Endosonography and pretreatment tumor profiling

- from sampling, staining, to sequencing

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Cover illustration: Endosonography-guided sampling of a gastrointestinal stromal tumor subsequently subjected to Ki-67-indexing and Sanger sequencing. Graphics by Martin Hedenström.

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To Anna, Klara, Tove, and Gustav

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ABSTRACT

Background and aims: Endosonography-guided fine needle aspiration (EUS-FNA) is imperfect in diagnosing solid pancreatic lesions (SPL) and subepithelial lesions (SEL) including gastrointestinal stromal tumors (GIST). In GISTs, imatinib therapy is effective only in variants of oncogenes *KIT* and *PDGFRA*. The global aim was to improve the EUS-diagnostics and study a biopsy approach (EUS-FNB) to obtain a reliable diagnosis of SPLs and SELs. In GISTs, the aim was to evaluate pretreatment samples for tumor risk assessment and the guidance of down-sizing imatinib therapy.
Methods: In two prospective, single-center studies (2012-2015), SPLs (n=68,*Paper I*) and SELs (n=70,*Paper II*) were sampled with EUS-FNA and EUS-FNB. A reference cohort (2006-2011) was used for comparison. The FNB-tissue of all GISTs (n=44) was subjected to Ki-67-indexing and DNA-sequencing of *KIT* and *PDGFRA* (*Paper III*). In a last study (*Paper IV*), pretreatment sequencing of GISTs (n=59) was performed.
Results: *Paper I*: In SPLs, EUS-FNB and EUS-FNA had a comparable diagnostic accuracy (69 % *vs* 78%, p=0.31). The combination EUS-FNA+FNB was superior to EUS-FNA alone in pancreatic non-adenocarcinoma neoplasms (89% *vs* 69%, p=0.02). *Paper II*: In SELs, EUS-FNB had a higher accuracy compared with EUS-FNA (83% *vs* 49%, p<0.001) leading to the reduced need for additional diagnostic procedures (14% *vs* 53%, p<0.001). *Paper III*: The EUS-FNB-tissue was diagnostic for GIST in 98%, accurate for Ki-67-indexing in 92%, and adequate for successful sequencing in 98% of the cases. In patients treated with down-sizing imatinib [*KIT* exon 11 (n=9); *PDGFRA* exon 12 (n=1)], the Ki-67-index was significantly higher in pretreatment FNB-tissue compared with resection specimens: $Ki\text{-}67_{DIFF}$ = 2.3 (95% CI: 0.67-5.37, p=0.005). *Paper IV*: Pretreatment sequencing, compared with no sequencing, lead to a higher rate of accurate down-sizing therapy (97% *vs* 70 %, p<0.001) and to the increased preoperative tumor size reduction on CT scan (32% *vs* 22%, p=0.036).

Conclusions: Endosonography-guided fine-needle biopsy sampling has a significant diagnostic and clinical value in subepithelial lesions; especially in gastrointestinal stromal tumors. The acquired tissue is also accurate for the early tumor proliferation rate assessment and genetic profiling of GISTs. This work-up approach facilitates the guidance and evaluation of down-sizing tyrosine kinase inhibitor therapy.

Keywords: endosonography, fine-needle biopsy, pancreatic neoplasms, gastrointestinal stromal tumors, *KIT*, *PDGFRA*, Ki-67, imatinib, neoadjuvant therapy

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SAMMANFATTNING PÅ SVENSKA

Bakgrund: Korrekt behandling kräver tillförlitlig diagnos. Via röntgen eller endoskopi händer det tämligen ofta att man finner förändringar i bukspottkörteln eller förändringar under magtarmkanalens slemhinna där diagnosen förblir osäker. Ett vävnadsprov av hög kvalitet krävs då eftersom ett brett spektrum av diagnoser är tänkbara, t ex gastrointestinal stromacellstumör (GIST). Denna tumörtyp behandlas kirurgiskt men förbehandling med målinriktad så kallade tyrosinkinashämmare är inte sällan nödvändig. Sådan behandling är effektiv endast vid vissa mutationstyper i onkogenerna *KIT* och *PDGFRA*. Prognosen vid GIST bestäms även av tumördelningshastigheten.

Endoskopiskt ultraljud med finnålsaspiration (EUS-FNA) är en värdefull diagnostisk teknik för att inhämta cellprov från svåråtkomliga förändringar i bröstkorg och bukorgan. EUS-FNA har emellertid svagheter och bristande diagnostisk träffsäkerhet. En ny typ av biopsinålar (FNB) för inhämtning av sammanhängande vävnad är ett potentiellt bättre alternativ än EUS-FNA. EUS-FNB har dock inte utvärderats i prospektiva, jämförande studier.

Målsättning: Det övergripande syftet med denna avhandling var att studera möjligheten att förbättra EUS-baserad diagnostik av förändringar i bukspottkörtel och magtarmkanal, särskilt GIST. Ett specifikt syfte var att utvärdera EUS-FNB för vävnadsinhämtning och histologisk diagnostik. En avslutande strävan var att experimentellt utforska möjligheten att använda GIST-vävnad inhämtad via EUS för att redan före start av förbehandling skatta tumördelningshastighet och bestämma genetiska profil i enskilda tumörer.

Metod: På Sahlgrenska sjukhusets endoskopiavdelning inkluderades under åren 2012–2015 totalt 68 patienter med pankreasförändringar (*delarbete I*) och 70 patienter med förändringar liggande under magtarmkanalens slemhinna (*delarbete II*). Patienterna genomgick rutinmässig cellprovtagning via EUS-FNA men i tillägg även vävnadsinhämtning med EUS-FNB. Den diagnostiska träffsäkerheten jämfördes sedan de två teknikerna emellan. En historisk grupp patienter (2006-2011) från samma sjukhus användes också som jämförelse.

Hos 44 patienter som alla slutligen fick diagnosen GIST (*delarbete III*) färgades den inhämtade vävnaden för markören Ki-67 varpå tumörcellernas delningshastighet beräknades. Vävnaden analyserades sedan också med

gensekvensering av *KIT* och *PDGFRA*. I en avslutande studie på GIST-patienter 2014–2017 (*delarbete IV*) genomfördes all gensekvensering före start av preoperativ förbehandling med tyrosinkinashämmare, t ex imatinib.

Resultat: Vävnadsinhämtning via den nya provtagningstekniken (EUS-FNB) visade sig vara likvärdig med den gängse tekniken (EUS-FNA) vid utredning av förändringar i bukspottkörteln. Användandet av bägge teknikerna tillsammans ökade dock den diagnostiska träffsäkerheten från 69% till 89% vid vissa tumörtyper såsom neuroendokrin pancreastumör (*delarbete I*).

Användandet av EUS-FNB var en tydligt bättre metod än EUS-FNA vid utredning av förändringar liggande under magtarmkanalens slemhinna. Elakartade förändringar diagnosticerades korrekt i 90% av fallen (*delarbete II*). Den höga träffsäkerheten gjorde även att behovet av kompletterande utredning efter EUS minskade under åren 2012–2015 jämfört med 2006–2011.

Tumörvävnad från GIST inhämtad via EUS-FNB var väl lämpad för genanalys av *KIT* och *PDGFRA*, där mutationsprofilen klargjordes i 43/44 (98%) av fallen (*delarbete III*). Identiska mutationer hittades i operationspreparaten hos de 27 fall som sedan opererades. Hos patienter som inte fick förbehandling med imatinib stämde tumördelningshastigheten (Ki-67-index) i FNB-vävnad väl överens med tumördelningshastigheten i motsvarande operationspreparat. Hos förbehandlade patienter var däremot tumördelningshastigheten signifikant högre i FNB-vävnad jämfört operationspreparat.

Omedelbar genanalys av *KIT* och *PDGFRA* i FNB-vävnad (*delarbete IV*) ledde till att en högre andel GIST-patienter (97% jämfört 70%) kunde erbjudas korrekt förbehandling under åren 2014-2017 jämfört med perioden 2006–2013. De patienter som fick förbehandling med imatinib under åren 2014–2017 hade också ett bättre behandlingssvar jämfört motsvarande patienter 2006–2013.

Slutsats: Endoskopiskt ultraljud med vävnadsinhämtning via biopsinål är diagnostiskt och kliniskt värdefullt vid utredning av förändringar liggande under magtarmkanalens slemhinna; i synnerhet då man misstänker gastrointestinal stromacellstumör. Vid denna diagnos kan inhämtad vävnad även användas för att kartlägga mutationer i GIST-tumören, bedöma dess riskprofil och slutligen målinrikta tumörkrympande behandling före kirurgi.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I–IV):

I. EUS-guided reverse bevel fine-needle biopsy sampling and open tip fine-needle aspiration in solid pancreatic lesions - a prospective, comparative study.

Hedenström P, Demir A, Khodakaram K, Nilsson O, Sadik R.

Scand J Gastroenterol. 2018 Feb;53(2):231-237.

II. High clinical impact and diagnostic accuracy of EUS-guided biopsy sampling of subepithelial lesions: a prospective, comparative study.

Hedenström P, Marschall HU, Nilsson B, Demir A, Lindkvist B, Nilsson O, Sadik R.

Surg Endosc. 2018 Mar;32(3):1304-1313.

III. Characterizing gastrointestinal stromal tumors and evaluating neoadjuvant imatinib by sequencing of endoscopic ultrasound-biopsies.

