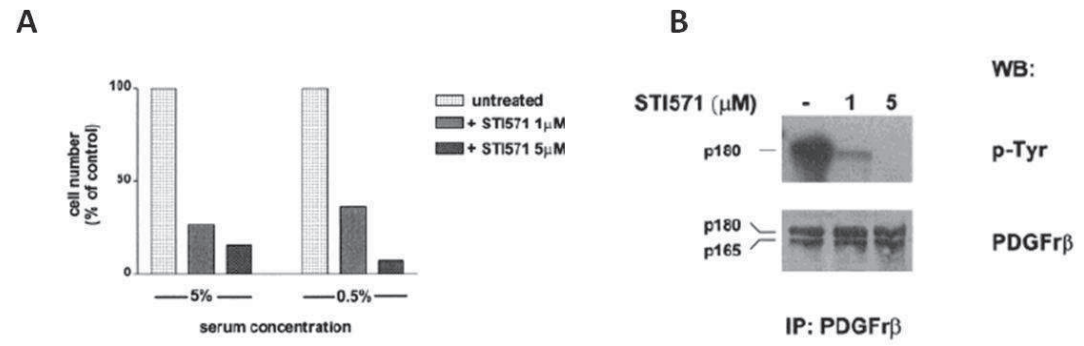

Figure 12 – Effect of imatinib on growth of 5A cells and on PDGFRB phosphorylation: (A) addition of imatinib (STI571) produced a significant (>60%), dose-dependent reduction of cell proliferation, and (B) PDGFRB phosphorylation was markedly reduced with 1 mM imatinib (STI571) and completely abolished with 5 mM (images A and B scanned and adapted from Greco et al., 2001).

In nude mice injected with 2 different DFSP-transformed cell lines (4A and 5A), a significant growth inhibition was observed after imatinib administration: tumor onset was delayed and the tumor weights at the end of the treatment were approximately between 50% and 80% lower than those of tumor-bearing control mice. After interruption of drug administration, the tumor growth restarted (Greco et al., 2001).

Frequent and continuous drug administration induced in early tumors inhibition of tumor weight of 90% compared to controls (more efficient than discontinuous – 80%). After continuous treatment, tumor growth restarted when imatinib treatment was discontinued. In late tumors, tumor weight was approximately 80% inhibited compared to the controls, which suggests an efficacy of imatinib on pre-existing tumors, and like observed in early tumors after imatinib removal, the growth rate restarted (Greco et al., 2001).

In the same way, in induced-mice tumors treated with imatinib, the phosphorylation of PDGFRB was measured by western-blot, and a decrease of PDGFRB phosphorylation was detectable after 2 hours of treatment, between 5-6 hours phosphorylation reached its lowest level and started to increase from 7 to 8 hours (Greco et al., 2001). The analysis of longer intervals showed that PDGFRB phosphorylation returned to the level of untreated tumors, which indicates that the *in vivo* imatinib-induced PDGFRB inactivation lasts no longer than 6 hours, suggesting that frequent administration may be required to maintain PDGFRB in the inactivated state (Greco et al., 2001).

In further studies in human DFSP and GCF tumor cells that harbour the *COL1A1/PDGFB* fusion gene were described a reduction of PDGFRB tyrosine phosphorylation to background levels after imatinib treatment (Sjoblom et al., 2001). At different concentrations and conditions, imatinib reduced the growth rate of DFSP cells, and lead to 65-70% reduction in

cell number, as compared with untreated cells. On the other hand, in normal fibroblasts which lack the PDGFRB stimulation, imatinib showed a smaller effect on the growth, the reduction of cell number was below 20% (figure 13.A) (Sjoblom et al., 2001).

On serially transplanted tumors (149333 cell line) with a DFSP-like histology, imatinib treatment induced a reduction of tumor volume to one-third the size of the control tumors (figure 13.B), decreased tyrosine phosphorylation without altering the expressing level of the PDGFRB and reduced the cell growth both *in vivo* and *in vitro* through induction of tumor cell apoptosis rather than inhibition of tumor cell proliferation (Sjoblom et al., 2001).

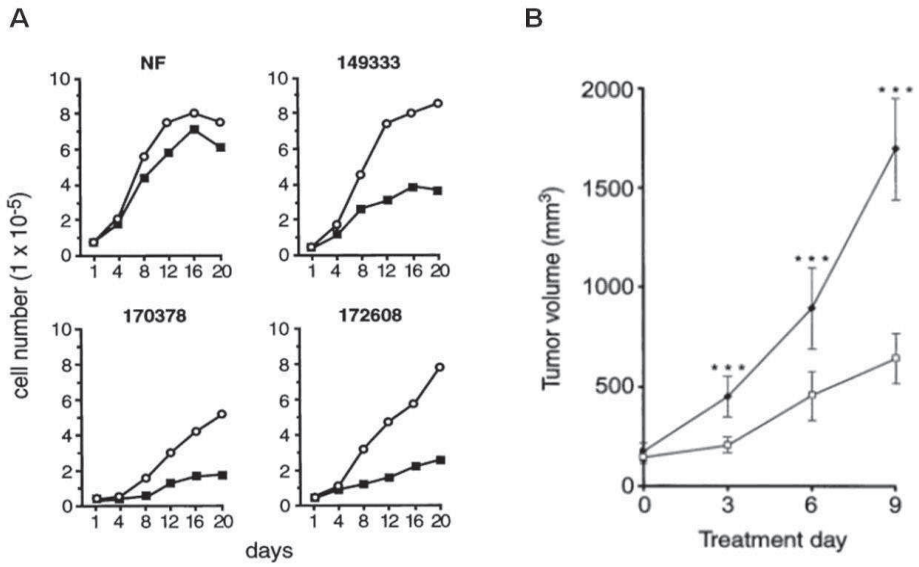

Figure 13 – Imatinib effects on DFSP/GCF primary cultures and tumors: (A) Normal fibroblasts (NF) and cells from three of the DFSP/GCF primary cultures were grown in the absence (○) or presence (▪) of 1 mM Imatinib mesylate. (B) Treatment with imatinib inhibits 149333 tumors growth (□) in one-third the size of control tumors (♦) (images A and B scanned and adapted from Sjoblom et al., 2001).

Imatinib effect on a patient with metastatic, inoperable DFSP was described for the first time by Rubin et al. (2002): the drug induced a clinical improvement in symptoms and strength, and coincided with a decrease in tumor uptake of positron emission tomography with 18F-fluorodeoxyglucose, with minimal toxicity (Rubin et al., 2002). A reduction of 75% of the size of the tumor was observed, and cytogenetic examination of a core biopsy revealed the presence of a translocation involving the 22q13 (the region of the *PDGFB* gene) and an unidentified translocation partner was seen (Rubin et al., 2002). Similar results were observed by Maki et al. (2002) and Labropoulos et al. (2005): in the former was observed partial response (shrinking of metastatic lung lesions), and in the latter a complete response

was observed in a patient with locally recurrent and metastatic DFSP and carrying the *COL1A1*/*PDGFB* rearrangement (Maki et al., 2002; Labropoulos et al., 2005).

McArthur et al. (2005) described a partial response and a complete response in 8 patients with locally advanced DFSP and in one patient with metastatic disease whose tumour harboured the *t*(17;22). On the contrary, in one patient with metastatic disease with a complex karyotype and absence of this specific rearrangement no clinical response to imatinib was observed (McArthur et al., 2005). In four patients, imatinib therapy accomplished substantial regression of locally advanced tumors, and effects of imatinib were also observed in histological biopsies, where reduced cellularity and development of hyaline changes were associated to pathological response (McArthur et al., 2005). However, evaluation of the level of PDGFRB activation reveals that value is substantially lower than the observed in a GIST with *PDGFRA* activating mutation, and suggests that autocrine/paracrine mechanism can be associated with substantially lower levels of TKR activation, and neither high levels of TKR activation nor TKR overexpression is required for clinical response to therapeutic inhibition of receptor signalling (McArthur et al., 2005).

Based on these results, imatinib efficacy was evaluated in phase II clinical trials:

- In the Imatinib target Exploration Consortium Study B2225, imatinib efficacy was evaluated in patients with malignancies associated to expression of imatinib-sensitive tyrosine kinases: clinical responses (complete and partial response) were observed in 90% of patients with DFSP with *t*(17;22), whereas one case that lack the PDGFB rearrangements has progressive disease (Heinrich et al., 2008b);

- Patients with locally advanced or metastatic DFSP that presented the *t*(17;22) and/or the *COL1A1/PDGB* rearrangement were treated in phase II trials of European Organization for Research and Treatment of Cancer (EORTC) and Southwest Oncology Group (SWOG) phase II trials (Rutkowski et al., 2010). The objective of both studies was similar, but the selection criteria, starting daily dose of imatinib, protocol treatment and primary end point were different. Partial response was observed in 46%, a median time to progression of 1.7 years and a 1-year progression-free survival of 58%. A global clinical benefit from therapy was seen in 70.9% (partial response plus stable disease) with minimal toxicity, and some patients with unresectable DFSP were able to undergo resection after imatinib treatment, leading to complete remission of the disease (Rutkowski et al., 2010). In patients that lacks the *t*(17;22) was observed progressive disease (including one patient with fibrosarcoma and 2 patients with fibrosarcomatous-DFSP) (Rutkowski et al., 2010);

- In a multicenter phase II study, imatinib efficacy as neoadjuvant treatment on patients with primary or recurrent DFSP was evaluated (Kerob et al., 2010). A clinical response (36%) was seen in 8 patients that harbour the *COL1A1/PDGFB* rearrangement and in one patient without this cytogenetic alteration, and was associated to a median relative tumoral decrease of 20%. Absence of response was observed in both patients whose tumors did not display the *t*(17;22). The clinical response was also correlated with a decrease in cell density, since previously described imatinib-induced hyaline fibrosis was observed in 19 of 25 patients (Kerob et al., 2010).

In 2006, Imatinib has been approved by the U.S. Food and Drug Administration (FDA) as a single agent for the treatment of advanced (inoperable and/or metastatic) DFSP and others eosinophilic/myeloproliferative diseases (FDA, 2006; McArthur, 2007) and since tumors lacking the *t*(17;22) translocation may not respond to imatinib, molecular analysis of *t*(17;22) of the tumor using cytogenetics is recommended prior to the institution of imatinib therapy (NCCN, 2014).

1.4.1.5 B-CATENIN

In epithelial cells, β-catenin is considered a cell-cell adhesion protein, mediating the connection of cadherins to the actin filament network and is thought to regulate thereby the strength of cadherin-mediated adhesiveness (Hoschuetzky et al., 1994). B-catenin has also a role as a signal transduction molecule in developmental systems (Polakis, 1999), since is a member of Wnt pathway that regulates cell fate determination during development (Miller, 2002).

In normal conditions, the modular protein axin provides a scaffold for the binding of glycogen synthase kinase-3 β (GSK-3 β), adenomatous polyposis coli (APC) protein, and β-catenin, which facilitates serine/threonine-phosphorylation in the amino terminus of β-catenin by GSK3-β and subsequent rapid degradation of β-catenin by a proteasome-dependent process (figure 14.A). Stabilization of β-catenin followed by nuclear translocation and subsequent T-cell factor/lymphoid enhancing factor (TCF/LEF)-mediated transcriptional activation has been proposed as an important step in oncogenesis (Polakis, 1999; Garcia-Rostan et al., 1999), and significant nuclear and/or cytoplasmic accumulation of β-catenin is considered a hallmark of deregulated β-catenin activity (Zhou et al., 2003).

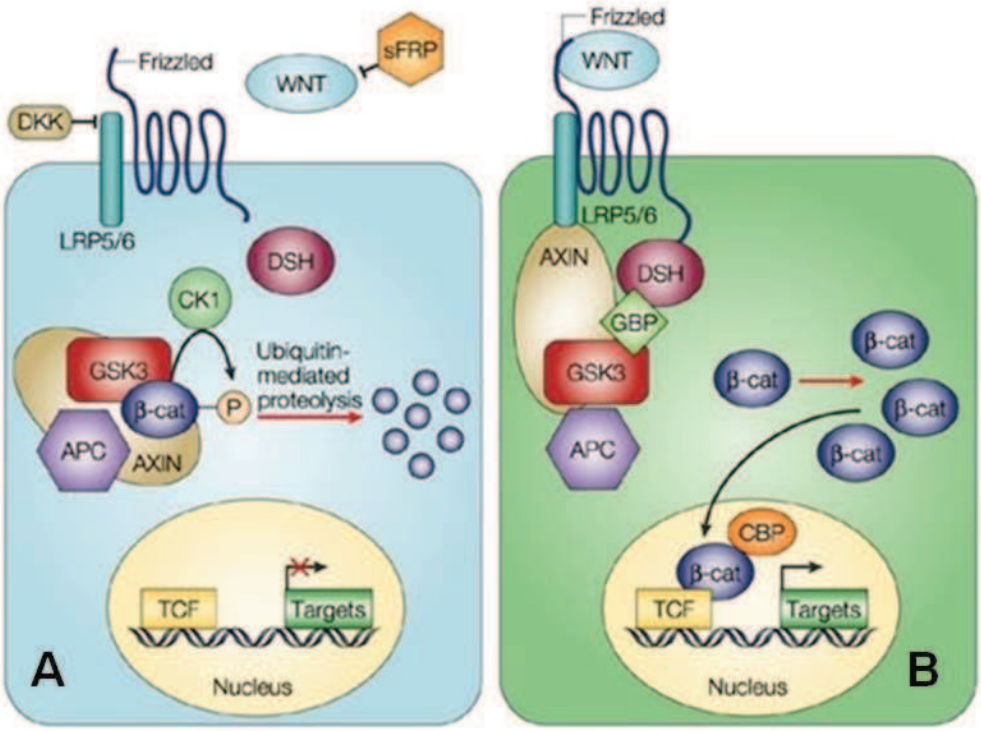

Figure 14 – B-catenin/Wnt signalling pathway: (A) In normal conditions, in the absence of active Wnt β-catenin is degraded; **(B)** If Wnt signalling is active, β-catenin degradation is reduced leading to accumulation of β-catenin, which enters to the nucleus, and binds to TCF/LEF-family transcription factors and activates transcription (image scanned and adapted from Moon et al., 2004).

B-catenin can be oncogenically activated by three main mechanisms that result in post-translational stabilization of the β-catenin protein:

1) **Wnt stimulation:** Different classes of Wnt can stimulate the Wnt/β pathway which antagonizes the destruction complex, leading to accumulation of β-catenin and activation of target genes: Wnt ligands initiate their signalling by binding to the frizzled receptors as well as the recently identified co-receptors LRP-5 and LRP-6, leading to phosphorylation of the dishevelled protein. It then associates with Axin and APC preventing GSK3β from phosphorylation B-catenin. Unphosphorylated β-catenin is stabilized by escaping recognition by ubiquitin/proteosome complex, and eventually translocates to the nucleus where it engages transcription factor LEF/TCF to activate the expression of downstream targets, such as c-myc and cyclin D1 (figure 14.B) (Polakis, 1999; Miller, 2002; Zhou et al., 2003; Rao et al., 2006; Takahashi-Yanaga and Sasaguri, 2007).

2) **Mutations on *APC* tumor suppressor gene:** more than 95% of germline and somatic mutations of the *APC* gene result in the synthesis of a truncated protein lacking the region of *APC* that is important for its function in the destruction complex. This truncation

in the *APC* removes binding sites for β-catenin and Axin, as well as putative phosphorylation sites for GSK3β, and as a result, the mutant APC protein cannot efficiently promote degradation of B-catenin (Polakis, 1999).

3) **B-catenin mutations:** mutations in the exon 3 of the human β-catenin gene (*CTNNB-1*) make it refractory for phosphorylation dependent degradation and lead to inappropriate accumulation of β-catenin, as observed on mammalian cells, colon cancer and melanoma cell lines (Polakis, 1999; Garcia-Rostan et al., 1999). Mutations of *CTNNB-1* are mutually exclusives in relation to *APC* mutations and can be found in several of human malignancies as: hepatocellular cancer, carcinomas of the ovarian, thyroid, prostrate and brain cancers (Garcia-Rostan et al., 1999; Polakis, 1999).

4) Other mechanisms can include inactivation of GSK3β and Axin/conductin proteins due to activating mutations, that has been show to downregulate β-catenin and therefore conceivable by tumor suppressors (Polakis, 1999), and also through oncogenic tyrosine kinases that have been reported to directly promote tyrosine-phosphorylation of β-catenin in thyroid, melanoma, breast and pancreatic cancer, and in chronic myelogenous leukaemia and can represent a significant therapeutic target in these neoplasms (Coluccia et al., 2007; Camp et al., 2007).

A putative effect of imatinib on β-catenin was suggested by Zhou et al. (2003): in their experiments, a TCF4 responsive report was introduced in colon cancer cell lines HCT116 and SW480 that present an oncogenic β-catenin mutation and inactivating mutation of the *APC* gene respectively, and resulted in highly activation of TCF4 in these cell lines. When treated with imatinib, the TCF4 report activity in HCT116 cell line was significantly inhibited (figure 15.A), whereas treatment with a concentration of 15 μM of imatinib did not exert any detectable inhibition on TCF4 reporter on SW480 cell line (figure 15.B). However higher doses of imatinib produce a more pronounced effect in SW480 cells, when compared to HCT116 cells (figure 15.B), which suggests that imatinib can effectively inhibit the constitutive activity of β-catenin signalling in representative human colon cancer cell lines (Zhou et al., 2003).

Imatinib also inhibit Wnt-induced β-catenin signalling activity in soft tissue sarcoma cell lines: Wnt1-induced β-catenin activity decreased to 57-30% in one cell line, and 60-90% in another cell line at different concentrations (Zhou et al., 2003). In transformed human embryonic kidney cell line (HEK 293), Wnt1-induced β-catenin activity was inhibited by approximately 70 and 85% in the presence of 15 and 20 mM of Imatinib, respectively. These results confirm that imatinib can effectively inhibit the Wnt/b-catenin signaling pathway (Zhou et al., 2003).

Evaluation of effect of imatinib on proliferation showed that cell proliferation was inhibited in a time- and dose-dependent manner in each cell line (figure 15.C and D), suggesting that inhibition of β-catenin/TCF4 activity may be responsible for the imatinib-mediated growth suppression of human tumor cells (Zhou et al., 2003).

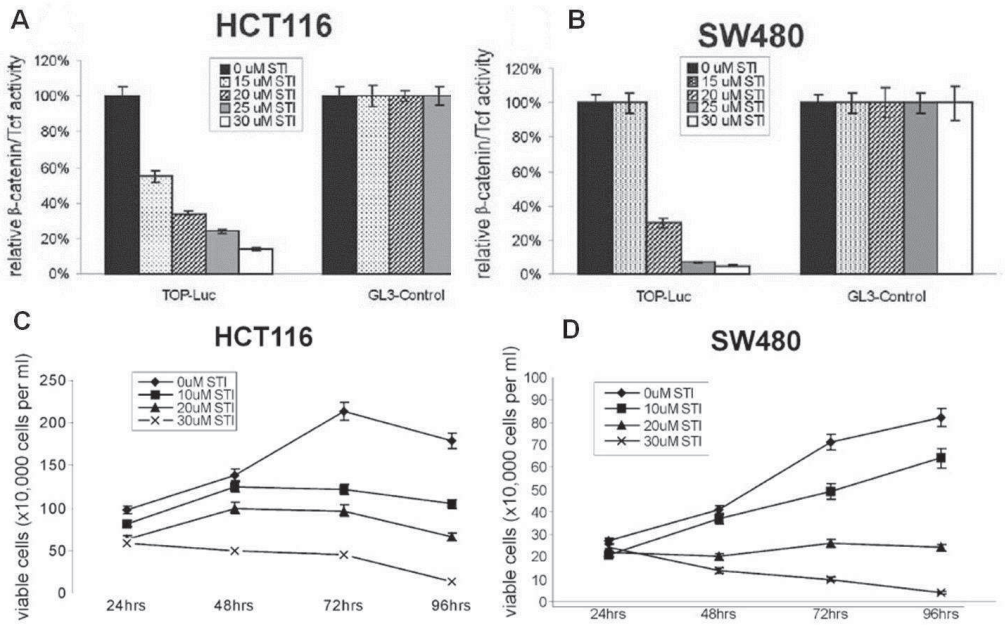

Figure 15 – Inhibitory effects of Imatinib on human colon cancer cell lines: Inhibition of B-catenin/TCF4-mediated transcription activity (A and B) and proliferation of human colon cancer cell lines (C and D) (images scanned and adapted from Zhou et al., 2003)

Inhibition of ABL kinase activity in anaplastic thyroid cancer (ATC) cell lines as a result of treatment with imatinib has already been described by Podtcheko et al. (2003) and Kurebayashi et al. (2006), as well an *in vivo* strong anti-tumor effect of imatinib in mouse models (Podtcheko et al., 2003; Kurebayashi et al., 2006), but contrasts with the lack of significant effect of imatinib in modulating the cell growth in ABL positive cell lines as described by Dziba et al. (2004) (Dziba and Ain, 2004).

Rao et al. (2006) in their experiments with ATC cell lines, demonstrated that β-Catenin and ABL tyrosine kinase co-immunoprecipitate with each other and treatment with imatinib (10 μM for 48h) reduced the co-immunoprecipitated β-catenin and ABL levels (figure 16.A) (Rao et al., 2006).

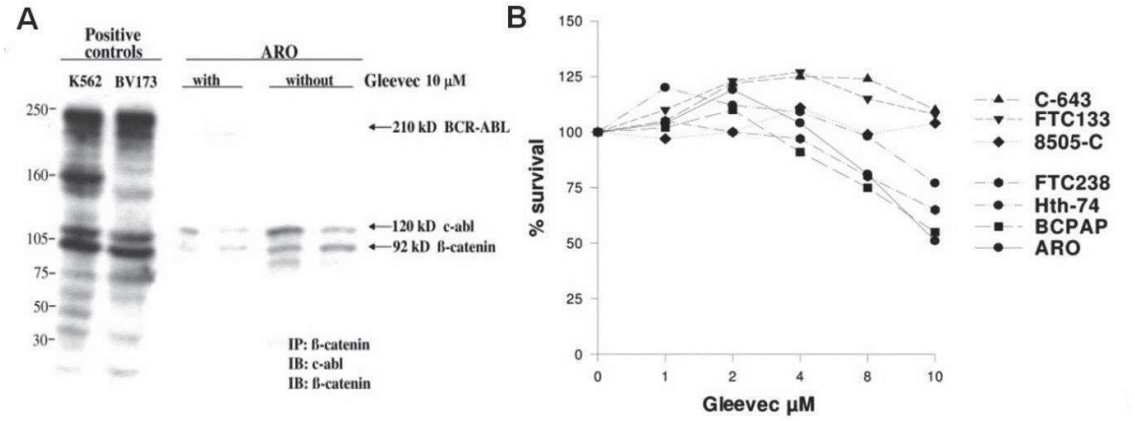

Figure 16 – Imatinib treatment effect on thyroid cell lines: (A) Co-immunoprecipitation of β-catenin and ABL and imatinib treatment effect; (B) Effect of imatinib on the cell proliferation of thyroid carcinoma cell lines (images scanned and adapted from Rao et al., 2006).

Imatinib dose dependent induced a decrease of β-catenin protein in cell lines expressing ABL, whereas in an ABL negative cell line (FCT133), β-catenin was unresponsive to imatinib. Transfection of FTC133 cells with wild-type ABL renders the cell line sensitive to imatinib, whereas cells transfected with dead kinase ABL remained insensitive. Treatment with imatinib also showed to promote β-catenin/e-cadherin binding at adherens junctions leading to a reduction in cytosolic/nuclear accumulation of free β-catenin: treatment of ARO cells (ABL positive) lead to a tyrosine phosphorylated β-catenin dose-dependently decrease, and in contrast to the tyrosine phosphorylated forms, the non-tyrosine phosphorylated β-catenin binds to e-cadherin, which was confirmed by confocal analysis and western blot that showed an imatinib-dependent relocalization of β-catenin and E-cadherin (Rao et al., 2006).

Assessment of putative role of imatinib in proliferation was evaluated in all cell lines that express TKR: in the two ABL positive cell lines incubation with imatinib showed a decline on growth (ARO and BCPAP) (figure 16.B). The Hth-74 cell line that expresses both PDGFRA and B showed intermediate sensitivity, whereas the two cell lines not expressing ABL (C-643 and 8505-C) had low sensitivity to imatinib (figure 16.B). In three ABL positive cell lines, incubation with imatinib showed a decreased proliferation by approximately 40% (Rao et al., 2006).

Imatinib also inhibits the β-catenin/TCF-LEF-dependent activity: ARO and FTC-133 cells (ABL positive and negative for all imatinib targets respectively) were transfected with TCF4 to block canonical Wnt signalling. The proliferation of both cell lines was reduced by about 50% in the transfected cells compared with the untrasfected controls. This suggests that irrespective of the ABL status, an active Wnt/β-catenin signalling is required for their basal proliferative activity in neoplastic thyroid cancer cell lines (Rao et al., 2006).

Imatinib treatment of ARO cells (ABL positive) showed a 40% decrease in the β-catenin TCF/LCF transcription levels, indicating that imatinib inhibits the Wnt/B-catenin signalling by specifically inhibiting TCF/LEF-dependent transcriptional activity. Western blot and reverse-transcription polymerase chain reaction analysis showed that imatinib also induced a reduction of cyclin D1 mRNA levels, compared to the controls (figure 17.A and B), and an effect on the invasiveness of the cells was observed after 48h of incubation with imatinib, which significantly dropped (Rao et al., 2006).

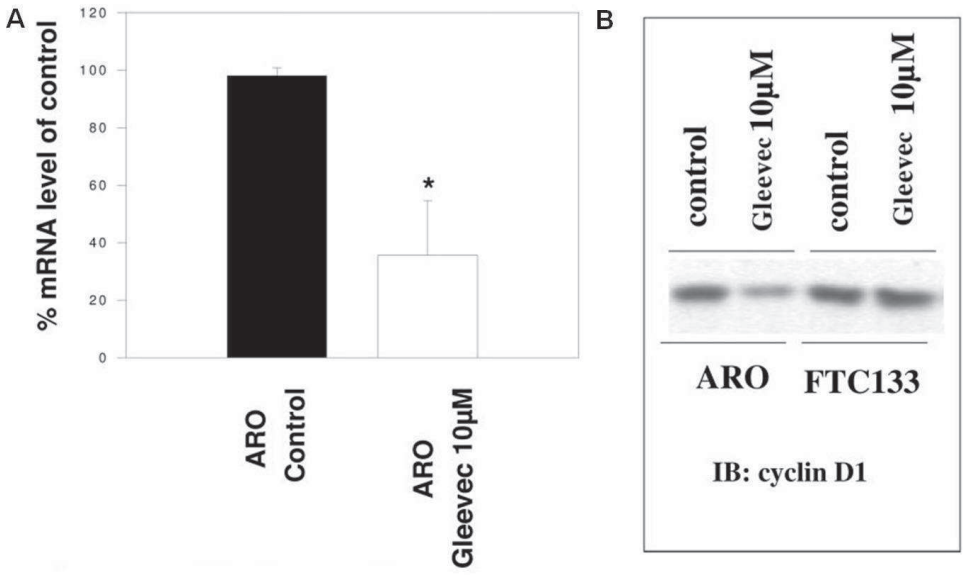

Figure 17 – Effects of imatinib on cyclin D1 expression: (A) Reduction of mRNA cyclin D1 levels in presence and absence of imatinib. (B) Western blot analysis after treatment with or without imatinib in ARO (ABL-positive) and FTC133 (ABL-negative) cell lines (images scanned and adapted from Rao et al., 2006)

Evaluation of possible relationship between TKR and β-catenin was also described by Kajiguchi et al. (2008). In their experiments, two mast cell leukaemia cell lines that harbour KIT activating mutations were used: HMC-1.1 (with V560G mutation) sensitive to imatinib and HMC-1.2 (with V560G and D816V mutations) resistant to imatinib. Both cell lines expressed β-catenin protein, and β-catenin was tyrosine phosphorylated in the absence of imatinib.

Treatment with imatinib induced a suppressed tyrosine-phosphorylation of β-catenin in imatinib-sensitive cells, but no decrease was observed in imatinib-insensitive cells (Kajiguchi et al., 2008). In HMC-1.2 cells β-catenin tyrosine-phosphorylation was suppressed by silencing the *KIT* gene, which support the hypothesis that tyrosine-phosphorylation of β-catenin depends on activated KIT in mast cell leukaemia cell lines (Kajiguchi et al., 2008).

Nuclear localization of β-catenin was observed in both cells lines, and after treatment of HMC-1.1 cells with imatinib, nuclear localization β-catenin was markedly decreased, as well the expression of both cyclin D1 and c-*myc* mRNA expression. Co-immunoprecipitation of KIT and β-catenin was observed in HMC-1.1 cell lines, whose association was significantly reduced in cells treated with imatinib (Kajiguchi et al., 2008).

In an *in vitro* kinase assay no tyrosine phosphorylation of β-catenin was detected in the absence of KIT protein, and addition of active KIT kinase induced tyrosine-phosphorylation of β-catenin, while inclusion of imatinib decreased tyrosine-phosphorylation of both KIT and β-catenin, which suggest that active TKR can directly phosphorylate tyrosine residues of β-catenin, in the same manner that observed in ABL positive ATC cell lines (Kajiguchi et al., 2008).

1.4.1.6 IMATINIB MESYLATE TREATMENT AND UTERINE SARCOMAS

In Sarcoma Alliance for Research through Collaboration – SARC phase II trial with the purpose to evaluate imatinib in different sarcoma subtypes, the four-month progression-free survival (PFS) rate was 20% (6/30) in the group of patients with uterine leiomyosarcomas. Beyond that, one patient had a complete remission for 11 months while four others had a PFS greater than one year (Chugh et al., 2004). In a recent multi-institutional phase II trial, imatinib showed a limited efficacy as single agent for treatment of recurrent carcinosarcomas that express KIT and PDGFRB (Huh et al., 2010).

In the case of endometrial stromal tumors (EST), few reports described attempts of treatment with imatinib on recurrent EST patients who express at least one of imatinib-targets (Salvatierra et al., 2006; Trojan et al., 2009; Kalender et al., 2009). Tumor shrinkage was the main indicator of objective response accompanied by stable disease in two cases (Salvatierra et al., 2006; Trojan et al., 2009) and in one patient with ESS who progressed on chemotherapy, a complete metabolic response was observed after treatment with imatinib (Kalender et al., 2009). Another report described a unique case of UES with EGFR expression and *EGFR* amplification which temporarily responded to imatinib (Mitsuhashi et al., 2007).

1.4.2 GEFITINIB AND ERLOTINIB

Gefitinib (Iressa™; AstraZeneca, Macclesfield, UK) and erlotinib (Tarceva™; OSI-Pharmaceuticals, New York, NY) are selective and reversible inhibitors that block the catalytic activity of EGFR (Mamot and Rochlitz, 2006).

Antiproliferative activity of gefitinib was demonstrated in various tumor cell lines and xenografts alone or in combination with other cytotoxic drugs (Moyer et al., 1997; Ciardiello et al., 2000). Inhibition effects of gefitinib were also observed in HER2-overexpressing breast tumor cells that also express EGFR (Moulder et al., 2001). Good tolerability and antitumor activity of gefitinib in malignant solid tumors was demonstrated in a phase I trial (Ranson et al., 2002).

In two phase II trials (Iressa Dose Evaluation in Advanced Lung Cancer [IDEAL]-1 and -2) treatment with gefitinib of patients with advanced non-small cell lung cancer (NSCLC) after failure of chemotherapy results in response rates ranged from 9%–19% (Kris et al., 2003; Fukuoka et al., 2003). Erlotinib efficacy was demonstrated in a phase II study with NSCLC patients expressing EGFR, previously treated with chemotherapy that showed an objective response rate of 12.3% (Perez-Soler, 2004).

In single arm studies increased benefit of gefitinib was seen in cases with EGFR high copy number (Cappuzzo et al., 2005a; Hirsch et al., 2005). In a series of 102 patients, amplification or high polysomy of the EGFR gene and also high protein expression detected by immunohistochemistry (IHC) was significantly associated to better response (36% versus 3%), disease control rate (67% versus 26%), time to progression (9.0 versus 2.5 months) and survival (18.7 versus 7.0 months) in patients with advanced NSCLC treated with gefitinib (Cappuzzo et al., 2005a).

High *EGFR* copy number was associated with improved survival after gefitinib therapy in a series of 89 patients with advanced-stage NSCLC (Hirsch et al., 2005). In both Iressa Survival Evaluation in Lung Cancer – ISEL and BR21 phase III trials was showed that high *EGFR* gene copy number measured by FISH was a predictor of a gefitinib and erlotinib-related effect versus placebo on OS (Tsao et al., 2005; Hirsch et al., 2006) and EGFR overexpression was associated to outcome in both studies. While in a recent meta-analysis *EGFR* gene copy number was described as a predictive marker for survival among patients receiving TKI monotherapy for advanced or recurrent NSCLC (Dahabreh et al., 2011), EGFR IHC present conflicting results (Perez-Soler et al., 2004; Parra et al., 2004) and is not yet established the value of EGFR protein expression as predictor of response to EGFR TKIs (John et al., 2009).

After discovery of *EGFR* somatic mutations that have shown *in* vitro increased sensitivity to gefitinib (Lynch et al., 2004; Paez et al., 2004) and erlotinib (Pao et al., 2004), association between the presence of activating *EGFR* mutations and response rate to TKI was demonstrated in phase II/III trials (Cappuzzo et al., 2005a; Eberhard et al., 2005; Hirsch et al., 2006). In phase II trials with selected NSCLC patients with *EGFR* somatic mutations, treatment with gefitinib showed responses rates between 55-75% in patients that harbour *EGFR* mutations (Sunaga et al., 2007; Sequist et al., 2008; Tamura et al., 2008). A prospective trial of the Spanish Lung Cancer Group showed that NSCLC patients with *EGFR* mutations had a PFS and OS of 14 and 27 months respectively (Rosell et al., 2009).

Additionally, in a phase III trial, patients with *EGFR* activating mutations showed a superior PFS with gefitinib (Mok et al., 2009) and erlotinib (Neal, 2010) than patients with wild-type *EGFR*, which state *EGFR* mutational status as an important predictive marker for TKI therapy (Dahabreh et al., 2010).

1.5 METHODOLOGICAL ASPECTS OF ANALYSIS OF TYROSINE KINASE RECEPTORS

Molecular techniques such as Immunohistochemistry (IHC), Fluorescence *in situ* hybridization (FISH) and polymerase chain reaction (PCR)-based methods applied in formalin-fixed paraffin embedded (FFPE) tissues are widely used in surgical pathology and nowadays are considered the *gold standard* techniques on evaluation of specific prognostic and predictive biomarkers as the TKR (Srinivasan et al., 2002; Xie et al., 2011).

Numerous variables can influence in different manners the result of a specific molecular technique, leading to result discrepancies between intra/inter-laboratories and lack of agreement between experimental studies (von Wasielewski et al., 2002; Goldstein et al., 2007a). They can be divided into three major groups (Hewitt et al., 2012):

- **Pre-analytic factors** (e.g. patient characteristics, specimen handling, fixation and processing procedures)
- **Analytic factors** (e.g. technical procedures)
- **Post-analytic factors** (e.g. interpretive and analytic factors)

1.5.1 PRE-ANALYTIC FACTORS

The pre-analytic factors include the patient population and intrinsic characteristics, surgery particularities, etc. which are uncontrolled (Chau et al., 2008; True, 2008) and the variables related with the collection and preservation of samples until to analytic phase (Hewitt et al., 2012). Absence of standardized protocols of handling, fixation and processing of specimens among different laboratories and also within laboratories, has been well recognized as contributing to variability in both diagnostic histopathology and advanced molecular studies (Srinivasan et al., 2002; Hewitt et al., 2008; Xie et al., 2011; Engel and Moore, 2011).

1.5.1.1 COLLECTION AND SAMPLE PRESERVATION

1.5.1.1.1 Specimen Handling

The prefixation time is the time between the surgical excision of the specimen and immersion on the fixative (Srinivasan et al., 2002). During this period, called “warm ischemia,” the tissue lacks oxygen and anoxic metabolic pathways can occur (Chung et al., 2008), thus the

prefixation time should be minimal to avoid DNA, RNA and protein degradation (<1hour) (Tokuda et al., 1990; Srinivasan et al., 2002; Wolff et al., 2007). In the same manner grossing of the specimen should be performed immediately to prevent loss of enzymes and decrease in histological mitosis and other artefacts (Cross et al., 1990; Williams et al., 1999; Srinivasan et al., 2002).

1.5.1.1.2 Tissue Fixation

Fixation is the most important step in the processing of a sample and an adequate and appropriate fixation is the basis of all histological and molecular procedures (Hewitt et al., 2008; Chung et al., 2008). A good fixation is the balance between under-fixation and over-fixation (Bancroft and Gamble, 2007), and the main factors that influence this step are: (1) choice of fixative; (2) concentration and pH of fixative solution; (3) thickness of tissue and volume of fixative and (4) duration and temperature of fixation (De Marzo et al., 2002; Hewitt et al., 2008).

Choice of fixative: to date a universal or ideal fixative has not been yet identified (Bancroft and Gamble, 2007) and formalin is the most common fixative used in diagnostic pathology due to the good morphological preservation (Shi et al., 1997; Bancroft and Gamble, 2007; Hewitt et al., 2008; Leong et al., 2010; Engel and Moore, 2011). In addition the characteristic immunoreactivity of neoplasms and lesions are based mainly in IHC assays performed in formalin fixed tissues (Shi et al., 1997; Goldstein et al., 2007a; Dunstan et al., 2011).

The principal effect of formalin fixation consists in the cross-linking between protein amino acid residues by methylene bridges (Fraenkel-Conrat and Mecham, 1949), that lead to the loss of antigen immunorecognition (Ben-Ezra et al., 1991; Shi et al., 1997; Miething et al., 2006), nucleic acids degradation and insertion of DNA sequence artefacts (Tokuda et al., 1990; Williams et al., 1999; Srinivasan et al., 2002; Akbari et al., 2005; Miething et al., 2006; Koboldt et al., 2010; Nothnagel et al., 2011).

Nonetheless, use of non formalin fixatives is strongly discouraged, tissues fixed in an alternative non-formalin fixative can produce similar appearance, but this does not mean similar antigen expression (Shi et al., 1997; Nadji et al., 2005; Goldstein et al., 2007a; Dunstan et al., 2011). In FISH experiments, use of different fixatives can lead to failures on nucleic acids hybridization success (Varella-Garcia et al., 2009; Moatamed et al., 2011).

Concentration and pH of fixative solution: formalin is recommended to be used in the form of 10% neutral buffered formalin (10% NBF). Utilization of different concentration and

pH of formalin can affect the morphology and the quality of immunostaining in FFPE specimens (Bancroft and Gamble, 2007; Engel and Moore, 2011) and induce nucleic acids modification and poor preservation of high-molecular weight nucleic acids (Cross et al., 1990; Noguchi et al., 1997; Douglas and Rogers, 1998; Srinivasan et al., 2002).

Thickness of tissue and volume of fixative: fixation depends of coefficient of diffusibility[5] of the fixative and the rate of reaction between the fixative and the tissue components (Srinivasan et al., 2002; Miething et al., 2006). 10% NBF has a coefficient of diffusibility of 0.79 (can vary depending on tissue type), and in contact with water becomes hydrated to form methylene glycol. The immerged tissues will be penetrated quickly by methylene glycol but fixed slowly by carbonyl formaldehyde (Srinivasan et al., 2002). To facilitate the penetration and consistent fixation it is imperative to fix small volumes of tissues (5 mm to 1cm) (Tokuda et al., 1990; Srinivasan et al., 2002; Hewitt et al., 2008). The volume ratio specimen/fixative should be 1:10 to 1:20 (Hewitt et al., 2008; Hammond et al., 2010).

Duration and temperature of fixation: fixation times remain poorly controlled in general practice (Hewitt et al., 2008). In general it is recommended the tissue fixation in 10% NBF for a minimum of 6-8 hours and a maximum of 72 hours after sectioning (von Wasielewski et al., 2002; Goldstein et al., 2007a; Wolff et al., 2007; Varella-Garcia et al., 2009; Hammond et al., 2010), although 24h is considered the ideal time to achieve reliable results in both biopsies and surgical specimens (Goldstein et al., 2003; Engel and Moore, 2011).

A fixation time inferior to 6-8 hours (under-fixation) is countered by relatively greater alcohol dehydration fixation and will produce differences in staining characteristics by IHC and nucleic acids degradation (Goldstein et al., 2007a), whereas long fixation (over-fixations) periods can lead to loss of immunoreactivity of some antigens and decrease the average size of DNA extracted, as well the deterioration of PCR signal (Ben-Ezra et al., 1991; Karlsen et al., 1994; Srinivasan et al., 2002; Hewitt et al., 2008; Leong et al., 2010). In extreme cases it can lead to loss of nuclear DNA (Selvarajan et al., 2002; Miething et al., 2006).

High temperatures lead to a decrease of tissue immunoreactivity (Shi et al., 1991) and was showed that use of low temperatures on tissue fixation with 10% NBF allows a better preservation of nucleic acids, through retarding the action of nucleases (Tokuda et al., 1990).

[5]Coefficient of diffusibility: the distance in millimetres that the fixative has diffused into the tissue in 1 hour (Srinivasan et al., 2002).

1.5.1.1.3 Tissue Processing

The tissue processing consist in a three-step process: sequential dehydration from an aqueous environment to an alcohol environment, clearing (replacement by xylene or xylene substitute), and impregnation (replacement of the xylene with paraffin) (Hewitt et al., 2008). Beyond insufficient, inconsistent, and different types of fixation, tissue processing is also a key factor behind suboptimal and inconsistent molecular studies (Goldstein et al., 2007a; Leong et al., 2010), and is suggested that any changes in the processing system have the capacity to alter the chemical makeup of the tissue specimen (Hewitt et al., 2008).

Although the contribution of tissue processing to loss of immunorecognition in tissue sections has not been completely characterized (Grizzle, 2009), tissue processing time directly affects protein quality (Xie et al., 2011): short processing times are associated to inadequate tissue dehydration and poor impregnation and consequent loss of antigenicity and nucleic acids degradation (Werner et al., 2000; Xie et al., 2011), whereas longer tissue processing time results in better quality of RNA, DNA and proteins suggesting a reduction in endogenous water in FFPE tissue (Hewitt et al., 2008; Chung et al., 2008; Xie et al., 2011).