Hedenström P, Nilsson B, Demir A, Andersson C, Enlund F, Nilsson O, Sadik R.

World J Gastroenterol. 2017 Aug 28;23(32):5925-5935.

IV. Pretreatment mutational analysis of *KIT* and *PDGFRA* optimizes down-sizing imatinib therapy of gastrointestinal stromal tumors

Hedenström P, Andersson C, Sjövall H, Enlund F, Nilsson O, Nilsson B, Sadik R.

Manuscript

TABLE OF CONTENTS

ABBREVIATIONS

DNA	Deoxyribonucleic acid
EUS	Endosonography
FDG-PET	Fluorodeoxyglucose positron emission tomography
FNA	Fine needle aspiration
FNB	Fine needle biopsy
GIST	Gastrointestinal stromal tumor
IMA	Imatinib
KIT	KIT proto-oncogene receptor tyrosine kinase
MA	Mutational analysis
MI	Mitotic index
NGS	Next generation sequencing
PDGFRA	Platelet-derived growth factor alpha
SEL	Subepithelial lesion
SPL	Solid pancreatic lesion
TKI	Tyrosine kinase inhibitor
TUS-NB	Transabdominal ultrasound needle biopsy

1 INTRODUCTION

"To treat or not to treat?" – that is actually quite often the question in modern healthcare. If the answer is "yes", the obvious next questions would be "whom and how to treat?".

The intent of this thesis was to elaborate diagnostic methods addressing the above questions and by that facilitate the management of patients with suspected neoplasms. The improved diagnostics can be one valuable step towards what is called personalized medicine[1,2].

1.1 DNA and tumor biology

Deoxyribonucleic acid (DNA) is the construction code of advanced lifeforms[3,4]. In humans, the DNA molecule is a double helix formation built upon nucleotides. Each nucleotide contains a sugar component (deoxyribose), a phosphate group, and one of four nitrogen-containing nucleobases - cytosine (C), guanine (G), adenine (A) or thymine (T). Two nucleobases of the two opposing DNA-strands (base-pairs) are bound together according to the strict rule: A-T and C-G.

Each individual aminoacid, which are the building blocks of proteins, is encoded by a triplet of nucleotides, **Appendix**. The protein-coding fraction of the human genome is small (1.5%)[5] including some 19 000 genes[6]. The *exons* are the parts of the genome encoding the actual protein, while the *introns* are the fragments removed at transcription.

1.1.1 How to unveil the base-pair sequence of DNA and why?

In numerous malignancies such as breast cancer and lung cancer, the tumor biology, the prognosis, and the recommended treatment depend on the DNA-sequence of certain genes[7,8]. Luckily enough, elegant methods, i.e. *sequencing*, have been developed to decipher the base-pair sequence hidden within the DNA. The *Sanger sequencing* method, outlined in **Figure 1**, is still in use after initially being elaborated in the nineteen seventies[9,10]. During the last two decades there has been an increased need for fast and large-volume DNA-sequencing. As a result, more advanced methods have been developed, which

are mainly entitled next generation sequencing (NGS). The specific NGS-method used in this thesis will be further described in chapter *Methods*.

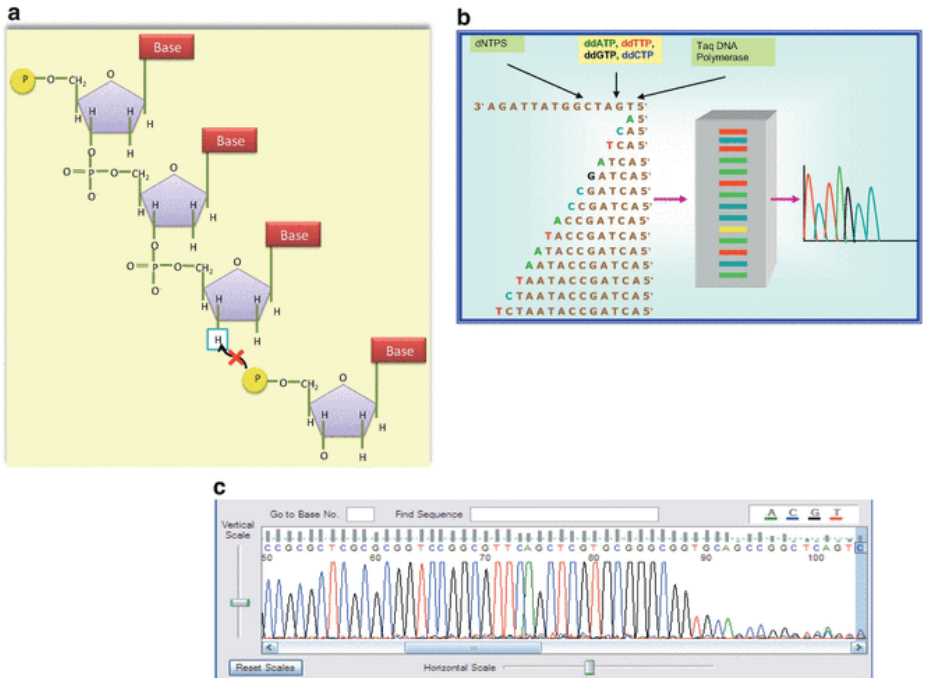

Figure 1. *Sanger sequencing - the dideoxy chain termination method.* ***a)*** *DNA synthesis of the gene of interest by PCR and the addition of regular deoxynucleotides (dNTPs) and fluorescently dye-labeled dideoxynucleotides (ddNTPs). The ddNTPs lack the 3' OH-group, which prevents a phosphodiester bond with the next dNTP whereupon further synthesis stops (red cross).* ***b)*** *Gene fragments of all possible lengths are formed, everyone ending with a fluorescent ddNTP. Subsequently, the fragments are separated by capillary electrophoresis, in which the shortest fragments move the fastest. At the end of the capillaries a laser beam makes the ddNTPs emit fluorochromes of pre-defined wave-lengths which are recorded by a detector.* ***c)*** *Finally, an electropherogram of the DNA-sequence can be produced. Adapted from Verma et al and reprinted with permission from Springer Nature.*

1.1.2 Mutations and the formation of neoplasms

A mutation is defined as an alteration of the normal DNA-sequence in a certain gene[11]. Mutations can be *acquired* (only found in a specific cell population) or *inherited* (found in all somatic or germ cells)[12]. There are different types of mutations such as point mutations, deletions, and insertions, which all change the base-pair sequence[13]. Consequently, the mutated DNA may lead to an altered gene expression and protein product.

A neoplasm is an abnormal growth of tissue, which often forms a mass or a tumor[14]. Due to a mutation or a chromosomal translocation, a so-called *proto-oncogene* can evolve into an *oncogene*, which is a type of gene that has the potential to stimulate the formation of a neoplasm. In general, proto-oncogenes are involved in cell-growth and cell differentiation. A famous example of an oncogene is the *Bcr-Abl* gene. The *Bcr-Abl* gene codes for a permanently active tyrosine kinase, which leads to the uncontrolled cell proliferation seen in chronic myeloid leukemia[15]

The development of a neoplasm is a multi-step process, in which normal cells gradually transform into cells with increasing abnormal properties such as decreased cell differentiation, increased cell division, and loss of apoptotic control. Often, sequential, acquired mutations in multiple genes of the DNA contribute to the neoplastic process[16].

A carcinoma is a neoplasm of epithelial origin. It is the most common type of malignancy exemplified by lung cancer, breast cancer, and gastric cancer. A sarcoma is a neoplasm of mesenchymal origin, i.e. the supporting tissue such as bone, cartilage, and connective tissue.

1.2 Solid pancreatic and subepithelial lesions

1.2.1 Solid pancreatic lesions – not only adenocarcinomas

Neoplasms in the form of solid lesions in the pancreatic parenchyma can be detected by radiology either incidentally or in the work-up of symptomatic patients. Even by the means of modern cross-sectional imaging and transabdominal ultrasound, it can be difficult to firmly diagnose the underlying diagnostic entity[17,18].

Among different neoplasms, pancreatic ductal adenocarcinoma (PDAC) is the most common one[19], while pancreatic neuroendocrine tumors (PNET) and metastases have a similar appearance at imaging[17]. The diagnostics is also complicated by the fact that a focal pancreatitis may imitate a neoplasm and present as an SPL. Therefore, the sampling of numerous SPLs is warranted for the microscopic assessment of the cellular morphology. In addition, the reliable diagnosis of neoplasms other than PDAC, requires complementary immunostaining for entity-specific tumor markers[20-22]. Problematically, the pancreas is located deep within the abdomen and challenging to reach by a transabdominal approach.

1.2.2 Subepithelial lesions – a wide spectrum of entities

Subepithelial lesions (SEL) are common incidental findings at routine endoscopy [23]. A SEL can be defined as ”*any intramural growth underneath the gastrointestinal mucosa, where the etiology cannot readily be determined by diagnostic endoscopy or barium radiography*”[24]. An extramural lesion originating from outside the wall may also present as a SEL during endoscopy[25,26], **Figure 2**. The expression subepithelial lesion is purely descriptive and it provides no information on the diagnostic entity of the underlying lesion. Under the SEL-umbrella, there hide lesions ranging from highly malignant sarcomas to completely benign duplication cysts[27]. Consequently, and as with SPLs, the sampling of these lesions is more or less required.

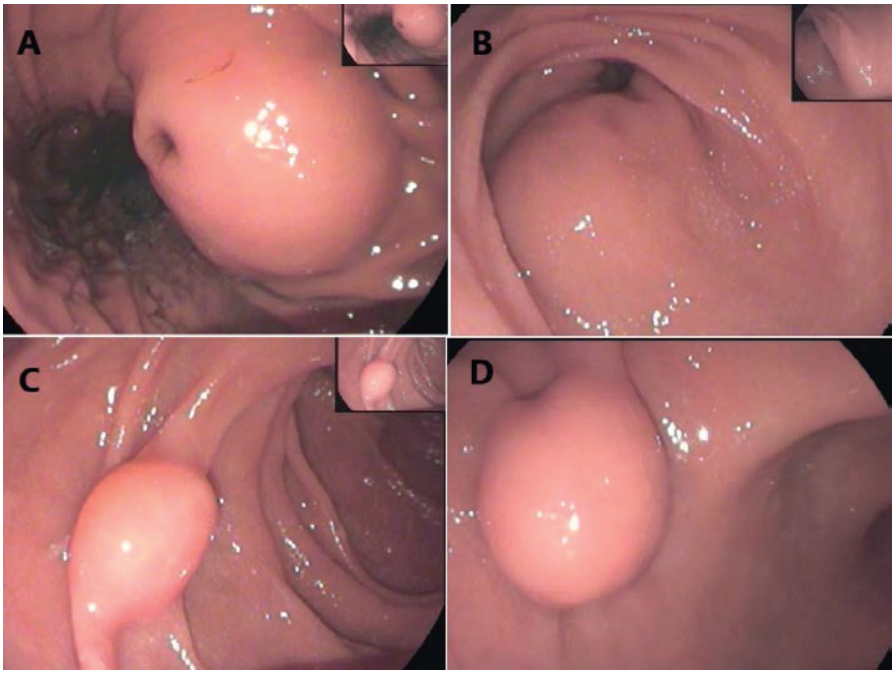

***Figure 2**. Four subepithelial lesions symbolizing the complexity of diagnostics by routine endoscopy. Images by the author.*
***A)** A schwannoma of the minor curvature of the gastric body.*
***B)** An extramural structure, in this case the benign gall bladder of a young woman, mimicking a true subepithelial tumor in the antral part of the stomach.*
***C)** A lipoma situated in the second part of the duodenum.*
***D)** A gastrointestinal stromal tumor originating from the distal part of the gastric body.*

However, numerous subepithelial lesions are difficult to discriminate from one another only by their cellular morphology, which indicates the need for immunochemistry[25]. The expected immunostaining pattern of three common SELs is presented in **Figure 3**. The rational clinical management of SELs requires a reliable diagnosis, which unfortunately cannot be obtained by routine gastroscopy[23,28,29] or by PET-CT[30,31]. To date, the challenging diagnostics of SELs has led to a wide spread surgical approach with the resection of these lesions to obtain the diagnosis[23]. That is not optimal management since surgery is associated with morbidity and since entirely benign lesions should not be resected.

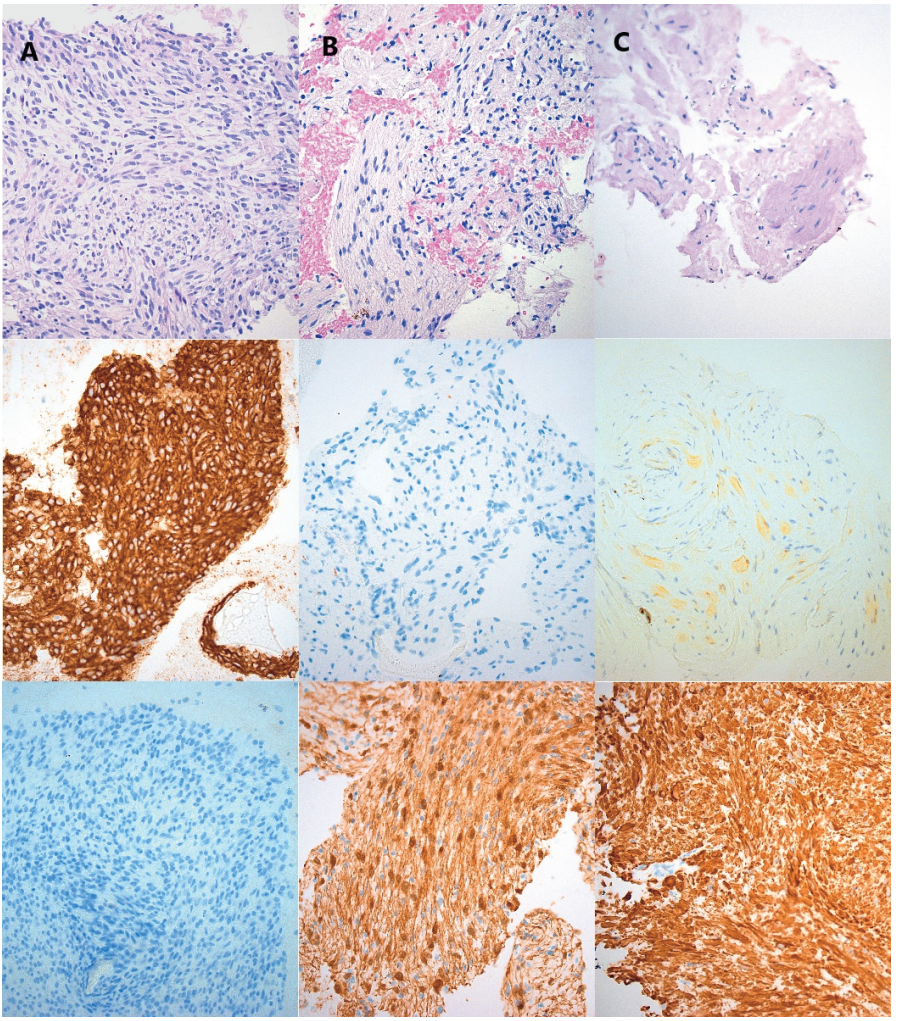

Figure 3. *EUS-FNB-tissue histology slides (magnification x 20) of a gastrointestinal stromal tumor (**A**); a schwannoma (**B**); and a leiomyoma (**C**). **Top row**: Routine hematoxylin and eosin staining. **Middle row**: c-KIT immunostaining (CD117). The GIST-tissue is positive (brown color) while the schwannoma and the leiomyoma are negative (blue color). **Bottom row**: The GIST-tissue is negative (blue) for DESMIN immunostaining, while the leiomyoma is positive (brown). The schwannoma in the middle stains positively for S-100 (brown). Images by the author.*

1.3 Gastrointestinal stromal tumors (GIST)

Gastrointestinal stromal tumor is one entity commonly presenting as a subepithelial lesion. As described below, a GIST constitutes a most challenging and demanding neoplasm both from a diagnostic, prognostic, and a therapeutic point of view.

1.3.1 Epidemiology

Gastrointestinal stromal tumors (GIST) are exotic in the sense that this diagnostic entity did not exist until some twenty years ago. Instead GISTs were regarded as smooth-muscle cell neoplasms originating from the stomach and incorrectly named gastric leiomyomas[32]. A true GIST is a neoplastic entity of its own. It is hypothesized that it originates from the interstitial cells of Cajal (ICC)[33], "the gut pacemaker cells", which are responsible for the initiation of the contractile bowel movements [34].

GIST is a relatively rare tumor, but the most common mesenchymal neoplasm of the gastrointestinal tract[35]. A clinical incidence of approximately 0.8 in 100.000 has been suggested[36] with similar numbers recorded in a large Swedish cohort[37]. Hereditary GISTs may appear at young age in individuals with germline mutations[12,38], while sporadic GISTs are diagnosed at a median patient age of 70 years without a clear sex predominance[28,36]. GISTs most commonly arise from the stomach (~50 %)[39,40] or the small bowel (20-30 %), but may also originate from the large bowel (<10 %) or rarely the retroperitoneum. The clinical presentation of GIST is diverse[28] but the incident detection during upper GI endoscopy is common[37].

1.3.2 Pathogenesis and molecular pathology

To a large extent, the pathogenesis of sporadic GISTs can be explained by mutually exclusive mutations in any of two proto-oncogenes - *KIT* and *PDGFRA*[41].

The *KIT*-gene is located in the long arm (q) of chromosome 4 and includes 21 exons of 34 kB[42]. The gene encodes a 145 kDa transmembrane tyrosine kinase receptor, TRK (also referred to as c-Kit or CD 117 in immunostaining)[43]. The receptor has an extracellular binding site of the *agonist* ligand SCF (stem cell

factor), **Figure 4**. The attachment of the SCF-ligand results in receptor dimerization, phosphorylation of tyrosines by the intracellular receptor domain, and finally activation of subsequent down-stream signaling pathways leading to cell proliferation and reduced cell apoptosis[44,45].

The c-Kit-receptor is expressed by the interstitial cells of Cajal, but also by hematopoetic cells. In 1993, mutations in *KIT* were identified as the cause of ligand-independent tyrosine kinase activity resulting in mast cell leukemia[46]. Later Hirota and co-workers described such "gain of function-mutations" as the key driver of the oncogenesis also in GIST[47]. Commonly mutated exons of *KIT* are exon 11 (~75 %), exon 9 (~15 %), exon 13 (~2 %), and exon 17 (~1 %)[48-50].