The type of paraffin wax seems to have no impact on results, although a melting point of 55ºC to 58ºC was recommended to avoid decrease and inadequate deparaffinization, and also yield reduction of nucleic acids (Hewitt et al., 2008). Quality of reagents has also impact on sample integrity, as well the adequate replacement of each auxiliary reagent (xylol, alcohols) (Hewitt et al., 2008; Engel and Moore, 2011;).

1.5.1.1.4 Slide-age and block storage

In clinical practice and predictive biomarkers studies is common practice to provide cut slides rather than the original block for additional molecular studies (Leyland-Jones et al., 2008).

Antigen and nucleic acids degradation is more common to occur in unstained slides (Fergenbaum et al., 2004; Mirlacher et al., 2004; Eberhard et al., 2008; Hewitt et al., 2008; Varella-Garcia et al., 2009), mainly due to exposure to air, resulting in *oxidation* (Blind et al., 2008). Several storage methods have been advocated, but none prevents loss of antigenicity (Jacobs et al., 1996; DiVito et al., 2004; Engel and Moore, 2011) and on the other hand, nucleic acid stability and antigen preservation are in general well-preserved in long-term stored paraffin blocks, if they are properly fixed and processed (Camp et al., 2000; Ellis et al., 2000; Cronin et al., 2004;).

Due to this, is recommended that policies should be developed to include FFPE tissue block or tissue core submission (if not longer required for clinical diagnosis) for clinical studies/trials (Leyland-Jones et al., 2008).

1.5.2 ANALYTIC AND POST-ANALYTIC FACTORS

Optimization is the process in which the laboratory serially tests and modifies component procedures with the end-point to produce consistently high-quality assays (Goldstein et al., 2007a). This step is essential to validate any molecular assay, since is the process whereby the parameters the assay are established: accuracy, reliability and reproducibility (Taylor, 2000; Goldstein et al., 2007a; Dunstan et al., 2011).

Different variables can influence the results, and while fixation and processing effects are transversal to each molecular technique, the analytic and post-analytic factors seem to be specific of each molecular assay (Goldstein et al., 2007a).

1.5.2.1 IMMUNOHISTOCHEMISTRY

Despite the widespread use of IHC, there is a surprising lack of standardization among IHC laboratories (Hsi, 2001). The overwhelming pre-analytic variable that influences IHC results is the fixation time (Chung et al., 2008), but other variables have been shown to have a substantial impact on assay performance, reproducibility and quantitative value: choice of antibody and technical issues, and staining interpretation (Shi et al., 1997; Hewitt et al., 2008; Mathieu et al., 2010), resulting in poor concordance and validation (Hsi, 2001; Goldstein et al., 2007a).

1.5.2.1.1 Analytic factors

Factors that could influence the IHC reproducibility and performance include:

a) Selection of appropriate primary antibody clone;
b) Selection of antigen retrieval procedure;
c) Selection of detection system and chromogenic detection system;
d) Selection of an appropriate test tissue, whose should contain the target antigen.

Selection of antibody clone: the specific antibody clone selected for the IHC assay should be selected on the basis of intended clinical use of the IHC assay and the established record of immunoreactivity in published studies (Goldstein et al., 2007a). In general, antibodies bind to a small region of the target antigen, and different antibody clones bind to regions on a target antigen that may be distinct and separate areas, overlap to some extent, or be nearly identical (Goldstein et al., 2007b), and although for some antigens, there are no detectable differences in immunoreactivity between antibody clones, for other the antigenic binding site of the specific clone has crucial importance (Goldstein et al., 2007a).

Selection of antigen retrieval method: antigen retrieval (AR) is recognized to ‘‘restore’’ the antigenicity after the formalin fixation through break of formalin-induced cross-linkages and subsequent exposition of antigenic epitopes (Shi et al., 1997; Eisenthal et al., 2008). The exact mechanism of antigen retrieval is unknown (Eisenthal et al., 2008), and although various hypothesis have been proposed (Eisenthal et al., 2008), is mainly achieved by hydrolytic break of the intra- and intermolecular cross-links (Shi et al., 1991; Shi et al., 1995).

The advantages of AR include: (1) lowering the antigenic detection threshold, (2) use of higher antibody dilutions, (3) reduction the possibility of background staining and increase the labelling specificity (Shi et al., 1991; Eisenthal et al., 2008) and (4) decrease of false negative results which raises the reproducibility of results and, thus the diagnostic accuracy (Shi et al., 1991; Shi et al., 1997) .

The parameters of an AR protocol must be balanced to match the unique length and type of tissue fixation of the individual laboratory and the characteristics of the individual antibody (Shi et al., 1997). The common methods of AR are enzyme digestion and heat-induced epitope retrieval (HIER). The first acts through break of protein cross-links and different kinds of enzymes are available, although not all antigens can benefit from this method (Leong et al., 2010).

HIER was considered the milestone in IHC (Boenisch, 2006), since allows the demonstration of several formalin-masking antigens, leading to enhanced tissue antigenicity (Shi et al., 1997; Leong et al., 2010). Diverse solutions of HIER (low and pH buffers) and various types of heating devices, can be used depending on the optimization parameters of individual antibodies (Goldstein et al., 2007a).

Different factors can influence the success of the HIER technique, namely temperature, pH, metal ions composition and molarity of the HIER solution (Shi et al., 1997; Eisenthal et al., 2008). Temperature and pH of AR are critical factors of a well succeeded HIER (Shi et al., 1995; Emoto et al., 2005).

High-temperatures in general yield better results and inversely correlates with the time of HIER treatment (Shi et al., 1997). The heating method used to apply energy (pressure cooker, microwave, water bath or autoclave) is substantially less important than the amount of energy applied to the slides by the HIER system (Taylor et al., 1996; Rhodes et al., 2000; Hsi, 2001).

The pH value of the HIER solution is antigen-dependent and more important than the composition of the buffer per se (Shi et al., 1995). Although there is no universally optimal pH of the retrieval medium, Shi et al. (1996) described best results with low pH solution associated to regular heating (e.g. MW heating) or high-pH with intense heating (e.g. pressure cooker or autoclave) (Shi et al., 1996).

Nonetheless, an adequate and standard fixation is the cornerstone to optimize AR and avoid inconsistent and unreliable IHC assays (Goldstein et al., 2007a): over-AR relative to the amount of formalin fixation produces high-background staining, section fall-off, tissue section holes and rents, indistinct nuclear detail, etc, while under-AR normally results in false-negative results (Goldstein et al., 2007a).

Detection system and chromogenic detection system: actually new amplification techniques are available, including dextran polymer conjugates and the tyramide system, which can potentially increase sensitivity by factors of 100 or more. The chromogenic detection system peroxidase/DAB based seems to be more sensitive than phosphatase/fast red (Hsi, 2001).

Selection of appropriate tissue control: the expression level of some antigens can vary between neoplasms from different patients, between benign and malignant tissues, and between different types of neoplasms (Yaziji and Barry, 2006). Antibodies optimized with tissues that express high levels of antigen expression may prove inadequate when staining tissues with low antigen expression. It may be necessary to optimize an antibody for a variety of cell types, accomplished by varying the concentration (dilution) or, if using a pre-diluted antibody, by varying the incubation time (Goldstein et al., 2007b). E.g. KIT optimization should be performed with a weak-CD117 positive control. If a higher-expression tissue control is used, weakly CD117 expressing tissues (e.g. GIST) may fall below the threshold of immunoreactivity and be interpreted as CD117-negative (Goldstein et al., 2007a).

1.5.2.1.2 Post-analytic factors

In an ideal paradigm an IHC assay would be interpreted with a universal standardized scoring system (Shi et al., 2007). The IHC assay immunoreactivity should be assessed with a quantitative scoring system (Wells et al., 2004), which should include two separate components: the extent or proportion of the target antigen that was immunoreactive and the intensity of the immunoreactivity (Goldstein et al., 2007a; Leong et al., 2010).

There is no universal scoring system, and also no common cut-point threshold for a positive result that can be applied across all clinical situation and types of samples. Substantial variability in interpretation can be due to several factor as clinical situation, type of specimen, amount of available target tissue in the specimen and mainly to the adequacy of specimen fixation and processing (Rudiger et al., 2002; Wick and Swanson, 2002). The positive result cut-point threshold must be applied to the result that is clinically appropriate and takes into account the expected level of immunoreactivity, specimen size, and amount of target antigen in the specimen (Goldstein et al., 2007b).

1.5.2.1.3 Immunohistochemistry on evaluation of tyrosine kinase receptors expression

KIT: immunohistochemical detection of KIT is a hallmark in the diagnosis of a GIST, which frequently present strong and uniform positivity with a membrane Golgi zone–like or cytoplasmic staining pattern (Miettinen, 2002). Considered a sensitive but not specific marker of KIT activation, its expression can be seen in different neoplasms including seminoma, melanoma and some carcinomas (Arber et al., 1998). While consistent expression of KIT in GIST is widely recognized (Miettinen and Lasota, 2006; Turner and Goldsmith, 2009), in other solid tumors was considered inconsistent (e.g. SCLC and melanoma), mainly due to the wide variety of different KIT antibodies, protocols, and scoring systems used to identify KIT-positive tumors (Went et al., 2004):

- Hornick et al. (2002) evaluated KIT expression in a series of other tumors than GIST, and conclude that KIT immunoreactivity is relatively limited in tumors other than GISTs (Hornick and Fletcher, 2002). In this study antigen retrieval was not used, and was suggested that absence of antigen retrieval may result in lower detection sensivity and a lower percentage of positive tumors (Miettinen, 2002);
- KIT expression in desmoid-type fibromatosis is controversial: Yamaguchi et al described expression up to 75% of cases (Yamaguchi et al., 2004), whereas others described

minimal cytoplasmatic expression (5%) after optimization of KIT immunodetection (Hornick and Fletcher, 2002; Lucas et al., 2003), which support the evidence that the KIT expression is highly dependent of establishment of patronized IHC procedures (Miettinen, 2002);

- In a large study that include more than 3,000 tumors cores from more than 120 different tumor categories, was analyzed the expression of KIT by IHC in a tissue microarray format. The antibody A4502 (DAKO) was used due the highest sensivity observed after a pre-selection between seven commercially available anti-KIT antibodies. With the exception of GIST, seminoma and melanoma, in the other tumor entities the KIT-positivity was below 30% (Went et al., 2004);
- In two KIT surveys was evaluated different anti-KIT clones associated to HIER (most commonly steamer or automated system). The staining concordance was up of 95% in GIST cases (Dorfman et al., 2006), whereas in three of the tissue cores from cases of leiomyosarcoma, peripheral nerve sheath tumor, and uterine stromal sarcoma, achieved staining concordance was about 81%: the authors indicates that positivity in cases of leiomyosarcoma and uterine stromal sarcoma, may be due to misinterpretation of immunostaining, KIT-positive mast cells were considered as positive staining of tumor, since no significant differences in false-positive staining were identified either due to specific antibodies or the antigen retrieval methods used (Dorfman et al., 2006);
- In a clinical and molecular study of imatinib effect on aggressive fibromatosis, KIT expression was variable accordingly the use of AR: in most cases the majority of tumor cells showed KIT staining when epitope retrieval was used (Heinrich et al., 2006).

Platelet-Derived Growth Factor Receptor, α-Polypeptide: antibodies against both PDGFRA and PDGFRB exist, but the results of IHC assays are hard to interpret (Turner and Goldsmith, 2009). In a large series of GIST, PDGFRA expression was observed in all KIT negative GISTs (8/8), whereas only 25% (2/8) of cases presented expression for PDGFRB (Rossi et al., 2005). In another series of GIST, was suggested that PDGFRA expression predicts the presence *PDGFRA* mutation (Miselli et al., 2008). However due the absence of reliable antibody and few data about its staining profile, evaluation of PDGFRA expression is not routinely used in practice to diagnose KIT–negative GISTs (Espinosa et al., 2008) or other malignancies (Raymond et al., 2008; Heinrich et al., 2008b).

Epidermal growth factor receptor: The prognostic and predictive value of EGFR IHC expression seems to be controversial: in a retrospective analysis detection of high levels of EGFR protein expression by IHC in univariate analysis was associated to survival in NSCLC

patients treated with erlotinib, but not in multivariate analysis (Tsao et al., 2005), whereas other studies failed to demonstrate a relationship between results of EGFR-immunohistochemistry and response to TKIs (Parra et al., 2004; Herbst et al., 2005).

The major factors pointed for these discrepancies were (i) sample characteristics and selection (Scagliotti et al., 2004), (ii) impact of pre-analytic factors, (iii) analytical factors: different EGFR antibody clones affinities and non-standardized IHC procedures (Lee et al., 2010), and (iv) the use of distinct scoring methods of IHC which makes difficult to interpret the results (Meert et al., 2002):

- In a prospective study it was observed that (a) quality and quantity of EGFR immunoreactivity in HNC, NSCLC and colorectal adenocarcinomas is in part influenced by the fixative used, (b) that expression is inversely correlated with storage time of unstained tissue sections, and (c) is dependent on the tumor entity tested (Atkins et al., 2004);
- Frequency of EGFR expression in STS was reported to range between 0.3% and 52.9%, depending of antibody and score system used and only one antibody (clone EGFR.113, Novocastra) was associated to the clinical outcome (Kersting et al., 2006);
- Mathieu et al (2010) demonstrated that different antibody clones can give different results independently of sample origin: EGFR status of 634 NSCLC was evaluated with the most commonly used antibody EGFR pharmDx^{TM} kit (DakoCytomation, Carpinteria, CA, USA) and the results were compared with those obtained by applying other anti-EGFR antibodies used in daily practice in 16 pathology laboratories (Mathieu et al., 2010). EGFR expression was seen in 525 (83%) tumours with the Dako pharmDx^{TM} kit, and the Zymed, Novocastra and NeoMarkers antibodies showed positive results in 384 (86%), 115 (90%) and 41 (68%) tumours, respectively, showing that the choice of the antibody indeed may be of importance and can alter the number of patients that would qualify for treatment with anti-EGFR agents (Mathieu et al., 2010). Sample type seems also influence the results: biopsy samples were more frequently positive than cytological and surgical samples (Mathieu et al., 2010), whose was previously observed in other studies (Meert et al., 2004) and might be related to heterogeneity distribution of EGFR protein expression throughout the tumour (Suzuki et al., 2005; Italiano et al., 2006) and to the different fixation methods between different types of samples (biopsy vs surgical specimens) (Mathieu et al., 2010);
- Lee et al (2010) compared also four commercially available EGFR antibodies using the same scoring system the modified Colorado scoring method (combination of the percentage of positive cells and staining intensity with a cut-off value of 101) (Cappuzzo

et al., 2005b), and to compare those results with FISH. They found that 56% percent of tumor samples were scored as positive with Zymed (Clone 31G7), 51% were scored as positive with Dako EGFR pharmDx™ (Clone 218C9) and presented a good correlation with high *EGFR* gene copy number and *EGFR* gene amplification, whereas a percentage lower than 20% were scored as positive with Dako (Clone H11) and with Novocastra (Clone EGFR.113). The authors conclude that both Zymed and Dako EGFR pharmDx™ tests were more sensitive than the Dako and Novocastra antibodies ($p<0.001$) (Lee et al., 2010), and that sensitivities of each antibody are regulated by the determination site of the antigen epitope (Lee et al., 2010).

1.5.2.2 FLUORESCENCE *IN SITU* HYBRIDIZATION

Fluorescence *in situ* hybridization (FISH) is a powerful molecular cytogenetic tool based on the hybridization of specific DNA sequences to the target genome, allowing the analysis of gene and chromosome copy number in normal and malignant tissue (Thompson et al., 1994; Selvarajan et al., 2002).

1.5.2.2.1 Analytic factors

Optimization of FISH experiments in FFPE tissues includes various requirements and is associated to technical limitations as type of fixative used, artefacts caused by sectioning of tissue blocks, thickness of sections, storage of cut sections, and probe penetration (Thompson et al., 1994; Ellis et al., 2000; Schurter et al., 2002; Ventura et al., 2006; Martin et al., 2009).

Formalin fixation induces cross linking in proteins and increases the chromatin condensation turning difficult the probe penetration into nucleus (Thompson et al., 1994), and knowledge of previous formalin fixation helps to establish proper pre-treatment of tissues (Varella-Garcia et al., 2009).

The desmasking steps that include heat pre-treatment and proteolytic treatment are critical for obtaining readable FISH results (Ventura et al., 2006) Tissue permeabilization and enzyme digestion depends of the extension of tissue fixation (Varella-Garcia et al., 2009), and should be standardized to maintain nuclear morphology and integrity. Insufficient pre-treatment can result in weak or absent hybridization, whereas extended pre-treatment can

cause damages on tissue morphology (Ventura et al., 2006; Martin et al., 2009). The hybridization and washing steps should be standardized (Ellis et al., 2000).

1.5.2.2.2 Post-analytic factors

Expertise on results interpretation: it is advisable to locate areas of tumor to be scored using a previous section stained with hematoxylin and eosin (Ellis et al., 2000). In evaluation of copy number changes should be take in account the sectioning artefacts (e.g. nuclei overlapping and/or nuclei truncation) (Martin et al., 2009), as well the influence variables such as ploidy, nuclear size, and chromatin condensation which can vary between normal control samples and tumor specimens (Ventura et al., 2006).

Choice of a reliable scoring method: the most critical factors affecting a proper interpretation of FISH signals is the establishment of cut-off values for the different probes used and for all signal patterns that might appear with a given assay (Ventura et al., 2006). FISH evaluation of gene *HER-2* is associated to a well-defined scored system (Wolff et al., 2013), but the same is not well established for other genes (e.g. *EGFR*).

1.5.2.2.3 Evaluation of *EGFR* gene by Fluorescence *in situ* hybridization

Evaluation of the pattern of *EGFR* gene alterations by FISH in solid tumours is complex and varies in different tumour types (Martin et al., 2009), and the evaluation criteria for the definition of *EGFR* gene amplification not always correlate with the response to target therapy (Martin et al., 2009). In NSCLC was evaluated the *EGFR* status by FISH with a wide range of results, which was also observed for others malignancies (Martin et al., 2009).

Even when the same *EGFR* probe and analytical method is used, a range of variability is observed, which indicates that different factors can influence the results: (1) differences in patient population, different study designs and treatment and control arms; (2) differences in the laboratorial methodology (sample preservation and assay performance); and (3) no clear definition for the scoring of atypical signals and/or gene clusters and lack of a precise method for calculation of the EGFR ratio, which could lead to a different interpretation of signals among different labs and across different studies (Varella-Garcia, 2006).

A scheme for classifying NSCLC tumours as EGFR FISH positive and FISH-negative was developed at the University of Colorado and has been used in multiple clinical studies (Varella-Garcia, 2006).

1.5.2.3 PCR-BASED MUTATIONAL ANALYSIS

PCR based mutation analysis of paraffin-embedded tissues is an important method in cancer diagnostics and in genetic analysis of tumor cells (Akbari et al., 2005; Eberhard et al., 2008; Anderson et al., 2012). Dideoxy sequencing (Sanger method) is considered the *gold-standard* technique to screen somatic mutations on solid tumors, and although other techniques have been applied in the screening of biomarkers, all of them relied on confirmation by Sanger sequencing as a confirmatory approach (Eberhard et al., 2008; Hewitt et al., 2012).

In a quality assessment was described that accuracy of mutational analysis results is more dependent of the quality of sample than on the difference in molecular sequencing procedures between distinct centres (Bellon et al., 2011; Beau-Faller et al., 2011). However limitations and source of variability were associated to this techniques: induced-formalin DNA degradation and sequence artefacts (Koboldt et al., 2010; Lamy et al., 2011; Solassol et al., 2011; Do and Dobrovic, 2012), as well the low sensivity of dideoxy sequencing method (20-30%) that can lead to false negative results (Kotoula et al., 2009).

Macrodissection or microdissection may improve the accuracy of Sanger sequencing, mainly in cases with less than 50% of tumor content (Akbari et al., 2005; Eberhard et al., 2008; Anderson et al., 2012), although associated to some limitations: low tumor content, impossibility to perform macrodissection in every cases, and inaccuracy of pathological estimation of tumor content (Bellon et al., 2011), and thus do not ensure that a given sample will have the sufficient level of mutant alleles to be consistently detected (Anderson et al., 2012).

A meta-analysis of 3381 somatic *EGFR* mutations in 12,244 NSCLC patients reported that the majority of mutations have been reported on only one occasion (158 of 254, 62.2%), and only nine mutations occur at a rate of ≥1% (Murray et al., 2008), which was also reported by Marchetti et al. (2005). Sequence artefacts are common in poorly processed FFPE samples and when low template amounts are used for PCR amplification (Akbari et al., 2005), are a mechanism poorly understood, although may be associated to fixation and embedding procedures (Williams et al., 1999; Gallegos Ruiz et al., 2007; Solassol et al., 2011; Anderson et al., 2012; Do and Dobrovic, 2012).

Mechanisms that lead to these artefacts can be post-mortem deamination of cytosine or adenine (C>T/G>A or A>G/T>C transitions), resulting in uracil or hypoxanthine residues, respectively (Hofreiter et al., 2001). To turn-over this limitation, the template can be treated with uracil-N-glycosylase, which removes uracil from DNA, creating a strand break (Marchetti et al., 2006; Do and Dobrovic, 2012).

Other mechanism that can generate artefactual mutations corresponds to chemical modification of DNA that affects cytosine and guanine residues and result in G>A transitions due to nucleotide misincorporations by DNA polymerase during amplification and has been reported in olden degraded DNA (Lamy et al., 2011). DNA degradation can be overturned by the use of short amplicons in PCR and through PCR optimization (Gallegos Ruiz et al., 2007; Lamy et al., 2011; Anderson et al., 2012; Do and Dobrovic, 2012).

1.5.2.4 TISSUE MICROARRAY VERSUS WHOLE TISSUE SECTIONS

The recent development of tissue microarray (TMA) technology has potentiated large-scale retrospective cohort studies using archival formalin-fixed, paraffin-embedded tissues, and allows for standardized, rapid, and cost-effective assays (e.g. IHC and FISH) which is critical in epidemiological studies (Camp et al., 2000; Fergenbaum et al., 2004; Thomson et al., 2009) and biomarkers studies (Ilyas et al., 2013).

Developed by Kononen et al. (1998), the construction of a TMA consists of taking a core of tissue from a ‘donor’ tissue block with a needle and transferring it to an empty ‘recipient’ block (Kononen et al., 1998). Numerous donor cores can be arrayed in a single recipient block, allowing large numbers of cases to be tested very quickly. The size of the donor core is determined by the diameter of the needle and thus, with appropriately sized needles (Ilyas et al., 2013).

The small size of the tissue core (0.6 to 2 cm) is pointed as a disadvantage and a source of variability between studies, since small cores may not be representative of the whole tumor, and a tumor tissue may comprise of many different histological areas within itself, such as regions of apoptosis, necrosis or increased proliferation, etc. and it may not be possible to sample all areas in one tissue core (Camp et al., 2000; Mirlacher et al., 2004; Sharma et al., 2010). However several studies have validated that results obtained from analysis of tissue microarray cores and whole sections using immunohistochemical studies are identical and comparable (Thomson et al., 2009; Sharma et al., 2010) and many other such quality assurance studies have demonstrated that the intratumoral heterogeneity do not limit the effectiveness of TMA as a tool for molecular pathology studies (Sharma et al., 2010).

Other possible disadvantages may be the can include loss of core and absence of tumor due to errors during TMA construction, which is reported as minimal and overcome with the inclusion of duplicate and triplicate tissue cores (Sapino et al., 2006; Thomson et al., 2009).

Loss of staining intensity was also reported, and seems to be related with to the heating process used to fuse the transplanted cores in the TMA block and can damage the antigen integrity (Thomson et al., 2009).

The greatest advantage of TMA is that all the factors related with the analytic phase (section thickness, slide age, the type and concentration of all reagents, technical procedures) are absolutely identical and consistent (Mirlacher et al., 2004), which can allow to decrease the sources of variability (Sauter and Mirlacher, 2002). Quantification of IHC is improved because it is possible to directly compare staining intensities of the different specimens on the same TMA slides, and also facilitate the application of reproducible scoring criteria, as the entire tissue is always used for interpretation and the subjective selection of one tumor area for decision making is avoided (Torhorst et al., 2001).

2. AIM OF THE STUDY

The aim of the present work was to investigate the protein expression and gene status of TKR KIT, PDGFRA and EGFR in endometrial stromal tumors, in order to elucidate their possible roles as therapeutic targets in these tumors.

The specifics aims were:

1. To evaluate the mutational status and protein expression of KIT and PDGFRA in EST;
2. To correlate the immunohistochemical expression of KIT and PDGFRA with the mutational status of its related genes;
3. To evaluate EGFR protein expression and *EGFR* gene amplification status in EST, and correlate it with the mutational status of *EGFR* gene.

3. MATERIAL AND METHODS

3.1 STUDY DESIGN AND INCLUSION CRITERIA

The study was developed under the project GEIS-18 from the Grupo Español de Investigación en Sarcomas[6] (GEIS) and approved by the Ethics Committee of Hospital Germans Trias i Pujol (Badalona, Spain).

The selection of samples was made accordingly to the following inclusion criteria:

i) Previous diagnosis of EST (ESS or UES);
ii) Availability of histological material sufficient to perform the molecular study.

75 histological samples were retrieved from Spanish centres of GEIS and from 2 external Centres (Complejo Hospitalario Universitario de Badajoz, Spain and Hospital do Espírito Santo E.P.E, Évora, Portugal) in a total of 9 centres (Appendix 2), and sent to the Tumor Bank of the Centro de Investigación del Cáncer (CIC) – Salamanca, Spain, where the codification of the samples and the research work were made in accordance with the Declaration of Helsinki (World Medical Association, 2008) and the Spanish Biomedical Research Act (2007).

The reference Institutional Review Board (Research Ethical Committee of Hospital Trias I Pujol) approved the use of the material and data necessary only to accomplish the aims of the study and due to this no clinical data was included in the study.

3.2 HISTOLOGICAL ANALYSIS AND DIAGNOSIS REVIEW

To review the diagnosis and subclassification of the encoded samples, hematoxylin-eosin (H&E) slides of each paraffin block were made and analyzed by my supervisor Prof. Enrique de Alava. To exclude other types of tumors mimicking endometrial stromal and related tumors, immunohistochemical evaluation of CD45, CD99, and pan-cytokeratin were assayed in some cases.

[6] Grupo Español de Investigación en Sarcomas (GEIS): http://www.grupogeis.org/

The final diagnosis was based on the 2003 WHO classification (Hendrickson and Tavassoli, 2003):

- Low-grade endometrial stromal sarcoma
- Undifferentiated endometrial sarcoma

3.3 CONSTRUCTION OF TISSUE ARRAY

Described in 1998 by Kononen (Kononen et al., 1998), tissue microarrays (TMA) represent a high-throughput technology for assessment of histology-based laboratory tests including IHC and FISH. Suitable for the study of tumor biology, assessment of novel molecular markers and also laboratory quality assurance (Voduc et al., 2008), TMAs have as principal advantage the decreased technique variability during staining and interpretation process, allowing more rapid and consistent biomarker scoring, and the rapid assessment of individual molecular markers on large clinical cohorts (Voduc et al., 2008).

Before elaboration of TMAs, the most representative tumor areas from each case were selected on H&E slides and then marked on the donor paraffin block. From each donor paraffin block, two cylinders of 1 mm diameter were obtained and placed into a new recipient paraffin block using a tissue microarrayer (Beecher Instruments Inc. Sun Prairie, Wisconsin, USA). Two TMAs were constructed according to previously described protocols (Parsons and Grabsch, 2009).

3.4 IMMUNOHISTOCHEMICAL STUDY

Protein expressions of distinct TKR - KIT, PDGFRA and EGFR - were evaluated by IHC. Beside this, and with the aim to complement the histopathologic review, the expression of two markers most commonly used on EST, namely CD10 and Calponin were also evaluated.

IHC was performed on 3 µm TMAs sections. Antigen unmasking was done by HIER and enzymatic digestion accordingly to each target antigen (table 6). The detection of CD10, Calponin, KIT and PDGFRA antigens was performed through sequential incubation with primary antibodies (table 6), universal secondary biotinylated antibody (Discovery™ Universal Secondary Antibody, Ventana Medical Systems, Tucson, Arizona, USA), and streptavidin-biotin peroxidase detection system (DAB MAP system, Ventana Medical Systems, Tucson, Arizona, USA) in the automatic processing system Discovery® Ventana (Ventana Medical Systems, Tucson, Arizona, USA). For EGFR analysis expression, paraffin-

embedded sections were deparaffinized in xylene and rehydrated in downgraded alcohols and distilled water. Antigen retrieval was performed with Proteinase K solution (Dako, Carpinteria, CA, USA). The primary antibody (table 6) was detected using a secondary antibody-horseradish peroxidase polymer conjugate (Dako REAL™ EnVision™ Detection System, Dako, Carpinteria, CA, USA), and all incubations were done with the Dako Autostainer Plus system (Dako, Carpinteria, CA, USA). The sections were counterstained with hematoxylin, upgraded alcohols, and xylene, mounted, and analyzed by standard light microscopy.

Table 6 – Characteristics and conditions of primary antibodies

Antibody (Clone)	Cat. #	Company	Working dilution	Antigen retrieval technique
CD10 (56C6)	NCL-L-CD10-270	Novocastra Laboratories	1:100	HIER: Tris-EDTA buffer pH8.0
Calponin (CALP)	M3556	DAKO	1:100	HIER: Citrate buffer pH6.0
KIT	A4502	DAKO	1:100	HIER: Tris-EDTA buffer pH8.0
PDGFRA	RB-9027-P	Thermo Scientific	1:100	HIER: Tris-EDTA buffer pH8.0
EGFR (2.1E1)	AP10540	Gennova Scientific	*Ready-to-use*	ED: Proteinase K solution

Legend: HIER: Heat-induced epitope retrieval; ED: enzymatic digestion

3.4.1 IMMUNOHISTOCHEMICAL EVALUATION

Immunostainings were scored semiquantitatively under a light microscope by Prof. Enrique de Alava. The expression of CD10 and Calponin was considered positive when over 1% of the tumor cells showed cytoplasmic expression.

The TKR expression was detected in the cytoplasm of tumor cells, and the immunoreactivity was evaluated using a 0 to 3 semiquantitative scoring system for both the intensity of stain and the percentage of positive cells, as previously reported (de Alava et al., 2007). The multiplicative index of intensity and labelling was considered for statistical analyses and the expression was defined as weak and/or focal (1+), for a multiplicative index between 1-3,

moderate, focal or diffuse (2+) for a multiplicative index between 4-6, and intense and diffuse (3+) if the multiplicative index was >6.

3.5 MUTATIONAL ANALYSIS OF TYROSINE KINASE RECEPTORS

Dideoxy-terminating DNA sequencing based on Sanger's method (Sanger et al., 1977) was chosen to evaluate hotspots of *KIT* (exons 9, 11, 13 and 17), *PDGFRA* (exons 12 and 18) and *EGFR* (exons 18 to 21). Considered as the gold standard method for identification and interpretation of human sequence variations (Metzker, 2005; John et al., 2009), this widely used technique combines the dye terminator chemistry (2´,3´-dideoxynucleotides) with a cycle sequencing asymmetric polymerase chain reaction (PCR) (Alphey, 1997). The detection of this dye-labelled DNA fragments is performed in an automatic DNA sequencer, where dye-labelled DNA fragments migrate through thin-coated capillaries containing high-resolution denaturating acrylamide gel (capillary electrophoresis) and are separated according to their size. The emitted light of each dye laser-excited is detected by a photomultiplier tube, which converts it into a digital signal and subsequently generates a DNA electropherogram. Despite the lower sensitivity when compared with targeted methods and the time-consuming, DNA sequencing permits the detection of all possible mutations, including novel variants, and identifies the exact mutation in the sample (Pirker et al., 2010). The next sections summarize all steps needed to perform a mutational analysis of a specific gene from a tissue sample.

3.5.1 DNA EXTRACTION

DNA extraction was performed by a spin-column technology (*QIAamp® DNA Mini Kit*, Qiagen, Hilden, Germany), which combines selective binding properties of a silica-based membrane with flexible elution volumes**,** and allows rapid purification of genomic DNA from solid tissue.

Ten serial 5 µm sections were cut from each paraffin block and transferred into eppendorf tubes. Dewaxing and hydration of the samples were performed with sequential washings in xylene (two times for 5 minutes) and absolute ethanol (two times for 5 minutes) at 4000 rpm, followed by drying of the samples at room temperature until the ethanol evaporated.

The tissue pellets were incubated overnight with tissue lysis buffer (Buffer ATL, Qiagen, Hilden, Germany) and proteinase K (QIAGEN Proteinase stock solution, Qiagen, Hilden,

Germany) at 56ºC in a water bath with agitation. The subsequent steps (additional lysis, purification and elution) were performed according to QIAamp® DNA Mini Kit protocol (Qiagen, Hilden, Germany). An elute of 100 µl of genomic DNA was obtained of each sample and stored at -20ºC until further analysis.

3.5.2 DETERMINATION OF DNA YIELD AND PURITY

To assess both DNA yield and purity, measurement of DNA absorbance (optical density) was carried out using spectrophotometry. Absorbance reading was performed at 260nm (A_{260}), where DNA absorbs light most strongly, allowing to estimate the DNA concentration. DNA purity was evaluated by the ratio 260/280 obtained in each sample.

To analyze the DNA yield and purity, each DNA sample was diluted to 1:20 in water to a total volume of 70 µl, and quantified with the SmartSpec Plus Spectrophotometer (Bio-Rad Laboratories Inc., CA, USA) accordingly the manufacturer's instructions.

3.5.3 PRIMER DESIGN

Specific primers for *KIT*, *PDGFRA* and *EGFR* were designed as described in previous studies (table 7) (Corless et al., 2002; Heinrich et al., 2003c; van Zandwijk et al., 2007; Jang et al., 2009). Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) was used to confirm the specificity of the primers.

Table 7 – Oligonucleotides sequences for PCR

Gene, Exon	Specific Primers	PCR Product
KIT		
Exon 9	F: 5'-TCCTAGAGTAAGCCAGGGCTT-3'	283 bp
	R: 5'-TGGTAGACAGAGCCTAAACATCC-3'	
Exon 11	F: 5'-CCAGAGTGCTCTAATGACTG-3'	284 bp
	R:5'-AGCCCCTGTTTCATACTGAC-3'	
Exon 13	F: 5'-GCTTGACATCAGTTTGCCCAG-3'	193 bp
	R:5'-AAAGGCAGCTTGGACACGGCTTTA-3'	
Exon 17	F: 5'-GGTTTTCTTTTCTCCTCCAACC-3'	203 bp
	R:5'-GATTTACATTATGAAAGTCACAGG-3'	
PDGFRA		
Exon 12	F: 5'-TCCAGTCACTGTGCTGCTTC-3'	260 bp
	R: 5'-GCAAGGGAAAAGGGAGTCTT-3'	
Exon 18	F: 5'-ACCATGGATCAGCCAGTCTT-3'	251 bp
	F: 5'-TGAAGGAGGATGAGCCTGACC-3'	
EGFR		
Exon 18	F: 5'-GCTGAGGTGACCCTTGTCTC-3'	225 bp
	R: 5'-CTC CCC ACC AGA CCA TGA-3'	
Exon 19	F: 5'-CAT GTG GCA CCA TCT CAC A-3'	230 bp
	R: 5'-CAG CTG CCA GAC ATG AGA A-3'	
Exon 20	F: 5'-CAT TCA TGC GTC TTC ACC TG-3'	377 bp
	R: 5'-CAT ATC CCC ATG GCA AAC TC-3'	
Exon 21	F: 5'-GCT CAG AGC CTG GCA TGA A-3'	348 bp
	R: 5'-CAT CCT CCC CTG CAT GTG T-3'	

Legend: *F: forward primer; R: reverse primer*

3.5.4 *KIT* AND *PDGFRA* GENE ANALYSIS

3.5.4.1 PREPARATION OF REACTION MIXTURE AND PCR CONDITIONS

Prior to molecular analysis, all the samples underwent PCR amplification of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to confirm integrity and quality of extracted DNA, and therefore a standard PCR master mix was established for *KIT*, *PDGFRA* and *GAPDH* gene amplification.

To obtain a total volume of 25 µl was added 1X PCR buffer, 2 mM MgCl2 (Roche Applied Science, Mannheim, Germany), 0.15 mM of each primer, 200 µM of each dNTP (GeneAmp® dNTP Blend, 10 mM, ABI, Carlsbad, CA, USA), 1.5 U of Taq polymerase (Taq DNA Polymerase, Roche Applied Science, Mannheim, Germany) and 1-5 µl of genomic DNA. A negative control that contains everything but the DNA template was included in each PCR set for detecting contamination or non-specific amplification.

The PCR reaction was performed in a thermocycler (MyCycler™ Thermal Cycler System, Bio-Rad Laboratories Inc., CA, USA) with the following 3-step PCR program: 94°C for 10 min, 40 cycles of 1 min at 94°C, 1 min 30 sec to 56°C (KIT and GAPDH) or 65°C (PDGFRA) and 1 min at 72°C, and a cycle of 10 min at 72°C, followed by holding at 4ºC. The final PCR products were stored at 4ºC until further use.

3.5.4.2 NUCLEIC ACID ELECTROPHORESIS

PCR products were loaded onto an agarose gel in order to visualize them under UV-light using a fluorescence dye.

The 2% agarose gel (in 1X TBE buffer) was cast and pre-stained with ethidium bromide (0,5 µg/ml) for 30 min. 5 µl of each PCR product was mixed with 2.5 µl loading buffer and loaded into the gel slots; a 100 bp Ladder (Biotools, Madrid, Spain, P/N 31.006) was also loaded. Electrophoresis was carried out at a voltage of 130 V for approximately 30 min. Visualization of agarose gel was done with an imaging system Gel Doc™ XR+ System and Quantity One® 1-D analysis software (Bio-Rad Laboratories Inc., CA, USA).

3.5.4.3 PURIFICATION OF PCR PRODUCTS

A pre-requisite of DNA sequencing is the need to start the sequencing reaction with a pure template. Thus, PCR products were first purified to remove unincorporated primers, nucleotides and buffer components, non-target amplification products and compounds that may inhibit the subsequent analysis (McPherson and Moller, 2006). In the present study a commercial spin-column based-method (*QIAquick® PCR Purification Technology Kit*, Qiagen, Hilden, Germany) was used for purification of PCR products. This method is based on the retention of DNA fragments in a solid support (e.g. silica membrane), which are submitted to washing steps to remove dNTPs, buffer components and primers, and a final elution step that allows recovery of the bounded DNA.

20 µl of PCR product was added to 100 µl of Buffer PB. The mix was transferred to a spin-column and centrifuged at maximum rotation during 1 min. After discarding the flow-through, the QIAquick column was replaced into the same collection tube, and was washed with Buffer PE (máx. rotation - 1 min). A second centrifugation was carried out to ensure complete elimination of residual ethanol from Buffer PE. The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube, and the purified DNA was eluted with 50 µl of nuclease free water. The samples were stored at 4ºC until further analysis.

3.5.4.4 CYCLE SEQUENCING PCR

For this purpose, a mix with 6.3 µl of diluted purified PCR product (1:2 in water) and 1.7 µl of PCR primer was prepared in duplicated (forward and reverse primer) for each sample. Then the samples were sent to Genomic Department of CIC, where the cycle sequencing PCR was performed with the ABI PRISM® BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) in a thermocycler according to the manufacturer instructions.

3.5.4.5 ETHANOL PRECIPITATION

The amplified sequences were submitted to ethanol precipitation to remove precipitate salts and remove the unincorporated label, which can obscure data in the early part of the sequence and interfere with basecalling (Alphey, 1997; McPherson and Moller, 2006).

Samples were incubated with 65 µl of precipitation solution (sterile 3M sodium acetate, 125 mM EDTA and ice-cold 95% ethanol) during 15 min. The samples were centrifuged 40 min at 4300 rpm in a refrigerate microcentrifuge. After removal of the precipitation solution, the pellets were washed with 150 µl of ice-cold 70% ethanol and centrifuged 15 min at 4300 rpm in a refrigerate microcentrifuge. The supernatant was removed and the samples were allowed to dry in the dark at room temperature for 10 min. Then the samples were resuspended in 12 µl of formamide and placed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

3.5.4.6 DATA ANALYSIS

All sequences obtained were visualized and analyzed in the program *Sequence Scanner Software v1.0* (Applied Biosystems, Carlsbad, CA, USA), based on the reference sequences: *KIT* - ENSG00000157404 and *PDGFRA* - ENSG00000134853.

3.5.5 *EGFR* GENE ANALYSIS

3.5.5.1 PREPARATION OF REACTION MIXTURE AND PCR CONDITIONS

To obtain a total volume of 15 µl was added 1X PCR buffer (Applied Biosystems, Carlsbad, CA, USA), 2.5 mM MgCl2 (Applied Biosystems, Carlsbad, CA, USA), 0.17 mM of each primer (20 µM), 200 mM of each dNTP (GeneAmp® dNTP Blend, 10 mM, Applied Biosystems, Carlsbad, CA, USA), 2 U of Taq DNA polymerase (AmpliTaq Gold® DNA Polymerase, Applied Biosystems, Carlsbad, CA, USA) and 4 µl of genomic DNA. As described previously, a negative control (without template DNA) was also included in each PCR set to test for contaminating DNA.

The amplification reactions were carried out under the following PCR program: an initial heating step of 95ºC for 12 min, followed by 40 cycles of denaturation at 95ºC for 15 sec, annealing at 65ºC for 1 min, and extension at 72ºC for 1 min and a final extension step at 72ºC for 10 min.

3.5.5.2 NUCLEIC ACID ELECTROPHORESIS

The DNA electrophoresis of PCR products was carried out as described in section 3.5.4.2.

3.5.5.3 PURIFICATION OF PCR PRODUCTS

Purified PCR products were purified with *USB® ExoSAP-IT®* (Affymetrix, Inc., Cleveland, Ohio, USA) with the same purpose described in section 3.5.4.3.

USB® ExoSAP-IT® consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase. The former removes residual single-stranded primers and any extraneous single stranded DNA produced during the PCR reaction, while Shrimp Alkaline Phosphatase removes the remaining unincorporated dNTPs from the PCR mixture.

5 µl of USB® ExoSAP-IT® were added directly to each PCR products. The PCR tubes were placed in the thermocycler and submitted to the following PCR program: 15 min at 37ºC and 15 min at 80ºC. The samples were stored at 4ºC until further analysis.

3.5.5.4 *EGFR* DNA SEQUENCING

As described above, cycle sequencing PCR was also prepared to amplify forward and reverse sequences of *EGFR* gene.