The platelet derived growth factor alpha gene (*PDGFRA*) is also located on chromosome 4. It is suggested to have the same ancestral gene as *KIT*[42,51]. As with *KIT*-mutations, *PDGFRA*-mutations leads to a permanently active receptor **Figure 4**. *PDGFRA*-mutations located in exon 12, 14, or 18 are responsible for around 7-10 % of sporadic GISTs and are associated with gastric origin and less aggressive progression[52,53].

1.3.3 The histopathology of GISTs

A spindle-cell morphology is the typical microscopic appearance of GIST. However, positive immunostaining for hematopoetic progenitor antigen (CD34), c-KIT (CD117), or anoctamin 1 (DOG-1)[47,54,55] is required for a conclusive diagnosis, **Figure 3**. Importantly, the positive c-KIT-immunostaining is not caused by a *KIT*-mutation *per se*, which instead modifies the function of the c-KIT-receptor.

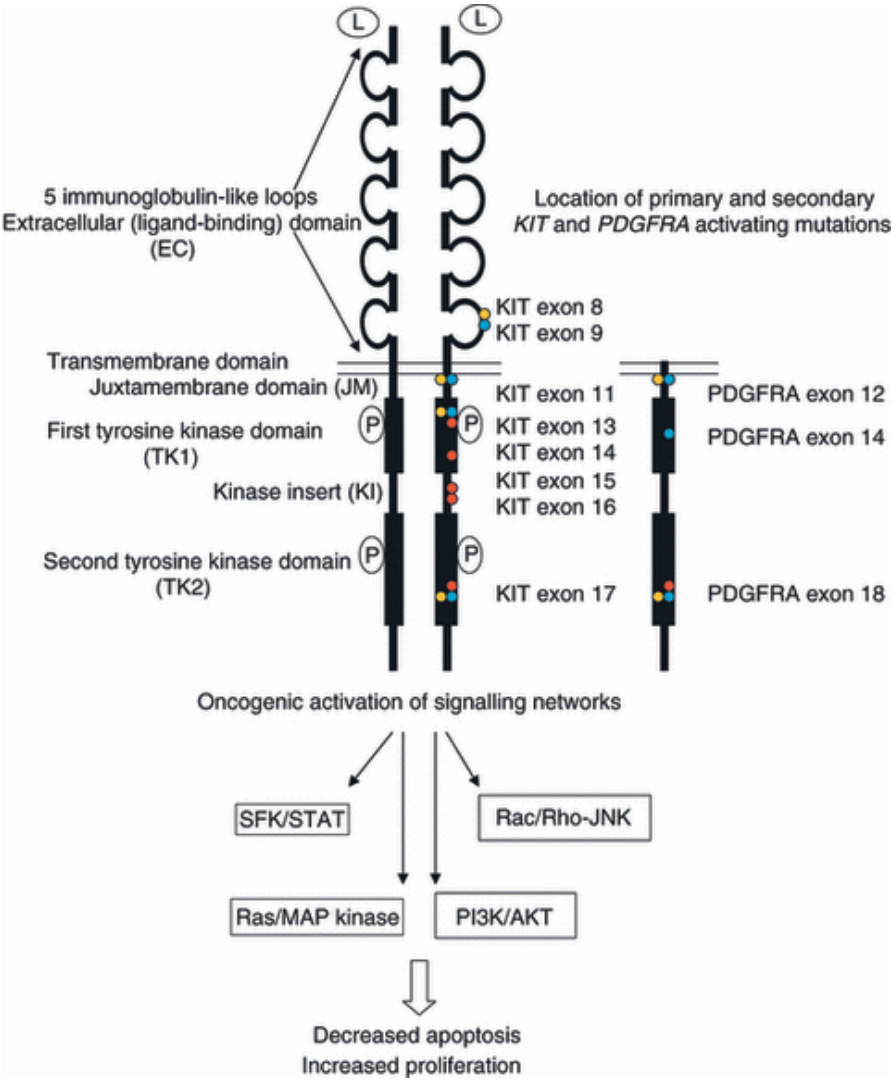

Figure 4. *A schematic outline of the c-KIT-receptor.*
A gain-of-function mutation (dots in blue, yellow, and red) leads to the ligand-independent dimerization and activation of the receptor resulting in autophosphorylation of tyrosines and activation of downstream signaling pathways. The location of primary, sporadic and primary, hereditary mutations in KIT and PDGFRA are indicated by blue and yellow dots respectively. The location of secondary mutations induced by TKI-therapy are indicated by red dots. Adapted from Lasota et al and reprinted with permission from John Wiley and Sons.

1.3.4 Prognostic risk and tumor proliferation rate

The prognosis of patients with GIST varies from excellent to poor depending on the tumor stage at the time of diagnosis[56]. The prognostic risk is based upon *a)* the tumor size and *b)* the tumor proliferation rate (mitotic index - MI); with both parameters included in the advocated risk score of the NIH (National Institutes of Health)[57], **Appendix**. The Ki-67-index is an alternative indicator of the tumor proliferation rate used in numerous neoplasms[58,59] and the level of the Ki-67-index strongly correlates with the prognosis also in GIST[37,60-62].

1.3.5 Treatment

Small GISTs (<1 cm) can be managed conservatively with watchful waiting, especially in elderly patients[63]. Otherwise, surgery is the primary treatment of resectable GISTs and can cure 60 % of the patients as the single therapy[56]. Tumor rupture during surgery significantly increases the risk for recurrence[64].

Tyrosine kinase inhibitors (TKI) and gene-driven targeted therapy

Imatinib (a 2-phenyl amino pyrimidine derivative) is a drug belonging to the family of tyrosine kinase inhibitors (TKIs). It was initially developed to treat chronic myeloic leukemia. In 2001, there was a first report on the efficacy of imtinib in GIST[65]. This breakthrough finding has revolutionized the treatment of GIST-patients[66-68]. Side effects related to imatinib therapy are however not rare[69]. Anemia, edema, nausea, diarrhea, and dermatitis are common reasons for dose reduction or for discontinuation of therapy[68,70]. As adjuvant or palliative therapy, the standard dose of imatinib is 400 mg daily[71]. High dose therapy (800 mg daily) is recommended for certain molecular subtypes, such as *KIT* exon 9-mutants[48,72], but it increases the risk of side-effects.

Tumor sensitivity to imatinib therapy

Imatinib therapy is genotype-driven[71] meaning that only tumors with certain mutations are sensitive to treatment. Almost all *KIT* exon 11-mutants are sensitive to imatinib as are *PDGFRA* exon 12-mutants[73]. However, specific subtypes of *KIT* exon 11-mutations such as the L576P-mutant respond poorly to imatinib[74]. Primary resistance or reduced sensitivity to imatinib, is also related to mutations in exon 9 or 17 of *KIT*, to mutations in exon 18 of

PDGFRA, or to the wild type profile (WT)[48,73,75-77], in which no mutations in *KIT* or *PDGFRA* are detected.

There is no indication for therapy in *PDGFRA* exon 18 D842V-mutants[78] and no obvious benefit has been found in WT-tumors[79]. Regarding *KIT* exon 13-mutants the knowledge is limited. These tumors are probably not resistant to imatinib but the response to therapy does not seem to be as good as in *KIT* exon 11-mutants[80,81]. So-called secondary mutations in *KIT* (exon 13, 14, 17) and *PDGFRA* can evolve during TKI-therapy leading to drug resistance[82].

Indications for imatinib therapy in GIST

While the benefits of surgery in metastasizing GIST remains unclear[83], imatinib given as a *palliative* treatment has dramatically improved the 5-year overall survival which is now approaching 85%[19]. This number can be compared with a median overall survival (OS) limited to 18 months in high-risk GISTs not available for radical surgery (R0) during the pre-imatinib era[37]. As another comparison, the prognosis of a regular leiomyoma is excellent with a very low risk for malignant transformation[84]. There is also solid support for 36 months of postoperative *adjuvan*t imatinib therapy in NIH high-risk tumors and some support considering intermediate risk tumors[72].

Preoperative imatinib treatment is called *neoadjuvant* or *down-sizing* therapy. Studies have proved that down-sizing imatinib is safe[85,86] and that it facilitates the resection of borderline resectable tumors[87]. There is also growing evidence and support for neoadjuvant imatinib in terms of disease free survival (DFS) and overall survival[87,88]. Guidelines recommend neoadjuvant imatinib to enable organ preserving, radical surgery[72].

1.3.6 Shortcomings in current GIST diagnostics and therapy

The complexity of GIST has resulted in severe shortcomings in current work-up and therapy. The need for sampling and immunostaining has left many GIST-patients without a preoperative diagnosis[23,89]. The prognostic risk has been speculative without serious attempts to assess the tumor proliferation rate at the preoperative stage. Finally, down-sizing imatinib therapy has been initiated purely by chance and not based on the results of mutational analysis[85].