6 µl of purified PCR product was added in duplicate to clean 0.2 ml PCR 8-tube strip, heated at 96ºC in a thermocycler for 3 min and then placed on ice. To each tube were added 2 µl of respective 3 mM primer and 12 µl of fresh prepared mix of GenomeLab™ DTCS Quick Start Kit (Beckman Coulter Inc, Fullerton, CA, USA) according to the manufacturer's instructions. The samples were placed in a thermocycler and subjected to 20 sec at 96ºC, 20 sec at 50ºC, and 4 min at 60 º C for 30 cycles, followed by holding at 4ºC.

3.5.5.5 ETHANOL PRECIPITATION

After the cycle sequencing PCR the amplified sequences were submitted for ethanol precipitation for post-sequencing reaction clean-up as described in the section 3.5.4.5.

A stop solution "premix" was made with 2 µl of sterile 3M NaOAc pH 5.2, 2 µl of 100 mM EDTA pH8.0 and 1 µl of 20 mg/ml glycogen, accordingly to the total of samples to clean-up. The samples were incubated with the 5 µl of stop solution and 60 µl of ice-cold absolute ethanol during 20 min at -20ºC. Then the samples were centrifuged 20 min at 4728 rpm in a refrigerated microcentrifuge. After removal of supernatant, the pellets were washed twice with 200 µl of ice-cold 70% ethanol for 10 min as described before. Subsequently, the supernatant was removed and the pellets were allowed to dry in the dark at room temperature for 35 min. The pellets were then resuspended in 30 µl of formamide and placed in a GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter Inc, Fullerton, CA, USA). The samples were run with LFR-b sequencing method, and with the default sequence analysis parameters sets (Sample Setup Module, Genome Lab Genetic Analysis System v10.0.30, Beckman Coulter Inc, Fullerton, CA, USA).

3.5.5.6 DATA ANALYSIS

The mutational analysis of the obtained sequences were performed using the *Sequence Analysis Module* of *Genome Lab Genetic Analysis System v10.0.30* (Beckman Coulter Inc, Fullerton, CA, USA), and compared with the reference sequence ENSG00000146648.

3.6 *EGFR* GENE AMPLIFICATION BY FLUORESCENCE *IN SITU* HYBRIDIZATION

The ploidy status of *EGFR* in each tumor was assessed by FISH on previously constructed TMAs. Considered as the most reliable method to evaluate *EGFR* gene amplification in patients candidates to treatment with EGFR TKIs (Hirsch et al., 2005; Eberhard et al., 2008), this method uses fluorescent-tagged DNA probes corresponding to *EGFR* to detect all cellular copies of the *EGFR* gene, and allows a direct correlation of numerical *EGFR* gene alterations with either morphological features or immunophenotypic tumors characteristics (Eberhard et al., 2008; Martin et al., 2009).

Gene amplification was determined by using an EGFR/CEN-7 FISH Probe Mix (Y5500, Dako, Carpinteria, CA, USA) containing Texas Red-labelled DNA probe covering the full EGFR region and a mixture of fluorescein-labelled PNA probes targeted at the centromeric region of Chr 7. The Histology FISH Accessory Kit (Dako, Carpinteria, CA, USA) was used for pre-treatment, pepsin digestion and stringent washes of TMA sections. All the pre-treatment steps, co-denaturation and overnight hybridization, and later stringent washes

were performed according to Step-by-Step FISH Procedure for Histological Samples (Code K5599, Dako, Carpinteria, CA, USA).

3.6.1 FLUORESCENCE IN SITU HYBRIDIZATION EVALUATION

Hybridization signals were visualized using a fluorescence microscope equipped with a *IAI monochrome progressive scan* (IAI Company, Taiwan) and ran by image analysis software *Cytovision*® (Leica Microsystems, Wetzlar, Germany). 20 tumour nuclei/case were scored, and the tumor cells in which the signals of *EGFR* and CEP7 were increased equally were classified as polysomy and those for which there was a double signal for *EGFR* or CEP7 were considered diploid. To assess gene amplification, the ratio of *EGFR* to CEP7 was calculated and evaluated in accordance with the criteria of FISH scoring system of Colorado Group (Varella-Garcia, 2006). Evaluation of the ploidy status of *EGFR* was performed by Prof. Enrique de Álava.

3.7 STATISTICAL ANALYSIS

The association between the expression of tyrosine kinase receptors and ploidy of *EGFR* with different histological types of EST, as well the mutational status of TKR were assessed. All statistical analyses were performed based on contingency tables, with *SPSS software v18.0 statistics* (Chicago, Illinois).

4. RESULTS

4.1 HISTOLOGICAL ANALYSIS

In the present study, 65 samples were considered valid and included in the study. Ten samples were not included because they did not accomplish the inclusion criteria: samples were too small to perform the proposed study, non-representative samples, or diagnosis different than EST.

ESS was the most frequent EST subtype, corresponding to 80% of cases (52/65), whereas UES encompassed 20% (13/65) of the total cohort. In the histopathologic review the classical pattern of ESS was observed in most of cases: proliferation of uniform tumor cells with oval to spindle nuclei was supported by a rich network of arterioles (figure 18.A), whereas UES cases showed pleomorphic features, atypical mitotic figures and no resemblance with endometrial stroma (figure 18.B). Distribution of cytoplasmic CD10 and calponin expression (figure 18.C-F) was similar in both histologic subtypes and is summarized in table 8.

Table 8 – Distribution and pattern of expression of CD10 and Calponin in Endometrial stromal tumors subtypes

EST subtype	Negative	Positive	Intensity 1+	Intensity 2+	Intensity 3+
CD10					
ESS	48.1% (25/52)	51.9% (27/52)	26.9% (14/52)	19.2% (10/52)	5.8% (3/52)
UES	76.9% (10/13)	23.1% (3/13)	15.4% (2/13)	7.7% (1/13)	0% (0/13)
Calponin					
ESS	55.8% (29/52)	44.2% (23/52)	23.1% (12/52)	13.4% (7/52)	7.7% (4/52)
UES	76.9% (10/13)	23.1% (3/13)	23.1% (3/13)	0% (0/13)	0% (0/13)

Legend: 1+: weak and/or focal expression; 2+ moderate, focal or diffuse expression; 3+ intense and diffuse expression

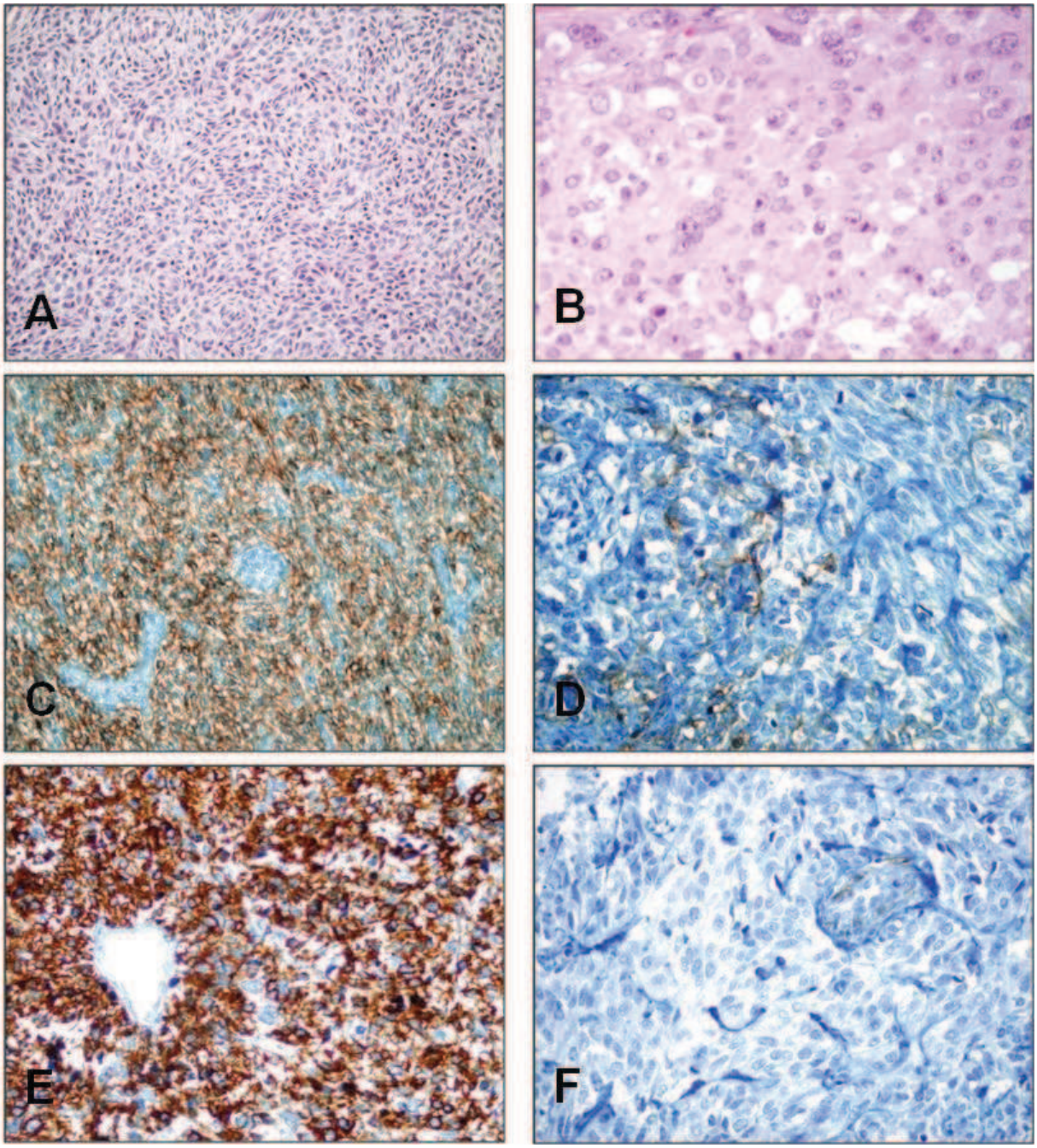

Figure 18 – Morphologic features of Endometrial stromal tumors and immunoexpression of CD10 and Calponin: (A) H&E stain of ESS: proliferation of uniform and well differentiated endometrial cells that resemble proliferative endometrium (magnification, 100x). (B) H&E stain of UES: cellular pleomorphism and atypical mitotic figures as normally observed in UES cases (magnification, 400x). CD10 immunostain: intense and diffuse cytoplasmic expression in ESS (C) (magnification, 100x) and moderate and focal in UES (D) (magnification, 400x). Calponin immunostain: intense and diffuse cytoplasmic expression in ESS (E) (magnification, 100x) and very weak and focal in a UES case (F) (magnification, 400x)

4.2 ANALYSIS OF EXPRESSION OF TYROSINE KINASE RECEPTORS

Evaluation of KIT and PDGFRA expression in EST was performed in previous series and reports. KIT expression presented a wide variation (0-100%, mean 25%), with a great percentage of cases showing focal or absence of expression. On the other hand, PDGFRA expression was normally found in these tumors (33-100%, mean 64%).

In our series KIT expression was present in only 2 cases and PDGFRA expression is observed in 35.4% (23/65) of EST cases with a similar distribution between the ESS and UES subtypes, as described in table 9. Regarding to the expression pattern, weak and/or focal expression was observed in both KIT positive cases. PDGFRA cytoplasmic expression was mostly weak and/or focal (27.7%; 18/65), and a lower percentage of cases presented moderate, focal or diffuse expression (7.7%; 5/65) (table 9 and figure 19.A-D). Intense and diffuse KIT and PDGFRA expression was not observed in any case.

Moinfar et al. (2005) described for the first time EGFR protein expression in EST (Moinfar et al., 2005), and based on his findings we decided to evaluate EGFR protein expression in our cohort. In the present study only 10.8% of cases (7/65) presented EGFR expression. Weak and/or focal EGFR expression was observed in 6.2% (4/65) of cases, while 4.6% (3/65) of cases presented moderate, focal or diffuse expression (table 9 and figure 19.E-F).

As described for the others TKR, in our series none of the cases showed intense and diffuse expression for EGFR. Co-expression of 2 or more TKR was observed in only 3 cases: one ESS present expression for the three TKR, other ESS present concomitant expression of PDGFRA and EGFR and one UES case present expression for both KIT and EGFR proteins.

Table 9 – Distribution and pattern of expression of each TKR in Endometrial stromal tumors subtypes

EST subtype	Negative	Positive	Intensity 1+	Intensity 2+	Intensity 3+
KIT					
ESS	98.1% (51/52)	1.9% (1/52)	1.9% (1/52)	0% (0/52)	0% (0/52)
UES	92.3% (12/13)	7.7% (1/13)	7.7% (1/13)	0% (0/13)	0% (0/13)
PDGFRA					
ESS	65.4% (34/52)	34.6% (18/52)	28.8% (15/52)	5.8% (3/52)	0% (0/52)
UES	61.5% (8/13)	38.5% (5/13)	23.1% (3/13)	15.4% (2/13)	0% (0/13)
EGFR					
ESS	88.5% (46/52)	11.5% (6/52)	5.76% (3/52)	5.76% (3/52)	0% (0/52)
UES	92.3% (12/13)	7.7% (1/13)	7.7% (1/13)	0% (0/13)	0% (0/13)

Legend: 1+: weak and/or focal expression; 2+ moderate, focal or diffuse expression; 3+ intense and diffuse expression

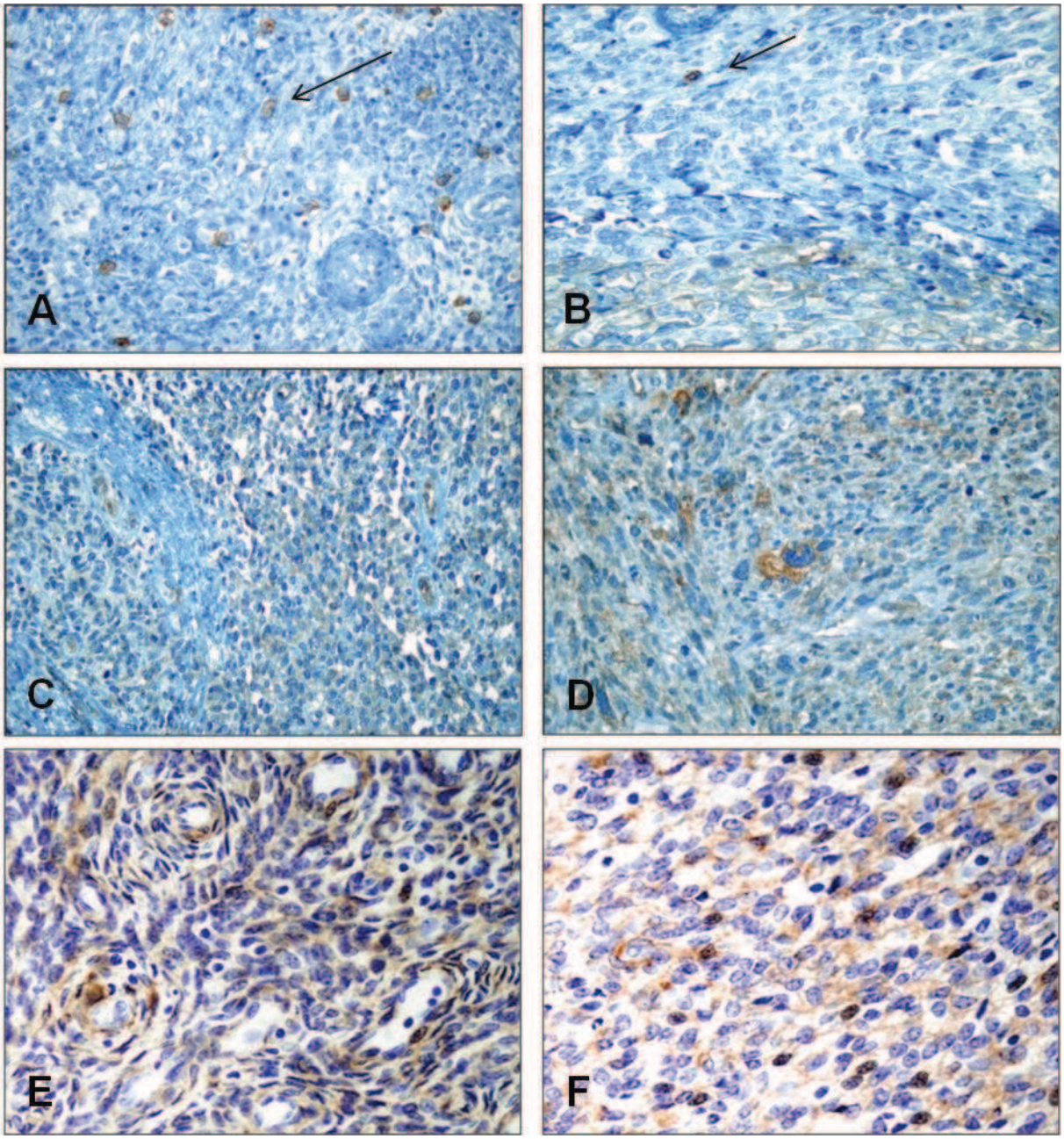

Figure 19 – Tyrosine kinase receptors immunoexpression in Endometrial stromal tumors: (A) ESS: absence of KIT expression. (B) UES: weak and/or focal cytoplasmic KIT expression (magnification 400x), the arrows indicate KIT cytoplasmic positivity in sparse mast cells. (C) and (D) Weak and/or focal cytoplasmic PDGFRA expression in ESS and UES (magnification 100x); (E) and (F) Weak and/or focal EGFR membranous and cytoplasmic expression in ESS and UES (magnification 400x)

4.3 ANALYSIS OF MUTATIONAL STATUS OF TYROSINE KINASE RECEPTORS

To evaluate the mutational status of TKR, previously described hotspots of each gene were evaluated by DNA sequencing. 62 samples were considered adequate to molecular study after successful PCR amplification of *GADPH* gene.

In the sequence analysis no mutations were detected in any exon of *KIT* and *PDGFRA* genes, but the presence of several single nucleotide polymorphisms (SNP) in both genes was observed. Two ESS showed a silent base substitution (ATC>ATT) in exon 17 of *KIT* gene at codon 798 (I798I) (figure 20.A). In *PDGFRA* gene analysis, all 62 samples presented the silent mutation (CCA>CCG) in exon 12 at codon 567 (P567P) (figure 20.B), 32.3% (21/65) showed a silent base substitution (GTC>GTT) in exon 18 at codon 824 (V824V) and 1 case (ESS) had a double silent base substitution (GTC>GTT and GGC>GGT) at codons 824 (V824V) and 838 (G838G) respectively, both in exon 18 (figure 20.C-D).

Mutational analysis of *EGFR* was performed only in cases with expression of EGFR, and in which DNA was available for molecular studies. In total, 5 cases were available for mutational analysis and two cases without EGFR expression were included as a negative control.

There were no mutations in *EGFR* exons 18, 19, 20 and 21. Silent base substitutions in exon 20 were in 7 cases found: in 6 cases silent base substitutions were identified at codon 787 (CAG>CAA; Q787Q) (figure 21.A), and one case showed also a silent base substitution at codon 790 (ACG>ACA; T790T) (figure 21.B).