1.4 Endosonography and tumor sampling

1.4.1 What is ultrasound?

Sound is mechanical energy appearing in the form of vibrations moving through a medium such as a gas or a liquid. The propagation of sound occurs when energy displace molecules from their original position and make them oscillate along the direction of what is described as the sound wave. The wave can be mathematically expressed as:

$$c = f \cdot \lambda$$

According to this equation, the wavelength (λ) is proportional to the velocity (c) of the wave propagation and inversely proportional to the frequency (f) of the molecule oscillations[90]. The formula above implicates that the wave frequency is the number of oscillations (cycles) per unit of time. A frequency of 1 cycle per second is expressed as 1 Hertz (Hz). Ultrasound is defined as frequencies greater than 20 kHz, i.e. waves inaudible by humans.

Ultrasound used in medicine normally operates within wave lengths ranging from 2–20 MHz[91]. An ultrasound wave is emitted from the so-called transducer, which also receives and analyses the reflected wave.

According to the equation of velocity, the distance (D) from the transducer to the organ or object reflecting the ultrasound wave is:

$$D = \frac{c \cdot t}{2}$$

where (c) is the wave velocity and (t) is the recorded time from the emission of the wave (speaking) to the return of the wave (listening).

Consequently, the ultrasound processor will be able to calculate the position of all reflecting objects within the examined part of the human body. Finally, all recorded echoes are reproduced in a two-dimensional fashion – the ultrasound image[92].

1.4.2 The rise of endosonography (EUS)

The first ever known outline of an endoscopic procedure was described by Hippocrates (460 – 375 B.C)[93]. It was not until 1881 that the era of flexible endoscopy started[94]. The potential use of ultrasound in medicine was proposed in the early 1940's[95]. Eventually, transcutaneous ultrasound was implemented into multiple areas of medical diagnostics. Nevertheless, the imperfection of transcutaneous ultrasound in the diagnosis of pancreatic diseases[96] lead to the idea that the attachment of an ultrasound transducer onto an endoscope would improve the imaging of the pancreas. After intense animal tests in the late 1970's the first primitive echoendoscope was launched in 1980[97]. The transducer of modern echoendoscopes is often of the type curved linear-array, which enables the sonographic visualization of the needle[98], **Figure 5**.

1.4.3 Endosonography-guided acquisition of tumor material

Certainly, the assessment of the ultrasound image is an important part of EUS-diagnostics but it alone cannot unveil the diagnosis in many lesions presenting as a SEL[99], **Figure 6**. Therefore, there is a frequent need for lesion sampling.

EUS-guided fine needle aspiration (EUS-FNA)

Almost thirty years ago, dr Peter Vilmann and colleagues were pioneers in the performance of EUS-guided sampling[100,101]. Early generation EUS-FNA-needles were designed for multiple use, **Figure 5**, while modern needles are disposable. In general, the FNA-needles are constructed with a so called "open-tip" design, **Figure 5**. In experienced hands, EUS-FNA is a safe procedure with a low risk for infection, bleeding, perforation, and pancreatitis[102]. EUS-FNA is well aimed for certain types of lesions, such as malignant lymph nodes[103,104]. Rapid on-site cytology evaluation (ROSE) may increase the yield of EUS-FNA but the diagnostic benefit is debated[105,106].

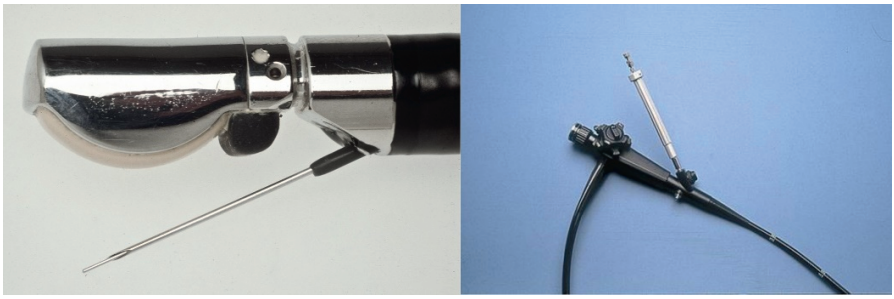

Figure 5. ***Left****: A first generation echoendoscope (Pentax FG 32 UA) with a 2 mm working channel. The transducer is of the type curved linear-array, which enables the ultrasonographic visualization of the open-tip FNA-needle (with a stylet) which protrudes from the working channel and appears below the transducer.* ***Right****: The first dedicated instrument for the performance of EUS-guided sampling mounted on an echoendoscope. The device was developed by the Danish surgeons dr Peter Vilmann and dr Soren Hanecke in the early 1990´s (GIP-Medizintechnik/Mediglobe GmbH, 1993). Photos reprinted with courtesy of Prof Peter Vilmann. Herlev Hospital.*

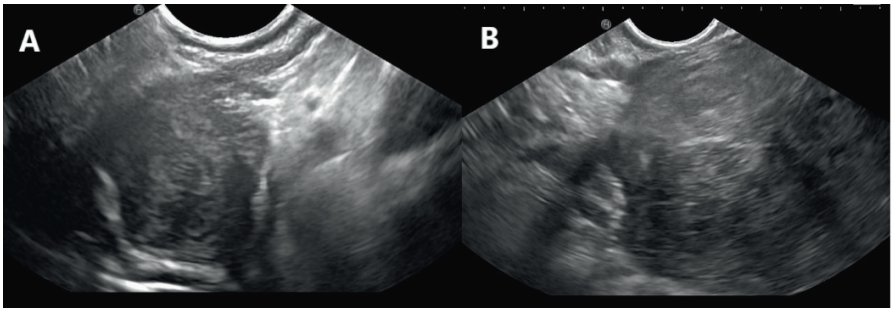

Figure 6. *The endosonography image is informative but cannot distinguish all diagnostic entities from one another.* ***Left*** *(A): A hypoechoic lesion arising from the forth wall layer of the gastric fundus.* ***Right*** *(B): A much similar lesion with the same echocharacteristics also situated in the gastric fundus. EUS-FNB including diagnostic immunostaining revealed that (A) was a benign leiomyoma and (B) was a gastrointestinal stromal tumor. The leiomyoma should be left without further treatment, while the GIST should be treated with surgical resection if possible. Images by the author.*

1.4.4 Unmet needs in EUS-FNA

However, EUS-FNA is imperfect. Performed in solid pancreatic lesions, EUS-FNA has only a moderate sensitivity for malignancy (~85 %) according to a large meta-analysis[107]. Moreover, the vast majority of publications have included mostly ductal adeocarcinomas while few studies have addressed other entities such as neuroendocrine tumors and metastases[105,108]. In the case of subepithelial lesions, EUS-FNA performs even worse with a low diagnostic accuracy leaving as much as every second patient without a diagnosis[109,110]. Lesions measuring < 2 cm is even more demanding[111].

In GIST, the preoperative diagnostics by EUS-FNA is difficult as such. The treating clinician is also burdened by the fact that a correct diagnosis is not enough. Preoperative information on the tumor proliferation rate and the genetic profile of *KIT* and *PDGFRA* is a must to offer patients a personalized care at an early stage.

Big effort has been invested in improving the accuracy of EUS-FNA. The use of suction during sampling is beneficial[112]. Unfortunately, most trials addressing other measures have been discouraging. The use of a 22 gauge needle, which is somewhat larger compared with the standard 25 gauge needle, is futile[113,114]. A number of FNA-passes beyond four is of no use[115,116]. Two passes is often sufficient if ROSE if performed[116]. Sampling with the use of a stylet[117] or by the use of slow stylet retraction ("slow-pull")[118] does not improve the yield of EUS-FNA. Obviously, there is a great need for alternative approaches.

1.4.5 EUS-guided fine needle biopsy sampling (EUS-FNB)

Due to the drawbacks of EUS-FNA there has been no lack of attempts to acquire whole tissue by EUS for the processing of histology specimens. Unfortunately, the first generation of EUS-needles aimed for histology (EUS-TCB) showed disappointing results with a high frequency of technical failures[109], low yield, and non-superior diagnostic accuracy compared with EUS-FNA[119]. Problematically, the lack of appropriate samples has also disabled the performance of preoperative Ki-67-indexing and DNA-sequencing of GIST.

In recent years a new generation of biopsy needles (EUS-FNB) has been developed. The tip-design differs somewhat in between these needles [120-122] but they are all, such as the side-fenestrated, reverse bevel FNB-needle[121], aimed for the acquisition of whole tissue.

Whether or not the use of the new FNB-needles and the processing of histology specimens can improve the diagnostics of SPL and SEL is not known. Prospective studies are lacking. Moreover, the patient safety of EUS-FNB has not been properly evaluated. Is there any clinical benefit motivating a shift from EUS-FNA? Finally, and maybe most worrying, there is a complete lack of studies addressing the important issue of pretreatment characterization of GISTs. Potentially, tissue acquired by EUS-FNB could be the valuable source for this information.

1.5 The research field in summary

In summary, despite modern diagnostic equipment the physicians and the surgeons of today still face numerous pancreatic and subepithelial lesions with unknown malignant potential and unclear prognosis. Concerning gastrointestinal stromal tumors, the preoperative lack of information on the tumor genetics and the tumor proliferation rate is equally problematic in the clinical context.

At present, and due to the lack of a definitive diagnosis, the clinical management of the above lesions is commonly erroneous with a substantial risk of maltreatment. In benign lesions, there is a high risk of unwarranted resection leading to patient morbidity. In malignant lesions, there is a risk of delayed, targeted therapy. The pretreatment acquisition of appropriate tumor material and the extensive analyses of this material would be a crucial step towards true personalized medicine.

2 AIMS

The global aim of the studies included in this thesis was to investigate a biopsy approach in EUS-guided sampling procedures with respect to the *patient safety* and the *diagnostic accuracy*. The focus was the sampling procedures performed in suspected neoplasms presenting as solid pancreatic lesions or as subepithelial lesions.

A complementary aim was to evaluate the *clinical impact* of using the biopsy approach as compared with routine EUS-guided fine-needle aspiration.

A final aim was to explore the *feasibilit*y and *clinical importance* of pretreatment genetic profiling and tumor risk assessment of gastrointestinal stromal tumors by the use of tissue acquired by EUS-guided sampling.

3 PATIENTS AND METHODS

The research presented in this thesis was performed using a deductive, empirical approach and a quantitative methodology meaning that first the study hypotheses were formulated and then they were tested by the measurement of different variables.

There are two main types of study design. In the *observational* study, the course of events within the study population is (presumably) not affected by the conduction of the study[123,124]. The observational study enables the researcher to identify *associations,* but not necessarily *causality*, in between the study variables. As an example, overweight has been found to be associated with an accelerated regional bowel transit[125]. Whether or not overweight was the cause of the detected fast transit remains however unclear (it could be vice versa).

The current thesis is based on *interventional* studies[124], which have the aim to draw conclusions on *causality*, i.e. if the study intervention (the independent variable) results in a detectable difference in the study outcome (the dependent variable). As an example, bowel cleansing with sodium picosulfate, as compared with polyethylene glycol, was found to result in less patient discomfort[126]. Some property of the cleansing fluid was the probable cause of the improved patient tolerance.

3.1 Study design and patient selection

The Sahlgrenska University Hospital (SU) is the tertiary center in the western part of Sweden (*Västra Götaland* and *Halland*) for patients with pancreatic malignancies, neuroendocrine neoplasms, sarcomas, and gastrointestinal stromal tumors. From centers within this region, a vast majority of new cases with such a suspected diagnosis is referred to SU for diagnostic work-up and care. The GEA endoscopy unit of SU (SU-GEA) is the tertiary endosonography center of the region performing all but few of the EUS-examinations in these patients.

The study characteristics of the four papers included in this thesis are presented in **Table 1**.

Table 1 *Study characteristics of the four papers*

	Paper I	**Paper II**	**Paper III**	**Paper IV**
Design	Prospective, interventional	Prospective, interventional	Prospective, exploratory	Prospective, interventional
Population	SPL	SEL	GIST	GIST
Patients (n)	68	70	44	59
Study aim	Improve diagnostics	Improve diagnostics	Explore new method	Implement new method
Intervention	EUS-FNB	EUS-FNB	(EUS-FNB)	Pretreatment sequencing
Comparison	EUS-FNA	EUS-FNA	(EUS-FNA)	Posttreatment sequencing
Ref cohort	2006-2011	2006-2011	2006-2011	2006-2013
Outcome(s)				
Primary	Diagnostic accuracy	Diagnostic accuracy	Diagnostic accuracy	Therapy adequacy
Secondary	Adverse events	Adverse events Clinical impact	Sample adequacy for Ki-67 and MA	Tumor response

*) MA=mutational analysis

Paper I-III

During 2006–2011, all patients subjected to EUS-guided sampling at SU-GEA were included in a EUS-quality research project with the intent to evaluate the performance of EUS-FNA (or EUS-TCB in few cases). This cohort was the *base-line cohort*, **Figure 7.** These patients were part prospectively (2009–2011), part retrospectively (2006–2008) included.

In 2011, and due to the drawbacks of EUS-FNA, we designed a single-center, prospective, interventional study on the diagnostic accuracy of reverse-bevel EUS-FNB amongst all in solid pancreatic lesions and subepithelial lesions including GISTs. The study was registered in the ClinicalTrials.gov database (NCT02360839).

The interventional study was launched in 2012 and continued until the end of 2015. This time frame was the *study cohort*. Patient recruitment and inclusion of the *study cohort* was exclusively performed at the SU-GEA. The patients were identified at referral and enrolled consecutively as study subjects by one of the endosonographers (RS/PH).

During a short *pilot phase* in the beginning of 2012, the new reverse bevel FNB-needle was tested by the endosonographers in some single needle EUS-FNB procedures (SPL: n=6; SEL: n=10). The specific inclusion and exclusion criteria (*Paper I-II*) are presented in **Figure 8**. Regarding Paper *III*, only lesions with a final diagnosis of GIST were included for further analyses.

The patients included in *Paper I* were not included in any of the other papers. Some patients included in *Paper II* were also included in *Paper III*. Some patients included in *Paper III* were also included in *Paper IV*.

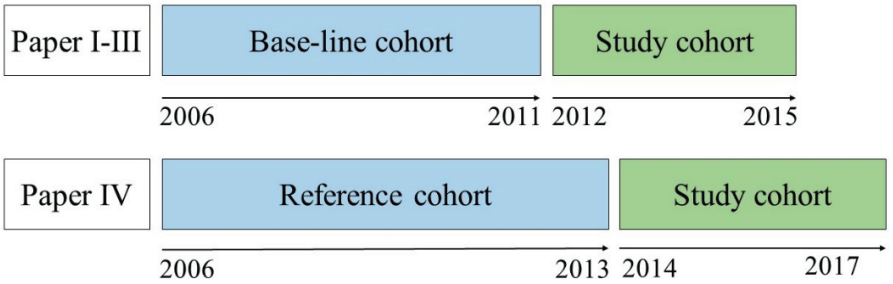

***Figure 7**. A timeline of the different study periods (Paper I-IV). Boxes in green symbolize the two main study cohorts, while the boxes in blue symbolize the historical cohort of patients of the same center used for reference and comparison.*

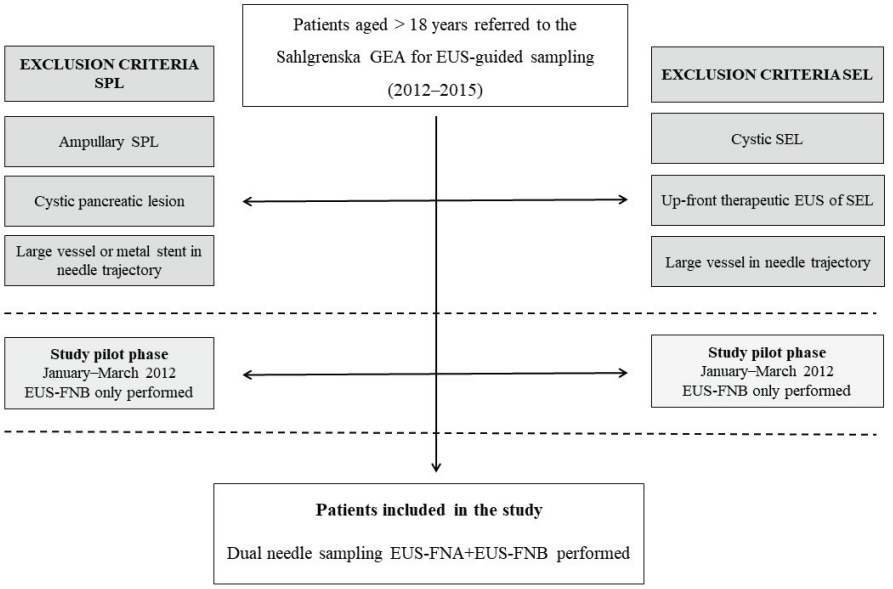

***Figure 8**. A flow-chart of the enrollment process (Paper I-II). Exclusion criteria in dark grey boxes. The study pilot phase within the dashed lines.*

Paper IV

Exploratory DNA-sequencing of GISTs by the use of FNB-tissue was initially performed (*Paper III*). Encouraged by the positive results, we decided in 2014 to initiate a single-center, prospective, interventional study investigating immediate, pretreatment sequencing of FNB-tissue as a routine analysis in the work-up of GIST-patients with a need for down-sizing imatinib therapy.

Eligible study subjects for prospective inclusion (2006–2017) were the patients with a highly suspicious GIST, who were referred to SU and evaluated for down-sizing imatinib therapy. The patients considered appropriate for up-front surgical resection or for watchful waiting were excluded as were patients in whom GIST was never confirmed by histopathology. Patient recruitment was performed at the SU-GEA or in some cases at the Surgery Department outpatient Unit by the study surgeon (BN)[37].

During 2006–2013 (the *reference cohort*, RC) no pretreatment sequencing of preoperative GIST-tissue or cells was performed, **Figure 7**. Sequencing was instead performed on surgical specimens or on EUS-samples late after the procedure.

During 2014–2017 (the *immediate sequencing cohort*, ISC) all eligible patients were subjected to high-priority, pretreatment EUS-FNB with immediate sequencing (<2 weeks) of the acquired tumor material, **Figure 7**. Single-needle EUS-FNB was performed after January 2016. In the few cases already diagnosed at the time of referral, i.e. by endoscopy forceps or transabdominal ultrasound-needle biopsy (TUS-NB), no EUS was performed but instead the obtained tumor material was used for sequencing.

3.2 Ethical considerations

This research project, including all the papers here presented, was reviewed and approved by the Regional Ethical Review Board of Gothenburg (REPN, Project ID: 573-09 and 1092-11).

In accordance with the Helsinki Declaration[127] written, informed consent was obtained from all study patients enrolled prospectively.