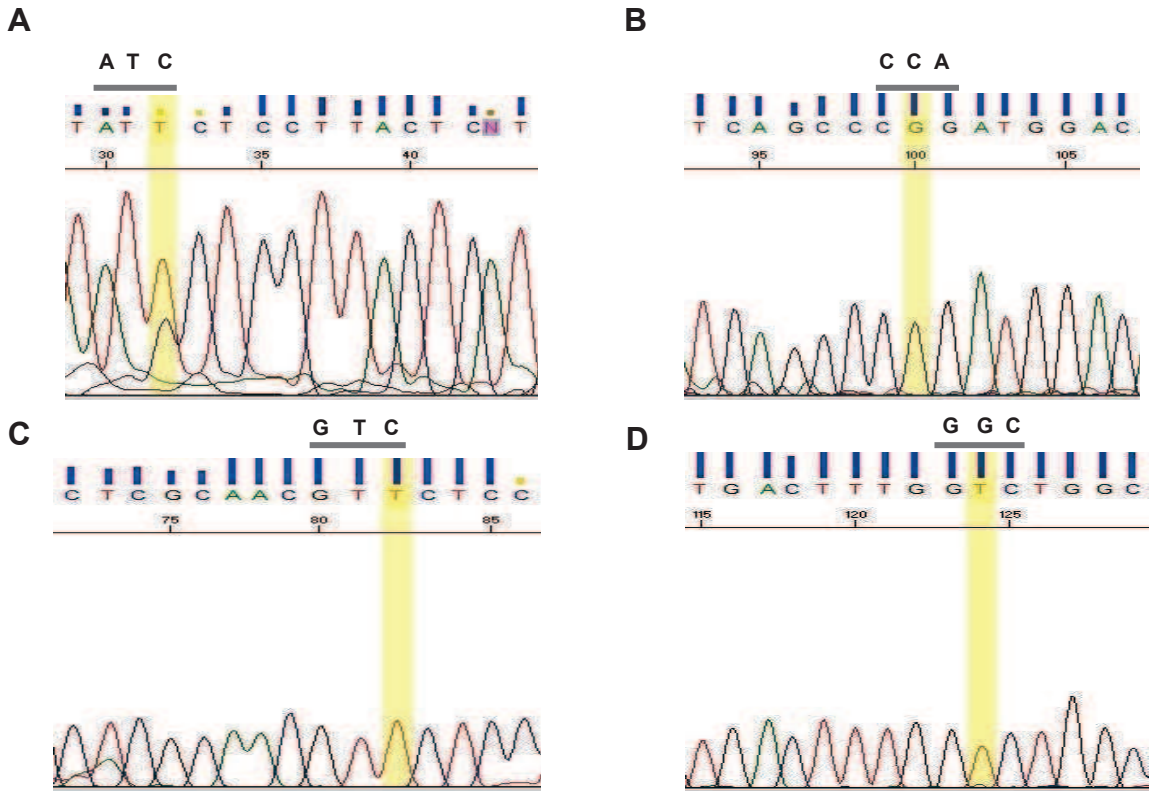

Figure 20 – *KIT* and *PDGFRA* single nucleotide polymorphisms: (A) ESS, KIT Exon 17 codon 798 (ATC>ATT). **(B)** ESS, PDGFRA Exon 12 codon 567 (CCA>CCG). **(C and D)** ESS with double PDGFRA SNPs: Exon 18 codon 824 (GTC>GTT) and codon 838 (GGC>GGT) (above the electropherogram the original codons)

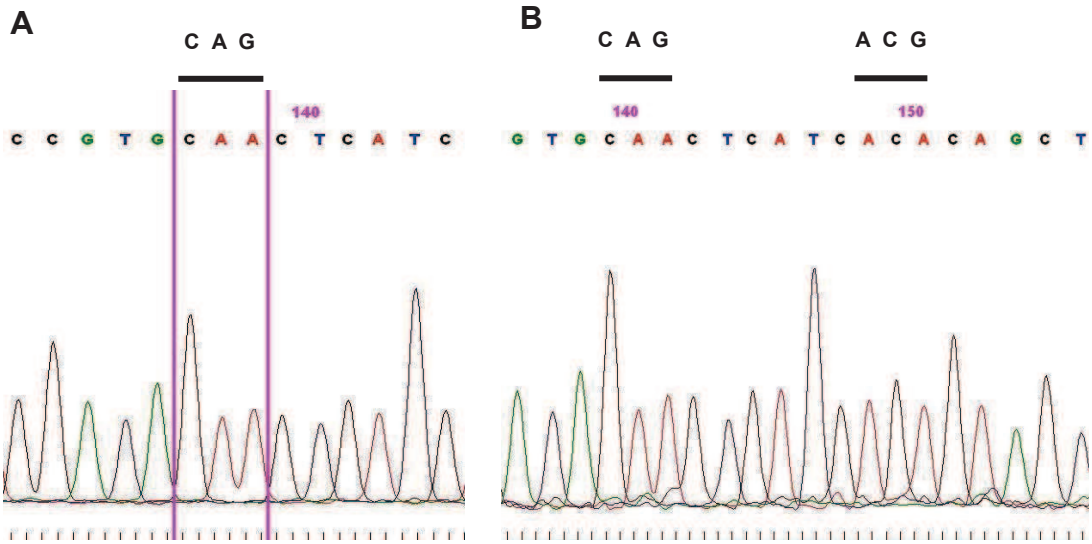

Figure 21 – *EGFR* single nucleotide polymorphisms: (A) UES with EGFR SNP: Exon 20 at codon 787 (CAG>CAA). **(B)** ESS with EGFR double SNP: Exon 20 at codon 787 (CAG>CAA) and codon 790 (ACG>ACA) (above the electropherogram the original codons)

4.4 *EGFR* AMPLIFICATION BY FLUORESCENCE IN SITU HYBRIDIZATION

Mitsuhashi et al. (2007) described *EGFR* amplification in a UES case which presented a temporary response to imatinib, and based on this finding we decided to evaluate *EGFR* gene amplification in our EST series. FISH was performed in the two previously constructed TMAs which encompass all cases included in the study, and in 64 cases the evaluation of gene amplification status was possible.

No amplification of *EGFR* gene was observed in any of the cases studied, which is consistent with the results of another previous series of 10 EST (Capobianco et al., 2012). Most cases (58/64, 90.6%) displayed a diploid status (1-2 copies) and a few cases (6/64, 9.4%) were considered polysomic (3-4 copies) (table 10 and figure 22). No relationship was observed between protein expression and gene status once all cases that presented EGFR overexpression were diploid for *EGFR*.

Table 10 – Amplification status of *EGFR* in Endometrial stromal tumors

EST subtypes	Diploid	Polysomic
ESS	92.2%	7.8%
	(47/51)	(4/51)
UES	84.6%	15.4%
	(11/13)	(2/13)

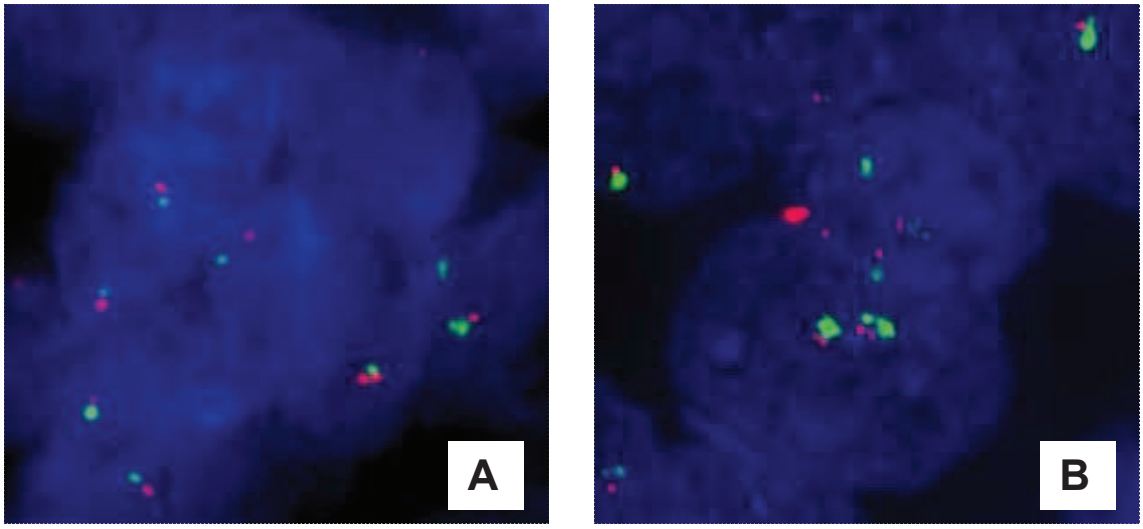

Figure 22 – FISH analysis of *EGFR* gene in Endometrial stromal tumors: (A) Representative image from *EGFR* gene diploidy, in each nucleus the ratio of red to green signal is equal to 1 (magnification, 1000x). (B) Representative image showing *EGFR* polysomy, in each nucleus the number of signals red and green is balanced (3-4 copies) (magnification, 1000x)

5. DISCUSSION

Low-grade endometrial stromal sarcoma (ESS) and undifferentiated endometrial sarcoma (UES) are endometrial neoplasms with distinct overall survival (OS) rates and metastasis pattern, which support the need for an appropriate classification (Bartosch et al., 2010). Adjuvant treatment differs between both entities (NCCN, 2013), and it is not well established mainly due the rarity of disease (only 15 % of uterine sarcomas), lack of randomized trials, and the limited number of cases reported. In the same manner, systemic treatment is a matter of debate due to the high relapse rate observed in these tumors (Schick et al., 2012).

Strategies for systemic treatment of ESS include ET with progestins due to the consistent expression of hormonal receptors in these tumors, and the light side-effects (Amant et al., 2009; Ioffe et al., 2009), whereas it has a limited role in UES. A few attempts with aromatase inhibitors, Gn-Rh agonists and PR modulators were also reported (Ioffe et al., 2009; Dahhan et al., 2009; Shoji et al., 2011), but similarly to progestin therapies, larger prospective trials are needed. On the other hand, chemotherapy showed a limited response rate in metastatic or recurrent ESS patients (Sutton et al., 1996; Leyvraz et al., 2006), and in a recent retrospective study including only 21 patients with UES a response rate of 19% in 2nd line or additional chemotherapy for progressive disease was reported (Tanner et al., 2012b). Treatment with radiotherapy seems to have a significant effect on local-regional control rate with a low impact on survival improvement (Ferrer et al., 1999; Weitmann et al., 2001; Valduvieco et al., 2010; Schick et al., 2012).

The possible efficacy of TKI treatment on these tumors, as imatinib, was suggested by several authors, based on i) expression of several target-TKR like PDGFRA/B, KIT and EGFR and ii) observation of clinical response to imatinib in recurrent EST patients whose express at least one TKR (Salvatierra et al., 2006; Mitsuhashi et al., 2007; Trojan et al., 2009; Kalender et al., 2009).

In fact, the possible clinical efficacy of imatinib in the treatment of EST and the contribution of its TKR-target in disease progression are the key questions to solve (Cheng et al., 2011). Until now, available data just refer a few case reports and small series (Appendix 1), which makes it difficult to draw conclusions. Based on this, we performed an extensive evaluation of molecular and immunohistochemical expression of TKR KIT, PDGFRA and EGFR in a large series of ESS and UES.

5.1 EXPRESSION AND MUTATIONAL STATUS OF KIT AND PDGFRA

In the literature KIT expression in EST has been reported to be quite variable (0-100%), and recently in two larger series, discordant results have also been reported: Cossu-Rocca et al. (2012) reported the absence of KIT expression in ESS cases (0/23) and positivity in 20% of UES cases (1/5), whereas Park et al. (2013) described expression in 82.1% (32/39) of ESS cases. In our series, KIT expression was always weak and focal and observed in only 3% (2/65) of total cohort, which is more in accordance with findings reported by Cossu-Rocca et al. (2012). We used the same antibody (polyclonal, DAKO) that Cossu-Rocca et al. (2012) and Park et al. (2013) used in their studies but at different dilutions and distinct methods of antigen retrieval. The highest KIT expression was observed in Park's series which used a higher antibody titration (1:400) and autoclave as antigen retrieval method, whereas in our series we used the lowest antibody titration (1:100) and performed the antigen retrieval in the slide heater of Ventana immunostainer.

Several factors can influence the results of a given IHC experiment, which include pre-analytical variables (fixation, tissue processing), analytical factors and post-analytical factors (Hewitt et al., 2012). In our case, we used TMA sections. Therefore section thickness, slide age, the type and concentration of all reagents, the temperature of incubation or during slide storage and all incubation times were absolutely identical. Thus, the discrepancies between the results of our and these studies may be due to other technical reasons than antibody election including sensitivity of immunostaining protocol (extension time of epitope retrieval, dilution and incubation time, sensitivity of detection system), population/sample selection and use of long-term storage paraffin blocks as suggested in previous studies (Hsi, 2001; Went et al., 2004; Nakayama et al., 2006).

Furthermore it is well established that IHC results depend not only on the technical methodology or quality of antibodies and reagents, but also on the reliability of the automated devices and the application of a rigorous scoring method (Miettinen, 2002; Eisenthal et al., 2008). In fact, KIT IHC and subsequent expression in mesenchymal tumors needs more studies, since questions related to sensitivity and specificity and also about standardization remains to be answered (Miettinen, 2002). However, there is no strict scientific basis for using this drug on the basis of KIT positivity alone, since immunohistochemical analysis cannot identify the tumor as driven by KIT signalling (Miettinen, 2002).

On the other hand, cytoplasmic PDGFRA expression was observed in 34.6% and 38.5% of ESS and UES, respectively. Expression in ESS, although mostly weak and/or focal, lies within the range reported in previous studies (33-100%, mean 64%), whereas expression in UES was less than the observed by Cossu-Rocca et al. (2012) and always with a weak

and/or focal pattern. Evaluation of PDGFRA expression is not routinely used (Raymond et al., 2008; Heinrich et al., 2008b), and since PDGFRA expression in UES was only reported in Cossu-Rocca's series and in our study, it is difficult to draw conclusions about PDGFRA expression in UES.

Physiologic activation of KIT and PDGFRA is, in general, due to autocrine and paracrine growth loops and genetic alterations such as gene amplification and activating mutations which can lead to constitutive activation of KIT and PDGFRA. However, as observed in previous studies, mutational analysis of *hotspots* of *KIT* and *PDGFRA* genes in our series did not reveal any somatic mutation. Presence of several SNP without clinical significance was detected, mainly in exon 12 and 18 of *PDGFRA* gene and was also observed in Cossu-Rocca's series. Nevertheless, the presence of a SNP at exon 17 (I798I), and a double SNP at exon 18 in EST cases were described for the first time.

In GIST, KIT expression is correlated with the presence of activating mutations in *KIT* and *PDGFRA* genes, and is characteristic of these tumors (Burger et al., 2005). Kang et al. (2005) showed that the relative KIT expression ratio was fivefold higher in cases with *KIT* mutation, than that of GISTs lacking *KIT* mutation, and the mutational status of *KIT* and *PDGFRA* was directly related to the different expression levels of activated KIT and PDGFRA (Kang et al., 2005). These facts support our results because KIT and PDGFRA expression observed was mostly weak and/or focal and activating mutations on its related genes were not found.

Autocrine/paracrine stimulation of the receptor by its ligand was observed in several human cancers and is related with their tumorigenesis (Hermanson et al., 1992; Inoue et al., 1994), and probably, the expression of related ligands of KIT and PDGFRA which were not evaluated in this series, might be the reason for expression of both receptors in these tumors, although more studies are needed to confirm this in uterine sarcomas.

5.2 EGFR PROTEIN EXPRESSION AND *EGFR* GENE ANALYSIS

In our study, IHC study of EGFR expression reveals lower cytoplasmic expression levels in EST cases than the observed in previous series: 11.5% and 7.7% in ESS and UES, respectively (Appendix 1). In the literature, several antibodies were used to assess EGFR expression, although the EGFR pharmDX^{TM} Kit (Dako, Carpinteria, CA, USA) and the monoclonal antibody 31G7 (Invitrogen, Carlsbad, CA, USA) are the most commonly used ones (Tsao et al., 2005; Cappuzzo et al., 2005b), since they yielded comparable results (Bhargava et al., 2006; Hirsch et al., 2008; Mathieu et al., 2010; Lee et al., 2010).

EGFR pharmDX™ Kit appears to better predict the survival outcome with gefitinib (Hirsch et al., 2008) and in both Moinfar and Cossu-Rocca series was the method used to detect EGFR expression, which explains the results concordance.

In our case, another EGFR clone (2.1E1, Gennova Scientific, Seville, Spain) was used and this fact may represent a limitation in our study, since different antibodies have different affinities and might differentially recognize wild-type EGFR and variants such as EGFRvIII and/or be affected by post-translational modifications of the EGFR such as glycosylation; thus, it is possible that different epitopes have different levels of sensitivity to degradation over time (Eberhard et al., 2008).

The lack of standardization in staining procedures and guidelines for interpretation of the EGFR assessment is pointed as a major reason for the conflicting results across studies (Cappuzzo et al., 2005b; Kersting et al., 2006; Eberhard et al., 2008; Mathieu et al., 2010). In fact, IHC relies on subjective judgement, which represents an intrinsic limitation to the technique (Suzuki et al., 2005) and a universal scoring system is still needed to better compare research results (Scagliotti et al., 2004). IHC is not a reliable approach for this determination because there are no established patient selection criteria for TKI therapy based on IHC (Ciardiello and Tortora, 2008; John, et al. 2009) and the association between EGFR protein expression and response to EGFR TKI presented inconsistent results (Hirsch et al., 2009).

Correlation between an increased *EGFR* copy number and gefitinib was proposed in NSCLC (Cappuzzo et al., 2005b) and based on that we decided to evaluate *EGFR* amplification status in our series by FISH. Our evaluation showed an *EGFR*/Chr7 ratio below 2, which accordingly with the Colorado Group EGFR scoring system (Varella-Garcia, 2006) it is indicative of non-amplification. These results are in accordance with described by Capobianco et al. (2012) which showed absence of amplification in their series of 10 ESS cases. A few cases showed polysomy, and unlike NSCLC EGFR overexpression, it had no influence on the gene status: all EST cases presenting EGFR overexpression displayed a diploid status.

In fact, in the literature only one UES case expressing EGFR presented a low-level amplification (mean ratio 2.9), according to the authors evaluation criteria (Mitsuhashi et al., 2007). Although no consensus has been reached on how to assess the presence and extent of *EGFR* status dysregulation in solid tumors by FISH analysis (Martin et al., 2009), it seems likely that gene amplification found in NSCLC and GBM is an uncommon event in EST.

Discovery of somatic mutations in the TK domain (exon 18 to 21) of *EGFR* (Lynch et al., 2004; Paez et al., 2004) was the cornerstone for selection of patients with NSCLC candidates to treatment with the EGFR TKI gefitinib and erlotinib (Pirker et al., 2010). Based on that, we evaluated the *EGFR* mutational status in our EST cases that expressed EGFR, since in some cases a correlation between EGFR protein expression and *EGFR* mutational status had been reported (Hirsch et al., 2006). Although mutational analyses were carried out only in cases that expressed EGFR, we did not find any *EGFR* somatic mutations. This is in accordance with results of Cossu-Rocca et al. (2012), which failed to find any genetic alteration in their series of 28 EST. We only detected the presence of SNP Q787Q at exon 20 as previously described (Cossu-Rocca et al., 2012b), and all these data confirm that EGFR activation does not play a role in EST tumorigenesis as observed in NSCLC.

5.3 IMATINIB AS A THERAPEUTIC TREATMENT FOR EST

Consistent expression of KIT and PDGFR receptors and their ligands was seen in a phase II trial in women with ovarian cancer, along with the absence of mutations in these genes and lack of response to imatinib (Schilder et al., 2008). In other two phase II clinical trials in SCLC (Krug et al., 2005) and carcinosarcomas (Huh et al., 2010), the high expression of KIT did not correlate with response to imatinib.

In our series, absence or minimal overexpression of KIT supports the hypothesis that overexpression of these TKR does not confer sensitivity to imatinib, which was previously suggested for other uterine sarcomas (Serrano et al., 2005). However, the expression of at least one of the imatinib targets (KIT, PDGFRA and PDGFRB) without evidence of activating mutations was reported in EST patients who responded to imatinib treatment (Salvatierra et al., 2006; Trojan et al., 2009; Kalender et al., 2009).

One possible explanation for these results may be due to the autocrine/paracrine signalling of PDGFRB. Dermatofibrosarcoma protuberans (DFSP) an infiltrating skin tumor with a high propensity for local recurrence, is characterized by *t*(17;22)(q22;q13) that results in a fusion between the *PDGFB* and the *collagen type 1A1* (*COL1A1*) genes (Simon et al., 1997). This cytogenetic abnormality is essential for the disease pathogenesis (Greco et al., 1998) and responsible for the constitutive activation of PDGFRB via autocrine and paracrine growth loops (Simon et al., 1997; Greco et al., 1998; Shimizu et al., 1999). Identification of this translocation as a key factor in PDGFRB activation, leads to postulate a possible inhibitory effect of imatinib in this tumor.

In vitro and *in vivo* studies showed that imatinib reduces PDGFRB phosphorylation and inhibits the growth rate of *COL1A1/PDGFB* expressing cells (Shimizu et al., 1999; Sjoblom et al., 2001) through apoptosis induction (Sjoblom et al., 2001). Further studies showed that imatinib treatment induced a reversion of the transformed phenotype in all of the DFSP-transformed cell lines, and the growth inhibition of mice tumors induced by DFSP-transformed cells (Greco et al., 2001). Both effects were reversible: two months after removal of treatment cells in culture regained their transformed morphology and mice xenograft tumor growth rates increased, suggesting an alternative pathway of inhibitory effect of imatinib in these tumors (Greco et al., 2001). Greco et al. (2001) suggested that since imatinib targets *COL1A1/PDGFB* genes it can represent an anti-tumor agent selective for this tumor, it might relevantly contribute to DFSP management, as well as recurrence rate reduction, when administered post-operatively (Greco et al., 2001).

In fact, several case reports suggested the efficacy of imatinib in metastatic and locally advanced DFSP (Rubin et al., 2002; Maki et al., 2002; Mizutani et al., 2004; Labropoulos et al., 2005). In the Imatinib Target Exploration Consortium Study B2225 that include 8 patients with locally advanced DFSP and 2 patients with metastatic disease, clinical responses were observed in 90% of patients: 4 clinical responses and 5 partial responses, although two of these patients subsequently experienced disease progression. All the tumors displayed the *t*(17;22)(q22;q13), whereas the only patient with metastatic disease whose DFSP lacked the *t*(17;22), had no clinical response to imatinib (McArthur et al., 2005). These data have led to approval of imatinib by the FDA for treating advanced (inoperable and/or metastatic) DFSPs (McArthur, 2007).

Further, in an open-label phase II study evaluation of imatinib activity in treating advanced, life-threatening malignancies expressing one or more imatinib-sensitive tyrosine kinases, complete and partial response were observed in 33.3% and 50% of DFSP cases, respectively (Heinrich et al., 2008b). These results were later confirmed in two single-agent, single-arm, open-label, multicenter phase II clinical trials that explored the efficacy of imatinib therapy in patients with locally advanced/metastatic DFSP: the European Organization for Research and Treatment of Cancer no. 62027 and the Southwest Oncology Group no. S0345. Although there were some differences in both trial designs, the results were similar: response rate of 46%, a median time to progression of 1.7 years and an overall therapeutical clinical benefit exceeding 70%, with good tolerability. Progressive disease was observed in one patient that did not harbour the *t*(17;22)(q22;q13), which pointed to the need of testing for the presence of this specific translocation before therapy with imatinib (Rutkowski et al., 2010).