3.3 Specific methodological considerations

3.3.1 Endosonography and EUS-guided sampling

After written, informed consent, all the patients fulfilling the inclusion criteria were examined by EUS as further specified in *Paper I*/*II*. Except for the *pilot phase* cases, the sampling of all lesions was performed with both EUS-FNA for cytology (*Paper I*: a 25 gauge needle; *Paper II/III*: a 22 gauge or a 25 gauge needle) and with reverse bevel EUS-FNB for histology (*Paper I*: a 22 gauge needle; *Paper II/III*: a 19 gauge or a 22 gauge needle). A photo of the needle tips is shown in *Paper II* (Figure 1).

By blocks of four and by using sealed envelopes the patients were randomized to first pass with FNA or FNB. The second pass was performed with the other needle. Further passes were performed by alternating the needles. A technical failure was defined as the non-ability to target the lesion. In both EUS-FNA and EUS-FNB, the needle tip was placed, if possible, in a non-necrotic part of the lesions. During 5–15 seconds and with suction applied (10 ml), the needles were moved 8-10 times to and fro in different directions during each pass, i.e. "fanning"[128], **Figure 9**. The suction was increased (20 ml) if poor initial yield. Rapid on-site evaluation (ROSE) was performed when the cytotechnician was available. The samples were then handled as further described in *Paper I/II*.

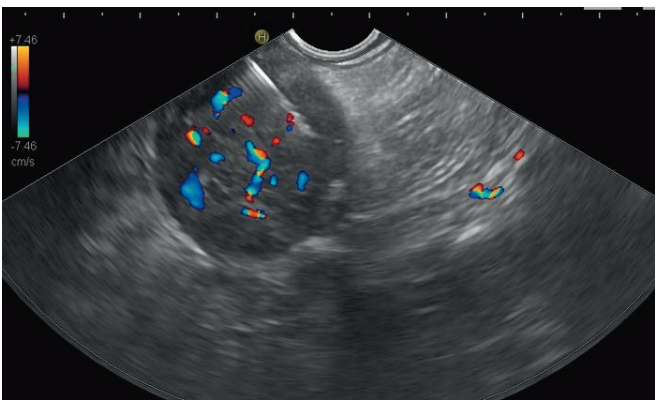

Figure 9*. Endosonography-guided fine-needle aspiration of a hypoechoic, intensely vascularized pancreatic neuroendocrine tumor. The EUS-needle presents as a thin white line and appears from the left in the upper part of the tumor. Image by dr Sadik.*

3.3.2 Cytology, pathology, and immunohistochemistry

The FNA-samples were directed to the study cytopathologist (AD) and the FNB-samples to the study pathologist (ON). After the preliminary diagnosis based on the cellular morphology (hematoxylin-eosin-staining), immunostaining was performed using entity-specific monoclonal antibodies as outlined in *Paper I-III*.

3.3.3. The assessment and classification of samples

Based on the cytomorphology, the FNA-samples and the FNB-samples were assessed as *representative* or *non-representative.* The *non-representative* samples, being defined as acellular aspirates or biopsy specimens, contaminated epithelium only, or obscuring artifacts, were per definition categorized as non-diagnostic. Only the samples containing adequate cells or whole tissue of the target lesion were regarded *representative*. Based on the cellular morphology and the immunostaining pattern, the *representative* samples were then further categorized as diagnostic or non-diagnostic according to the details and prerequisites in *Paper I-III*.

3.3.4 Follow-up, retrieval of clinical data, and reference standard

The study subjects were monitored post-EUS by visits to the outpatient unit of the Sahlgrenska University Hospital, which was responsible for the decision on surgical resection and on tyrosine kinase inhibitor therapy (GIST). The medical files of all cases were carefully reviewed post-EUS at least until the final diagnosis was established or until patient death. The diagnostic work-up before and after the EUS was recorded. In patients subjected to surgery, the pathology report of the resected specimen was used as the reference standard. In not resected cases, a conclusive (cyto)pathology report of the EUS-sampling itself or of an alternative sampling modality was accepted. If a tissue-based final diagnosis was not obtained, the clinical diagnosis at a minimum of 12 months follow-up was used.

The Standards for Reporting of Diagnostic Accuracy (STARD) protocol[129-131] was applied during the conduct of the research project.

3.3.5 Analysis approach

Two different approaches can be applied in the evaluation of diagnostic tests. In the *per-protocol analysis* only the evaluable cases count[132]. This implicates that only technically successful procedures and procedures with an adequate yield are included in the analysis. In the *intention-to-diagnose analysis* all cases count[133]. In the context of an EUS-sampling study, this means that both technical failure procedures and procedures with an inadequate or non-representative yield are included in the analysis. The intention-to-diagnose approach will in most scenarios lead to a lower diagnostic accuracy as compared with the per-protocol analysis[134]. Meanwhile, it provides a more realistic picture of the intrinsic potential and clinical utility of various diagnostic tests[133].

Repeated procedures should not be included in the calculation of the diagnostic accuracy since such a generous approach inevitably leads to the risk of overestimating the utility of a certain diagnostic test. In *this thesis*, a conservative approach was applied by using the *intention-to-diagnose analysis* and by including only the index-sampling procedures.

3.3.6 Patient safety (Paper I-III)

The medical files of the study subjects were carefully reviewed post-EUS to detect any adverse post-EUS. Via the digital system of the institution, any visit to a Swedish hospital can be detected. In addition, the referring institution was requested to report any adverse event. An adverse event was defined as a complication within 30 days (such as pancreatitis, infection, or bleeding), which resulted in patient contact with or patient care by any hospital department.

3.3.7 Measurement of the clinical impact of EUS-FNB (Paper II)

To analyze the clinical impact of EUS-FNB (2012–2015) we used as comparison the *base-line cohort* (2006–2011), **Figure 7**, during which routine EUS-FNA (or EUS-TCB in few cases) was performed. In the base-line cohort and the study cohort respectively, any additional, diagnostic procedure performed after a non-diagnostic EUS and any unwarranted surgical resection of the targeted lesions was recorded. A surgical resection was defined as

unwarranted if the subsequent pathology report demonstrated a diagnosis that should have been managed conservatively.

3.3.8 Sequencing of *KIT* and *PDGFRA* (Paper III & IV)

All the FNB-biopsies of the GISTs included in *Paper III* and *Paper IV* (2012–2017) were subjected to tumor DNA-sequencing of *KIT* and *PDGFRA*. Initially (2012–2013) the sequencing was performed on a research basis only and late after the date of the EUS. Later (2014–2017), the sequencing was performed immediately after the date of the EUS (<two weeks). In the cases not subjected to EUS (*Paper IV*), the pretreatment sequencing was instead performed on tissue acquired by transabdominal ultrasound (TUS-NB) or gastroscopy-forceps.

Two different methods for sequencing were applied. In 2012–2015, Sanger sequencing, **Figure 2**, was the method used as thoroughly described in *Paper III*. In 2016–2017, Next Generation Sequencing (NGS) was the method used as described in *Paper IV* and outlined in **Figure 10**.

All the corresponding resected specimens of the resected subjects included in *Paper III* were also subjected to sequencing. The mutations detected in the FNB-tissue were compared with the mutations detected in the corresponding surgical specimens in a case-by-case basis (*Paper III*). Based on the individual mutation profile and in accordance with the available literature of the field, each GIST-case was categorized concerning the sensitivity for imatinib (Table 1 in *Paper IV*).

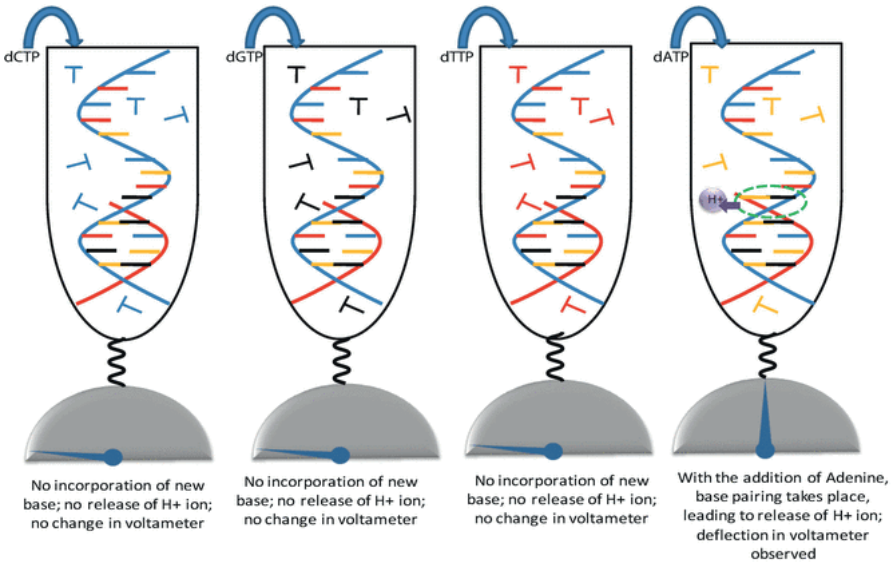

Figure 10. *Next generation sequencing by the Ion torrent technique (Life Technologies). After barcoding of DNA and emulsion PCR, the amplified tumor DNA-fragments are loaded on a semiconductor chip containing thousands of microwells. These wells are then flooded with DNA polymerase and the different dNTPs in a step-wise and repeated manner. With, and only with, the incorporation of a complementary base, a proton is released, which leads to the change in the pH of the micro-environment (far right) detected by an ion-sensitive transistor. Base by base, the sequence of the incorporated bases can then be deciphered. Adapted from Verma et al and reprinted with permission from Springer Nature.*

3.3.9 Measurement of the Ki-67-index (Paper III & IV)

The pretreatment EUS-FNA-aspirates (*Paper III* only), the EUS-FNB-tissue, and the corresponding surgical specimens of all GISTs were subjected to immunostaining of the proliferation marker Ki-67. First, the tumor cell count was categorized as adequate or non-adequate for the evaluation of the Ki-67-index by the study cytopathologist (AD) and pathologist (ON).