The role of imatinib as neoadjuvant treatment was also suggested previously (Han et al., 2009) since several patients with unresectable DFSP were able to undergo resection after imatinib therapy (Rutkowski et al., 2010). These findings were confirmed in a phase II multicentric study where imatinib was evaluated in the neoadjuvant setting (Kerob et al., 2010). Recently, striking results in advanced DFSP were described in a series of 15 patients treated with imatinib outside clinical trials (Rutkowski et al., 2011).

Indeed, in described EST cases tumor shrinkage was the main indicator of objective response to imatinib, accompanied by stable disease in two cases (Salvatierra et al., 2006; Mitsuhashi et al., 2007; Trojan et al., 2009). Interestingly, strong expression of PDGFRB was described in one case where remission and no progressive disease was reported after imatinib therapy (Trojan et al., 2009).

In the literature, PDGFRB expression in EST is controversial, Liegl et al. (2007) did not describe expression of PDGFRB in any of the 37 ESS cases, whereas Caudell et al (2005) reported expression in 8/8 ESS cases. Recently, in a series of EST where expression of several TKR in EST was evaluated, expression of this TKR was seen in 35% and 40% of ESS and UES cases, respectively (Cossu-Rocca et al., 2012b), and one ESS that presented PDGFRB protein overexpression at the immunohistochemical level also showed overexpression of *PDGFRB* by real time-PCR. Analysis of exons 12 and 18 of PDGFRB did not identify mutations in any of the investigated exons (Cossu-Rocca et al., 2012b), as observed in an previous study (Liegl et al., 2007).

Expression of PDGFB, natural ligand of PDGFRB, was observed in 22% of ESS cases (Liegl et al., 2006), and despite the initial negative results of Liegl et al. (2007), EST seems to present a consistent expression of PDGFRB (Cossu-Rocca et al., 2012b). Since no mutations were described in this gene, expression of PDGFRB may be due to the constitutive activation of PDGFRB through autocrine growth loops which could explain the efficacy of imatinib in these tumors. In fact, evaluation of level of PDGFRB activation on DFSP suggested that autocrine/paracrine mechanism can be associated with substantially lower levels of TKR activation than the observed when TKR are activated through mutation, and that the inhibitory effect of imatinib is not correlated with high levels of PDGFRB activation or overexpression (McArthur et al., 2005). Nonetheless, expression of PDGFB was only described in ESS cases (Liegl et al., 2006), enabling any conclusions drawing.

Conversely, in several human cancers β-catenin dysregulation has been explored as a target of imatinib. B-catenin is a multifunctional protein and has at least two distinct but interdependent functions: I) as an essential signal transducer of the Wnt/Wingless pathway

responsible for cell proliferation and differentiation (Miller, 2002) and II) as an intracellular stabilizer, allowing cadherins to form the adherent junctions (Hinck et al., 1994).

In the former, its activity is quite regulated by multiprotein complex that includes APC and glycogen synthase kinase-3b (GSK3B). Activation of the Wnt, inactivating mutations of the *APC* tumor suppressor gene or oncogenic mutations of the *CTNNB1* (β-catenin) gene, allows β-catenin levels to rise in the cytoplasm, and also to translocate to the nucleus. There, it activates the TCF/LEF transcription factors, which activate the expression of downstream targets, such as c-Myc and cyclin D1 (Polakis, 1999). On the other hand, tyrosine phosphorylation of β-catenin protein induced by PTK would result in its decreased association with e-cadherin, leading to the junctional assembly disruption and an increased propensity for cancer cells to become invasive and metastasize (Zhou et al., 2003).

B-catenin is involved in tumorigenesis of several cancers. Its role as an oncogene was shown in transgenic animals (Harada et al., 1999) and, nowadays, a significant nuclear accumulation of β-catenin is considered a feature of deregulated β-catenin activity (Zhou et al., 2003). While mutations of *CTNNB1* or *APC* genes are normally found in colorectal cancer, in other tumors the molecular mechanisms underlying β-catenin deregulation remain undefined (Zhou et al., 2003).

In human colon cancer cell lines harbouring an oncogenic mutation of the β-catenin gene and an inactivating mutation of the *APC* gene, the constitutive activity of β-catenin signalling is inhibited by imatinib in a dose-dependent manner. In human sarcomas cell lines with unknown β-catenin signalling deregulations, an inhibitory effect of imatinib at different concentrations was observed (Zhou et al., 2003). Growth inhibition mediated by imatinib was observed in different human cell lines, which suggested that β-catenin/TCF4 activity inhibition may be responsible for the imatinib-mediated growth suppressor in human tumor cells (Zhou et al., 2003; Rao et al., 2006).

On the other hand, several studies indicate that β-catenin can become a tyrosine-phosphorylated protein upon the activation of several PTK, which is consistent with the observation of β-catenin tyrosine phosphorylation induced by several growth factors such as EGFR (Hoschuetzky et al., 1994; Takahashi et al., 1997; Harada et al., 1999), hepatocyte growth factor (Shibamoto et al., 1994) and others. In *in vitro* studies with anaplastic thyroid cancer (ATC) cell lines coexpressing ABL, a known imatinib target, and β-catenin, results in sensitivity to imatinib, whereas cells expressing β-catenin but ABL negative showed no sensitivity to imatinib (Rao et al., 2006).

Immunofluorescence and immunoblotting studies supported an imatinib-dependent relocalization of β-catenin and E-cadherin from the nucleus to the cell membrane. This shift

was associated with a relative decrease in nuclear β-catenin staining, suggesting that imatinib promotes β-catenin/e-cadherin binding at adherent junctions leading to a reduction in cytosolic/nuclear accumulation of free β-catenin (Rao et al., 2006). Other cell lines expressing PDGFR (A and B) showed an intermediate sensitivity to imatinib, and the cell lines that did not express ABL had a low sensitivity to the drug, suggesting that only this subset of ATC cell lines expressing classical imatinib targets had enough sensitivity. In fact, Rao et al. (2006) suggest that imatinib may be effective in a subset of ATC patients expressing imatinib-sensitive targets and have antitumor activity acting through the β-catenin signalling pathway.

Nuclear β-catenin expression is well described in EST cases mainly in ESS and UES-U cases (Hrzenjak et al., 2004; Ng et al., 2005; Kurihara et al., 2008; Jung et al., 2008; Kildal et al., 2009b; Kurihara et al., 2010). However, despite the β-catenin expression, EST lacks of mutation in *CTNBB1* and *APC* genes and the mechanism of nuclear β-catenin accumulation remains to be explained (Kurihara et al., 2010). One explanation can be the activation of β-catenin through Wnt signalling pathway in EST, since they are characterized by the lower expression of putative suppressors of Wnt: secreted frizzled-related proteins SFRP1 and SFRP4 (Hrzenjak et al., 2004). B-catenin and cyclin D1 coexpression in UES-U supports this evidence raising the possibility that cyclin D1 may be upregulated through an activated Wnt/β-catenin, and may contributes to tumorigenesis of these tumors (Kurihara et al., 2010).

Other explanation for this phenomenon may be the tyrosine phosphorylation and subsequent nuclear expression in EST due to induced activation by PTK as ABL, which is also expressed in these tumors (Cheng et al., 2011; Cossu-Rocca et al., 2012a) or other PTK, which can lead to an inhibitory effect of imatinib observed in EST cases as observed in ATC cell lines. In fact, tyrosine phosphorylation of β-catenin through a PTK seems to be a reasonable cause for β-catenin nuclear accumulation and probably the role of β-catenin is not only limited to differential diagnosis (Jung et al., 2008), but it could also be a basis for a new therapeutic option in these tumors.

6. CONCLUSIONS

EST are rare uterine sarcomas characterized by a well-defined recurrence pattern and although several attempts were made to manage mainly in the recurrent setting, it is not established an appropriate adjuvant and systemic treatment for these tumors. Expression of molecular targets of TKI was described by several authors and descriptions of objective clinical responses to imatinib in some EST patients lead to postulate that target therapy can be an alternative treatment for these patients. Based on this, we performed an extensive molecular and expression analysis of KIT, PDGFRA and EGFR in the largest series of EST studied so far (Appendix 1).

Our study showed absence of significant expression, amplification and activating mutations on these TKR and related genes as observed previously by other authors, suggesting that it is unlikely that EST can benefit from therapies such as imatinib and EGFR TKI's in advanced disease and the molecular mechanism behind the few clinical responses to imatinib observed in EST patients may not be the same observed in other human malignancies as GISTs.

Imatinib efficacy on treatment of solid tumors is not only dependent of activating mutations on KIT and PDGFRA genes, but also of other molecular alterations that lead to constitutive activation of other imatinib-targets: in BCR-ABL-induced tumors, imatinib completely eradicated the tumors whereas in other tumors it represents a contribution to the clinical management by slowing down tumor growth and reducing rate of recurrence (e.g. DFSP) or by prolonging the overall survival (e.g. GIST).

In fact, Imatinib can interfere with different tyrosine kinases in different cellular contexts, with various *in vivo* and *in vitro* outcomes, and although imatinib does not provide eradication of tumors with specific imatinib-targets it may represent an anti-tumor agent selective for these tumors.

Thus, in the future, identification of specific post-translational abnormalities as autocrine or paracrine stimulation loops responsible for TKR activation on EST tumors are needed (e.g. PDGFRB activation), as well the mechanism behind nuclear β-catenin accumulation, in order to explain imatinib action in EST.

In the same way, imatinib efficacy in unresectable EST should be explored: centralized and intergroup studies are warranted to define the role of this targeted drug in the clinical management of EST.

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APPENDICES

APPENDIX 1: Tyrosine kinase receptor expression in Endometrial Stromal Tumors

Author	KIT (%)		PDGFRA (%)		EGFR (%)	
	ESS	UES	ESS	UES	ESS	UES
Oliva et al. (2002)	0/8 (0)	-	-	-	-	-
Hornick and Fletcher (2002)	0/10 (0)	-	-	-	-	-
Winter et al. (2003)	0/1 (0)	-	-	-	-	-
Wang et al. (2006)	3/11 (27)	2/3 (67)	-	-	-	-
Rushing et al. (2003)	2/2 (100)	-	-	-	-	-
Klein and Kurman (2003)	1/10 (10)	0/2 (0)	-	-	-	-
Leath et al. (2004)	3/3 (100)	-	-	-	-	-
Caudell et al. (2005)	0/8 (0)	1/4 (25)	-	-	-	-
Moinfar et al. (2005)	-	-	-	-	14/20 (70)	3/3 (100)
Salvatierra et al. (2006)	-	1/1 (100)	-	-	-	-
Nakayama et al. (2006)	0/5 (0)	-	-	-	-	-
Liegl et al. (2007)	0/37 (0)	-	22/37 (59)	-	-	-
Adams et al. (2007)	0/8 (0)	-	8/8 (100)	-	-	-
Mitsuhashi et al. (2007)	-	0/1 (0)	-	-	-	1/1(100)
Zafrakas, et al. (2008)	2/2 (100)	1/2 (50)	-	-	-	-
Martin et al. (2009)	1/1 (100)	-	-	-	-	-
Trojan, et al. (2009)	0/1 (0)	-	1/1 (100)	-	-	-
Koivisto-Korander et al. (2011)	2/9 (22)	-		-	-	-
Cheng et al. (2011)	1/12 (8)	-	4/12 (33)	-	-	-
Cossu-Rocca et al. (2012)	0/23 (0)	1/5 (20)	15/23 (65)	4/5 (80)	10/23 (43)	4/5 (80)
Park et al. (2013)	32/39 (82.1)	-	28/39 (71.8)	-	0/39 (0)	-

APPENDIX 2: List of participating centres

Centre	ESS	UES	Total
Hospital Clinic i Provincial de Barcelona, Barcelona, Spain	2	2	4
Hospital Universitario Central de Asturias, Oviedo, Spain	3	5	8
Hospital Universitari Germans Trias i Pujol, Badalona, Spain	9	0	9
Complejo Hospitalario Universitario de Badajoz, Badajoz, Spain	5	0	5
Hospital Son Espases, Palma de Mallorca, Spain	1	1	2
Hospital Espírito Santo, E.P.E, Évora, Portugal	2	1	3
USP-Institut Universitari Dexeus, Barcelona, Spain	14	1	15
Hospital Universitario de la Laguna, Canarias, Spain	7	0	7
Hospital Universitari Bellvitge, Barcelona, Spain	9	3	12
Total	**52**	**13**	**65**

ANNEX 1:

Original publication: Sardinha, R. et al. (2013). Endometrial stromal tumors: immunohistochemical and molecular analysis of potential targets of tyrosine kinase inhibitors

CLINICAL SARCOMA RESEARCH

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Endometrial stromal tumors: immunohistochemical and molecular analysis of potential targets of tyrosine kinase inhibitors

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Abstract

Background: The systemic treatment of malignant endometrial stromal tumors (EST) is not well established. A few reports describe objective responses to imatinib, which suggest a novel therapeutic strategy for these tumors. Due to these facts, we aimed to perform a retrospective analysis of possible molecular targets of tyrosine kinase inhibitors (TKI) in EST: KIT, PDGFRA and EGFR.

Methods: 52 endometrial stromal sarcomas and 13 undifferentiated endometrial sarcomas were examined and reviewed. Mutational analysis were performed for exons 9, 11, 13, and 17 of the *KIT* gene, exons 12 and 18 of the *PDGFRA* gene and exons 18, 19, 20 and 21 of the *EGFR* gene. The incidence and distribution of the KIT, PDGFRA, and EGFR expression were examined by immunohistochemistry, and *EGFR* amplification was assessed by fluorescence *in situ* hybridization.

Results: No mutations in *KIT*, *PDGFRA* and *EGFR* genes were detected. Overexpression of KIT, PDGFRA, EGFR, was detected in 2 (3%), 23 (35.4%), 7 (10.8%) cases respectively, whereas amplification of *EGFR* gene was not found.

Conclusions: Absence of significant expression, amplification and activating mutations on these tyrosine kinase receptors suggest that it is unlikely that EST can benefit from therapies such as TKI on the systemic setting.

Keywords: Endometrial stromal tumors, Tyrosine kinase inhibitors, KIT, PDGFRA, EGFR, Systemic treatment

Background

Endometrial stromal sarcoma, low grade (ESS) and undifferentiated endometrial sarcoma (UES) belong to the rare group of endometrial stromal tumors (EST), which represents 15% of uterine sarcomas [1]. ESS presents a cellular background similar to the cells of normal endometrial stroma in proliferative phase. In contrast, UES lacks specific differentiation and bears no histological resemblance to endometrial stroma. Tumor cells are high-grade spindle to polygonal-shaped, with marked nuclear pleomorphism and high mitotic activity. Necrosis and vascular invasion are commonly seen [2]. While ESS is characterized by indolent course and late recurrences, with a 5-year overall survival (OS) up 70%, UES is usually diagnosed at advanced stages, and has a high rate of distant metastasis and a 5-year OS ranging from 25-55% [3-6]. FIGO stage [7] is the strongest prognostic factor for these malignancies [5,8]. CD10 is the most sensitive marker for ESS [9,10]. Estrogen and progesterone receptors [11] and aromatase [12] are usually expressed in ESS, and less commonly in UES [13-15]. The rearrangement *t*(7;17)(p15;q21), which results in *JAZF1/JJAZ1* gene fusion, is the cytogenetic hallmark of ESS [16], although other translocations have been reported [17-19]. In contrast, UES is characterized by a complex karyotype [20,21]. Recently, the *t*(10;17)(q22;p13) that results in *YWHAE-FAM22A/B* gene fusion with oncogenic properties was reported in a subset of UES [22], which is associated to the expression of Cyclin D1 [23,24] and β-catenin [23];

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this supports the recent sub-classification of UES [15,25]. Surgery is the standard treatment, and includes total hysterectomy and bilateral salpingo-oophorectomy. However, due the rarity of these tumors, distinct clinical behavior, and lack of randomized studies including both categories, an appropriate systemic treatment of these malignancies was not been yet established.

Molecular targets of tyrosine kinase inhibitors (TKI) such as imatinib mesylate (Glivec®, STI-571, Novartis, Switzerland), gefitinib (Iressa®, AstraZeneca, Macclesfield, UK) and erlotinib (Tarceva®, OSI-Pharmaceuticals, New York, NY), which includes PDGFRA/B, KIT, C-ABL and EGFR, were reported to be expressed in ESS and UES by immunohistochemistry [26-40], although without presence of any activating mutations [36-39,41,42]. Interestingly, a few reports described objective responses with imatinib in patients who express at least one TKI target [36,37,43]. Another report described a unique case of UES with EGFR expression and *EGFR* amplification which temporarily responded to imatinib [42]. Based on these findings, an extensive evaluation of the molecular targets of TKI on EST was carried out to identify a novel therapeutic strategy for these malignancies. In the present study we analyzed the gene status and protein expression of KIT, PDGFRA, and EGFR in a large series of ESS and UES to evaluate their distribution among the distinct subgroups and correlate the immunohistochemical expression with mutational status.

Material and methods

Patient selection and study design

A series of 75 EST was retrieved from Spanish centers associated to Spanish Sarcoma Group (GEIS) and from the Pathology Departments of Complejo Hospitalario Universitario de Badajoz, Badajoz, Spain and Hospital do Espírito Santo E.P.E, Évora, Portugal, and sent to Tumor Bank of the Cancer Research Centre – Salamanca, Spain. The selection of patients was made according to the following inclusion criteria - previous diagnosis of EST (any histological grade) and availability of histological material sufficient to perform the study. After receiving and encoding the samples, cases were reviewed and subclassified by 1 co-author (EA) based on the current WHO classification [2]. The study was approved by the Ethics Committee of Hospital Germans Trias i Pujol (Spain), and was conducted in accordance with the Declaration of Helsinki and Spanish regulative law for Tumor Banks.

Tissue microarray and immunohistochemistry

65 samples were considered valid and included in the study. Ten cases were discarded because of a sample too small to perform the proposed study or non-representative sample or diagnosis different than EST. Before tissue microarray (TMA) construction, representative areas of tumor were selected on hematoxylin and eosin (H&E) section and marked on the paraffin block. For each sample were obtained two cylinders of 1 mm diameter and placed in a recipient block using a tissue microarrayer (Manual Tissue Array; Beecher Instruments Inc. Sun Prairie, Wisconsin, USA). In total two TMAs were constructed according to previously described [44].

The tyrosine kinase receptors (TKR) evaluated were KIT, PDGFRA and EGFR, and to confirm diagnosis the expression of two markers most commonly used on ESS [45,46], CD10 and Calponin were assessed in each tumor. Immunohistochemistry (IHC) was performed in 3 μm sections. KIT, PDGFRA, CD10 and Calponin immunostaining was performed using a Discovery® Ventana automated immunostainer (Ventana Medical Systems, Tucson, Arizona, USA). Heat-induced antigen retrieval was done with Tris-EDTA buffer (pH 8.0) for KIT, PDGFRA and Calponin and with citrate buffer (pH 6.0) for CD10. Sections were incubated with the primary antibodies Calponin (clone CALP; Dako, Carpinteria, CA, USA), KIT (polyclonal; Dako, Carpinteria, CA, USA), PDGFRA (polyclonal; Thermo Fisher Scientific, Fremont, CA, USA) and CD10 (clone 56C6; Novocastra Laboratoires, Newcastle upon Tyne, UK) at 1:100 dilution. Universal secondary biotinylated antibody (Discovery™ Universal Secondary Antibody, Ventana Medical Systems, Tucson, Arizona, USA) was used and developed for detection using the DAB MAP system (Ventana Medical Systems, Tucson, Arizona, USA). For EGFR analysis expression, a *ready-to-use* monoclonal antibody (clone 2.1E1; Gennova Scientific, Seville, Spain) was used. Briefly, paraffin-embedded sections were deparaffinized in xylene and rehydrated in downgraded alcohols and distilled water. Antigen retrieval was performed with Proteinase K solution (Dako, Carpinteria, CA, USA). The primary antibody was detected using a secondary antibody- horseradish peroxidase polymer conjugate (Dako REAL™ EnVision™ Detection System; Dako, Carpinteria, CA, USA), and all incubations were done with the Dako Autostainer Plus system (Dako, Carpinteria, CA, USA). All sections were counterstained with hematoxylin, upgraded alcohols, and xylene, mounted, and analyzed by standard light microscopy.

Immunohistochemical evaluation

The expression of CD10 and Calponin was considered positive when over 1% of the tumor cells showed cytoplasmic expression. The TKR expression was detected in the cytoplasm of tumor cells, and each case was interpreted for immunoreactivity using a 0 to 3 semi-quantitative scoring system for both the intensity of stain and the percentage of positive cells, as previously reported [47]. The multiplicative index of intensity and labeling was considered for statistic analysis and the

expression was defined as weak and/or focal for a multiplicative index 1–3, moderate local or diffuse for a multiplicative index 4–6 and intense and diffuse if the multiplicative index was >6. The IHC analysis was scored by the same co-author who performed the histologic review (EA).

Molecular analysis of tyrosine kinase receptors

DNA extraction

Ten serial 5 μm sections were cut and transferred into 15 ml conical centrifugation tubes and deparaffinized in xylene (two times for 5 minutes) and absolute ethanol (two times for 5 minutes) at 4000 rpm, followed by drying of the samples at room temperature. Subsequently, DNA extractions were performed according to QIAamp DNA Mini Kit protocol (Qiagen, Hilden, Germany), and then quantified by spectrophotometry (SmartSpec Plus Spectrophotometer, Bio-Rad Laboratories Inc., CA, USA).

KIT and PDGFRA

According to previous studies [48-50], exons 9, 11, 13, and 17 of *KIT* gene and exons 12 and 18 of *PDGFRA* gene were amplified in order to identify possible mutations. Amplification of *GAPDH* gene was performed to confirm integrity and quality of extracted DNA. PCR amplification of *KIT* and *PDGFRA* was performed using 1–5 μl of genomic DNA, 1X PCR buffer + 2 mM MgCl2 (Roche Applied Science, Mannheim, Germany), 0.15 mM of each primer, 200 μM of each dNTP (GeneAmp® dNTP Blend, 10 mM, ABI, Carlsbad, CA, USA), and 1.5 U of Taq DNA polymerase (Roche Applied Science, Mannheim, Germany) in a total volume of 25 μl. The PCR conditions were 94°C for 10 min, 40 cycles of 1 min at 94°C, 1min30sec to 56°C (*KIT*) or 65°C (*PDGFRA*) and 1 min at 72°C, followed by a cycle of 10 min at 72°C. The amplified products were visualized on agarose gel 2%.

Prior to sequence analysis, *KIT* and *PDGFRA* PCR products were first purified using a QIAquick® PCR Purification Technology Kit (Qiagen, Hilden, Germany). Direct sequencing of PCR products was performed using ABI PRISM® BigDye Terminator Cycle Sequencing Kit in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer instructions. Sequencing primers were the same as those used for PCR, and both strands (forward and reverse) were sequenced.

All sequences obtained were visualized and analyzed in the program Sequence Scanner Software v1.0 (Applied Biosystems, Carlsbad, CA, USA), based on the reference sequences: *KIT* - ENSG00000157404 and *PDGFRA* - ENSG00000134853.

EGFR

PCR amplification of exons 18, 19, 20 and 21 of the *EGFR* gene, which encompass most of the *EGFR* mutations [51,52], was carried out using previously described primers [53,54] (Table 1). Using 4 μl of DNA, PCR was performed in a reaction volume of 15 μl containing 1X PCR buffer (Applied Biosystems, Carlsbad, CA, USA), 2.5 mM MgCl2 (Applied Biosystems, Carlsbad, CA, USA), 0.17 mM of each primer, 200 mM of each dNTP (GeneAmp® dNTP Blend, 10 mM, Applied Biosystems, Carlsbad, CA, USA) and 2 U of Taq DNA polymerase (AmpliTaq Gold® DNA Polymerase, Applied Biosystems, Carlsbad, CA, USA).

The conditions for PCR were 95°C for 12 min, 40 - cycles of 30 sec at 95°C, 45 sec at 65°C and 1 min at 72°C, followed by a cycle of 10 min at 72°C. After visualization of amplified products by gel electrophoresis on a 2% agarose, these were purified with USB® ExoSAP-IT® (Affymetrix, Inc., Cleveland, Ohio, USA). Subsequently the purified products were precipitated and labeled with GenomeLab™ DTCS Quick Start Kit (Beckman Coulter Inc, Fullerton, CA, USA) according to the manufacturer's instructions and were sequenced using GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter Inc, Fullerton, CA, USA). As described above, sequencing was performed in both directions, and the same PCR primers were used.

The sequences were analyzed using Genome Lab Genetic Analysis System v10.0.30 (Beckman Coulter Inc, Fullerton, CA, USA), and compared with the reference sequence ENSG00000146648.

Fluorescence in situ hybridization - EGFR

The ploidy status of *EGFR* in each tumor was assessed by fluorescence *in situ* hybridization (FISH) on previously constructed TMAs. Gene amplification was determined by using an *EGFR*/CEN-7 FISH Probe Mix (Y5500, Dako, Carpinteria, CA, USA) containing Texas Red-labeled DNA probe covering the full *EGFR* region and a mixture of fluorescein-labeled PNA probes targeted at the centromeric region of chromosome 7 (Chr7).

Table 1 EGFR oligonucleotide sequences for PCR analysis

Exon	Specific primers	PCR product
Exon 18	F : 5′- GCT GAG GTG ACC CTT GTC TC -3′	225 bp
	R: 5′- CTC CCC ACC AGA CCA TGA -3′	
Exon 19	F: 5′- CAT GTG GCA CCA TCT CAC A -3′	230 bp
	R: 5′- CAG CTG CCA GAC ATG AGA A -3′	
Exon 20	F: 5′- CAT TCA TGC GTC TTC ACC TG -3′	377 bp
	R: 5′- CAT ATC CCC ATG GCA AAC TC -3′	
Exon 21	F: 5′- GCT CAG AGC CTG GCA TGA A -3′	348 bp
	R: 5′- CAT CCT CCC CTG CAT GTG T -3′	

F: *forward primer;* R: *reverse primer.*

The FISH was performed using the Histology FISH Accessory Kit (Dako, Carpinteria, CA, USA), according to the manufacturer's instructions.

Hybridization signals were visualized using fluorescence microscope equipped with a IAI monochrome progressive scan (IAI Company, Taiwan) and run by image analysis software Cytovision® (Leica Microsystems, Wetzlar, Germany). 20 tumour nuclei/case were scored, and the tumor cells in which the signals of *EGFR* and CEP7 were increased equally were classified as polysomy 7 and those for which there was a double signal for *EGFR* or CEP7 were considered diploid. To assess gene amplification, we calculated the ratio of *EGFR* to CEP7 and evaluated in accordance with the criteria of FISH scoring system of Colorado Group [55]. Evaluation of the ploidy status of *EGFR* was performed by a single pathologist (EA).

Statistical analysis

The association between the expression of TKR and ploidy of EGFR with different histological types of EST, as well the mutational status of TKR were assessed. All statistical analyses were performed based on contingency tables, with SPSS software v18.0 statistics (Chicago, Illinois).

Results

Classification of endometrial stromal tumors

From 65 EST cases, 80% (52/65) were diagnosed as ESS, and 20% (13/65) were UES. As expected, the majority of ESS presented expression of CD10, 51.9% (27/52), which was lower in UES cases (23.1%; 3/13).

Analysis of expression of tyrosine kinase receptors

The expression of KIT, PDGFRA and EGFR was evaluated in all cases of EST, and an example of the pattern of expression of these markers is shown in Figure 1.

KIT stromal expression was found in only 2 out of 65 cases, and it was weak and/or focal. In contrast, PDGFRA showed positive stromal expression in 35.4% of cases (23/65), mostly weak and/or focal; its distribution was similar in both histological subtypes. Interestingly, expression was not intense and/or diffuse in any case. For EGFR, a minority of cases were classified as positive, representing 10.8% (7/65).

Mutational status of tyrosine kinase receptors

The molecular study was performed in 62 cases. Sequencing of exons 9, 11, 13 and 17 of *KIT* only revealed the presence of a silent base substitution (ATC>ATT) in exon 17 at codon 798 (I798I) in two cases (ESS). The *PDGFRA* analysis showed the presence of several silent mutations, a base substitution (CCA>CCG) in exon 12 at codon 567 (P567P) in all cases, 32.3% (21/65) showed a silent base substitution (GTC>GTT) in exon 18 at codon 824 (V824V) and 1 case had a double silent base substitution at codons 824 and 838 (GTC>GTT and GGC>GGT; V824V and G838G respectively) both in exon 18. There were no somatic mutations in the genes *KIT* and *PDGFRA*.

EGFR mutational analysis was performed only in cases with expression of EGFR, and in which DNA was available for molecular studies. Two cases without EGFR expression were included as control. There were no mutations in *EGFR* exons 18, 19, 20 and 21. In 6 of 7 cases silent base substitution were identified at codon 787 (CAG>CAA; Q787Q), and in one of these cases were identified another silent base substitution at codon 790 (ACG>ACA; T790T) both in exon 20.

EGFR amplification by FISH

EGFR gene amplification analysis was performed in all cases included in the study (65 cases), except in one which was considered not evaluable. No amplification of *EGFR* gene was observed. Numerous cases (58/64, 90.6%) were diploid (1–2 copies), and few cases (6/64, 9.4%) were polyploid (3–4 copies), and the distribution was similar in both histological types.

Discussion

Systemic therapy in EST has a marginal efficacy. Low grade ESS can achieve control with hormonal treatments (progestins, aromatase inhibitors) and high grade undifferentiated uterine sarcomas are included in clinical trials together with leiomyosarcomas. Tanner *et al.* reported efficacy of docetaxel and gemcitabine or adriamycin for advanced cases of high grade endometrial tumors [56]. Indeed, the indication of systemic treatment of EST is controversial. Hormonal treatment can depend on hormonal receptor status and is associated to light side-effects and occasionally indicated. Responses were reported in a few ESS cases treated with progestin or aromatase inhibitors [57-64], but prospective larger trials are needed. Chemotherapy showed no apparent benefit in ESS [34,65-67]. In the case of UES some objective and partial responses were observed [68-70]. In a retrospective study including only 21 patients was described a response rate of 62% with gemcitabine/docetaxel and doxorubicin-based regimens in first-line, whereas in second- or additional chemotherapy for progressive disease, the response rate was around 19% [56]. On the other hand, radiotherapy seems to have a significant effect on local-regional control rate with a low impact on survival improvement [3,4,71-74]. Due to these facts several reports address the need to explore the role of TKR in these malignancies. The possible clinical efficacy of imatinib in the treatment of EST and the contribution of its TKR-

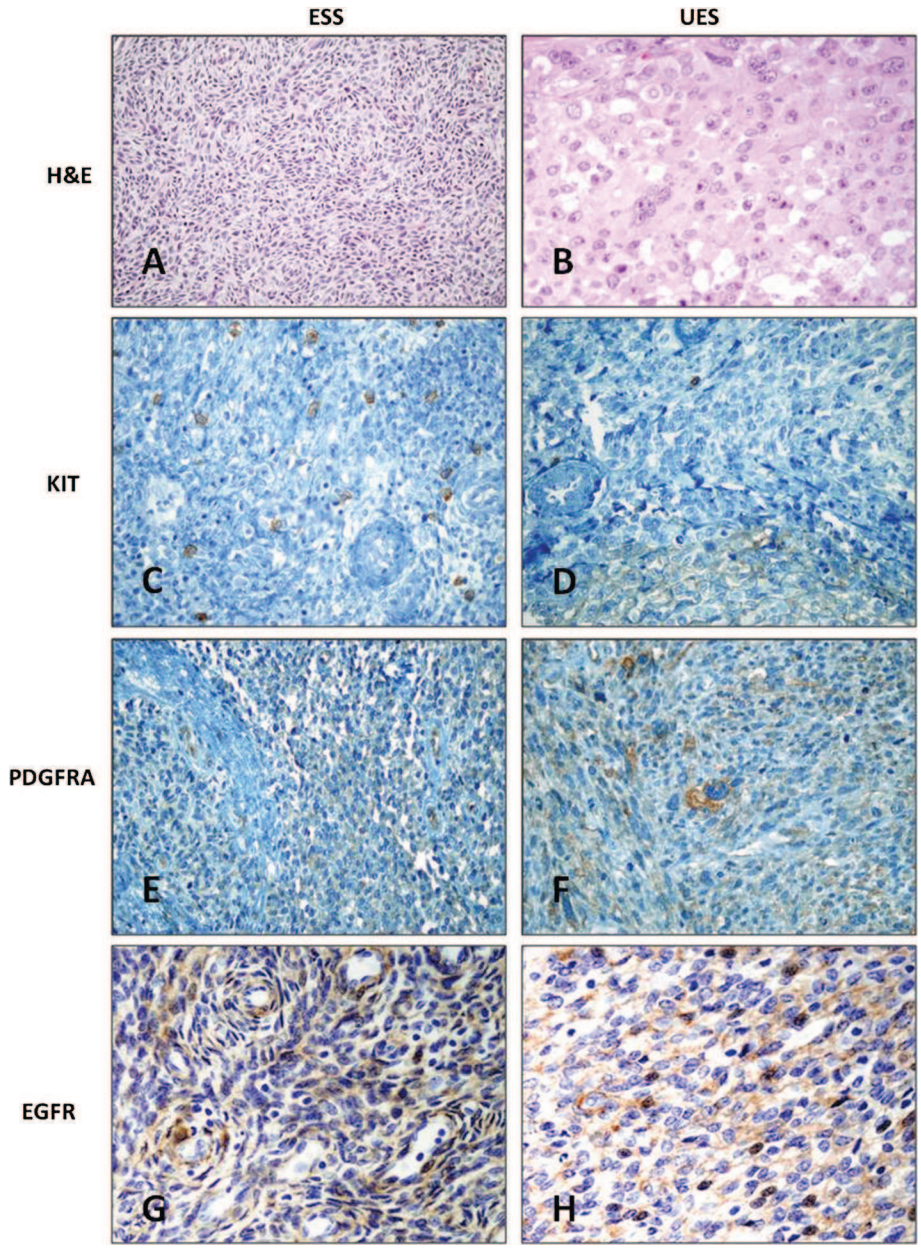

Figure 1 Immunohistochemical expression of tyrosine kinase receptors in endometrial stromal tumors. Endometrial stromal sarcoma, low grade (**A**) and undifferentiated endometrial sarcoma (**B**). Negative expression of KIT in ESS (**C**) and very focal expression in UES (**D**). Weak and/or focal PDGFRA and EGFR expression in ESS (**E, G**) and UES (**F,H**).

target in disease progression are the key questions to solve [34].

Previous reports showed the presence at least one TKR in EST, but available data just refer a few case reports and small series, which makes it difficult to draw conclusions. Based on this, we performed an extensive evaluation of molecular and immunohistochemical expression of TKR KIT, PDGFRA and EGFR in a large series of ESS and UES.

KIT and PDGFRA cytoplasmic expression was observed in 3% and 35.4% respectively. In the literature, KIT expression has been reported to be quite variable (0-100%) (Table 2) and this might be due to differences in the choice of antibody clones, tissue pretreatment, sensitivity of immunohistochemical procedures, sample selection and use of whole tissue sections or TMA sections, which was also suggested in previous studies [75,76]. On the other hand,

Table 2 TKR expression on endometrial stromal tumors

Author	KIT (%)		PDGFRA (%)		EGFR (%)	
	ESS	UES	ESS	UES	ESS	UES
Oliva, Young *et al.* [79]	0/8 (0)					
Hornick and Fletcher [80]	0/10 (0)					
Winter, Seidman *et al.* [81]	0/1 (0)					
Wang, Felix *et al.* [26]	3/11 (27)	2/3 (67)				
Rushing, Shajahan *et al.* [27]	2/2 (100)					
Klein and Kurman [28]	1/10 (10)	0/2 (0)				
Leath, Straughn *et al.* [29]	3/3 (100)					
Caudell, Deavers *et al.* [35]	0/8 (0)	1/4 (25)				
Moinfar, Gogg-Kamerer *et al.* [40]					14/20 (70)	3/3 (100)
Salvatierra, Tarrats *et al.* [36]		1/1 (100)				
Nakayama, Mitsuhashi *et al.* [76]	0/5 (0)					
Liegl, Gully *et al.* [38]	0/37 (0)		22/37 (59)			
Adams, Hickson *et al.* [39]	0/8 (0)		7/8 (88)			
Mitsuhashi, Nakayama *et al.* [42]		0/1 (0)				1/1(100)
Zafrakas, Theodoridis *et al.* [31]	2/2 (100)	1/2 (50)				
Martin, Ramesh *et al.* [32]	1/1 (100)					
Trojan, Montemurro *et al.* [37]	0/1 (0)		1/1 (100)			
Koivisto-Korander, Butzow *et al.* [33]	2/9 (22)					
Cheng, Yang *et al.* [34]	1/12 (8)		4/12 (33)			
Cossu-Rocca, Contini *et al.* [77]	0/23 (0)	1/5 (20)	15/23 (65)	4/5 (80)	10/23 (43)	4/5 (80)
Park, Kim *et al.* [78]	32/39 (82.1)		28/39 (71.8)		0/39 (0)	
Present series (2013)	1/52 (2)	1/13 (7.7)	18/52 (34.6)	5/13 (38.5)	6/52 (11.5)	1/13 (7.7)

cytoplasmic PDGFRA expression in ESS, although mostly weak and/or focal, lies within the range reported in previous studies [34,37-39,77,78].

In our series, mutational analysis of *hot spots* of *KIT* and *PDGFRA* genes did not reveal any somatic mutation, which is consistent with previous studies [36-39,41,42,77]. Constitutive activation via autocrine/paracrine stimulation of the receptor by its ligand was observed in several human cancers and is related with their tumorigenesis [82,83]. Probably, expression of related ligands of KIT and PDGFRA, which were not evaluated in this series, might be the reason for expression of both receptors in these tumors, although more studies are needed. Second, the work of Kang *et al.* demonstrated that relative KIT expression ratio was fivefold higher in cases with *KIT* mutation, than in GISTs lacking *KIT* mutation and the mutation status of *KIT* and *PDGFRA* was directly related to the different expression levels of activated KIT and PDGFRA [84]. Furthermore, with exception of GISTs, the presence of overexpression of KIT and PDGFRA in human cancers was not correlated with presence of activating mutations [85]. These facts support our results, once that KIT and PDGFRA expression observed was mostly weak and/or focal and lacks activating mutations on its related genes. Furthermore, the consistent expression of KIT and PDGFR receptors and their ligands was seen in a phase II trial in women with ovarian cancer, together with the absence of mutations in these genes and with lack of response to imatinib [86]. In other two phase II clinical trials in lung cancer [87] and uterine carcinosarcomas [88], the high expression of KIT did not correlate with response to imatinib. These clinical findings support the hypothesis that overexpression of these TKR does not confer sensitivity to imatinib, which was previously suggested for other uterine sarcomas [89].

However, the expression of at least one of imatinib-target (KIT, PDGFRA and PDGFRB) without evidence of activating mutations was reported in EST patients who responded to imatinib treatment [36,37,43]. Tumor shrinkage was the main indicator of objective response accompanied by stable disease in two cases [36,37]. One explanation for these results may be due to the autocrine/paracrine signaling of PDGFRB. In dermatofibrosarcoma protuberans (DFSP), *t*(17;22)(q22;q13) results in a *COL1A1-PDGFB* gene fusion. This cytogenetic abnormality is essential for pathogenesis of the disease and responsible by constitutive activation of PDGFRB. Imatinib

inhibits the activity of the dysregulated PDGFRB [90], decreases enzymatic activity in DFSP cells and inhibits their ability to divide and grow [91] and induces apoptosis in tumor cells [92], which may have effects on decreasing tumor size [93]. In fact, an effect of imatinib on tumor shrinkage was observed in patients with DFSP [94,95], and therefore imatinib was approved for unresectable, recurrent, and/or metastatic DFSP in adults. However, in ESS and UES cases reported as responsive to imatinib, PDGFRB was only determined in one case reported by Trojan *et al.* [37], which makes difficult to draw any conclusions.

Concerning EGFR expression, our study reveals lower cytoplasmic expression of EGFR in EST cases (around 10.8%), in contrast with the results obtained by other authors [40,77] (Table 2). This contradiction may be a consequence of the same variables indicated for the immunoexpression of KIT. Since i) a correlation between an increased *EGFR* copy number and gefitinib was proposed in non-small-cell lung cancer (NSCLC) [96] and ii) immunohistochemistry is not a reliable approach for this determination, we decided to evaluate *EGFR* amplification status in our series by FISH. The *EGFR*/Chr7 ratio was below 2, which is indicative of non-amplification, and only a small percentage of the cases presented polysomy. Previous evaluation of *EGFR* amplification was only performed in a UES case which presented a temporary response to imatinib. This case presented a low-level amplification (mean ratio 2.9) according to the evaluation criteria of the authors [42]. Although no consensus has been reached on how to assess the presence and extent of *EGFR* status dysregulation in solid tumors by FISH analysis [97], it seems likely that gene amplification found in NSCLC and glioblastoma is an uncommon event in EST. *EGFR* mutational status also did not show any somatic mutation in exons 18–21. In fact, all these data confirm that it is unlikely that EGFR activation could play a role in tumorigenesis of EST.

Conclusions

In summary, we performed an extensive molecular and expression analysis of KIT, PDGFRA and EGFR in the largest series of EST studied so far (Table 2). Our findings reveal a lack of significant expression, amplification and activating mutations on these receptors and related genes, which suggests that it is unlikely that EST can benefit from therapies such as imatinib or EGFR inhibitors in advanced disease. However, the role of imatinib in the management of unresectable tumors needs to be clarified through evaluation of expression of constitutive activation of PDGFRB by its ligands, or other genetic alterations in ESS and UES. The recent discovery of a chromosomal rearrangement *t*(10;17)(q22;p13) that results in a 14-3-3 fusion protein with oncogenic properties in a subgroup of UES [22], and the nuclear expression of β-catenin, a member of Wnt and E-cadherin signaling pathways in EST need to be better explored, since they can represent an entry point for targeted therapeutic strategies.

Competing interests
Support for this study was obtained from Novartis.

Authors' contributions
RS carried out the molecular genetic studies, performed the sequence alignment and data analyses and drafted the manuscript. TH and SF carried out the TMA construction and immunohistochemistry assays. TH carried out fluorescent hybridization assay and participated in the sequence alignment. FT, AV, MCG, AA, NH, JS, JO, LG and RR provided the samples for the study. CB and EA conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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