Second, manual counting of the Ki-67-index was carried out both in the FNB-tissue ($Ki\text{-}67_{EUS}$) and in the corresponding surgical specimens ($Ki\text{-}67_{SURG}$) as further described in *Paper III*. Finally, a case-wise comparison of the $Ki\text{-}67_{EUS}$ and the corresponding $Ki\text{-}67_{SURG}$ was performed.

3.3.10 Tumor response to down-sizing imatinib (Paper IV)

In both cohorts (ISC: 2014–2017; RC: 2006–2013), the evaluation and the measurement of the tumor response to down-sizing imatinib therapy was performed in the patients receiving standard dose therapy (400 mg daily). The evaluation was performed by the use of CT scan, ^{18}FDG-PET, and Ki-67-indexing (as above). The first two methods were performed twice at the preoperative stage; both before (base-line exam) and after (evaluation exam) the initiation of down-sizing imatinib. In CT scan, and in line with the RECIST-criteria, a positive tumor response was defined as a tumor size reduction of at least 30 % (partial response)[135]. In ^{18}FDG-PET, a positive tumor response was defined as a complete or a partial signal reduction, while a signal with no reduction was considered a negative tumor response[136]. The Ki-67-index reduction was measured by comparing the pretreatment sample with the resection specimen.

3.3.11 Outcomes

The study outcomes in each of the papers are specified in **Table1** and in each of the papers (*Paper I-IV)*.

3.4 Statistical considerations

3.4.1 Sample size calculations and study hypotheses

To prove or falsify the pre-study, suggested hypothesis one has to estimate the number of cases needed in the study. Three factors have to be taken into account and included in the *sample size calculation*:

1) A study including a small number of cases (observations) has the obvious risk of not detecting an actually existing difference between the compared groups. This error is called a type II-error (*beta*). In the papers of this thesis, the statistical power (1 – *beta*) was set at 0.8, i.e. there was an 80 % probability of detecting an actually existing difference.

2) The opposite error, i.e. the detection of a difference between groups not actually existing, is called a type I-error (*alpha*). In accordance with common

practice, the *alpha*-value was set at 0.05, i.e. there was a 5 %-risk of detecting a non-existing difference.

3) The third factor is the minimum difference between groups, which the researcher aims to detect. In the papers of this thesis the minimum detectable difference was determined based on historical data of our center.

The null hypothesis of *Paper I, II*, and *III* was that the diagnostic accuracy of EUS-FNB was equal to routine EUS-FNA. A sample size calculation based on this hypothesis and for the comparison of two paired proportions (Mc Nemar's test) was performed including the above three factors[137] and by using a web-based calculator (http://powerandsamplesize.com/Calculators). The following number of study cases required was returned: n=66 (*Paper I*), n=59 (*Paper II*), and n=33 (*Paper III*). Regarding *Paper IV*, a sample size calculation was performed likewise but for the comparison of two unpaired proportions[138]. The null hypothesis was that down-sizing therapy of GISTs guided by pretreatment sequencing is equally often correct as down-sizing therapy initiated by chance. By knowing the results and the fixed number of patients included in the reference cohort (RC), the sample size required in the immediate sequencing cohort (ISC) could be calculated. A number of 59 cases required (ISC) was returned.

3.4.2 The presentation of data and statistical tests

Descriptive, base-line data were mainly expressed as the median and the range or as the in-variable distribution in numbers and percentages (categorical variables).

Before choosing the appropriate statistical tests for the comparison of different study variables, an initial analysis of the data distribution and the independence of data was performed. Then, the appropriate parametric tests were applied for the continuous variables with a sufficient number of observations and with a normal distribution of data. Regarding the categorical variables (and continuous variables with few observations or with a skewed distribution) the appropriate non-parametric tests were used. Independent groups were compared using tests for unpaired data whereas dependent groups were compared using tests for paired data.

An overview of the statistical tests used in the different papers of this thesis is presented in **Table 2**. Details on when the different tests were applied are found in the respective paper (*Paper I-IV*).

The 95% confidence interval (95% CI) was calculated and presented when the number of observations was sufficient, since that, compared with plain p-values, gives a better perception of the reliability of a certain finding[139].

All statistical calculations were performed using the software SPSS Statistics for Windows (version 22.0, IBM Corp., Chicago, IL, USA). All tests were two-tailed and conducted at a statistical significance level of $p<0.05$.

Table 2 *Tests used for the statistical calculations*

	Paper I	**Paper II**	**Paper III**	**Paper IV**
Parametric tests				
Student's t-test		x		x
Non-parametric tests				
Mann-Whitney U-test (unpaired data)	x	x	x	x
Fisher's exact test (unpaired data)	x	x	x	x
Mc Nemar's test (paired data)	x	x	x	
Wilcoxon sign rank test (paired data)			x	

4 RESULTS

4.1 The diagnostic accuracy of EUS-FNB (Paper I-III)

4.1.1 Sampling of solid pancreatic lesions (Paper I)

A total of 68 study patients (m/f: 32/36; median age: 67) with SPLs were subjected to dual needle sampling EUS-FNA and EUS-FNB. EUS-FNB had a similar overall diagnostic accuracy and sensitivity for malignancy compared with EUS-FNA, **Figure 11**. The combined modality EUS-FNA+FNB had a higher sensitivity for malignant entities other than PDAC, but was not significantly superior to single EUS-FNA in PDACs. There was no technical failures recorded neither using EUS-FNB nor EUS-FNA.

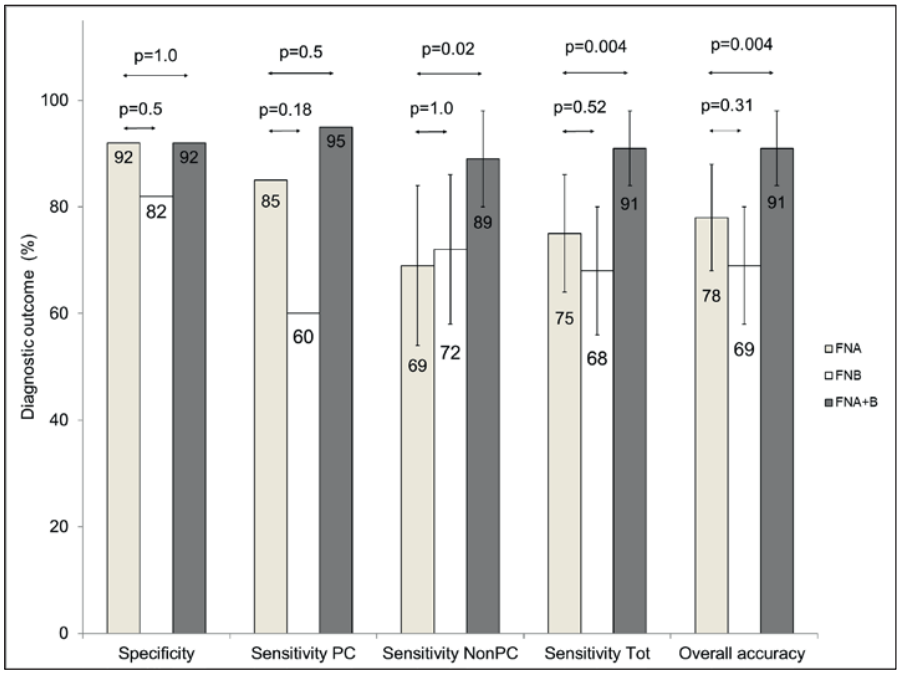

***Figure 11**. The diagnostic outcomes (%) of sampling of SPLs (n=68). EUS-FNA (bars in light grey), EUS-FNB (white), and the combined modality EUS-FNA+FNB (dark grey). The error bars equal the 95% CI. The diagnostic sensitivity for ductal adenocarcinoma (PC) is represented by bars second to the far left, for non-PC tumors in the center, and for the whole group of neoplasms by bars second to the far right.*

4.1.2 Sampling of subepithelial lesions (Paper II)

A total of 70 study patients (m/f: 34/36; median age: 68) with SELs were subjected to dual needle sampling EUS-FNA and EUS-FNB, **Figure 12**. EUS-FNB had a significantly higher overall diagnostic accuracy and sensitivity compared with EUS-FNA, **Figure 13**. EUS-FNA was non-diagnostic in all the lesions (n=12) in which EUS-FNB was not conclusive for the diagnosis.

There was one technical failure recorded regarding EUS-FNB. In one patient with a 50 mm duodenal tumor the 22 gauge FNB-needle was not possible to place in adequate position (failure rate: 1/86, 1.2%). There was no technical failure of EUS-FNA.

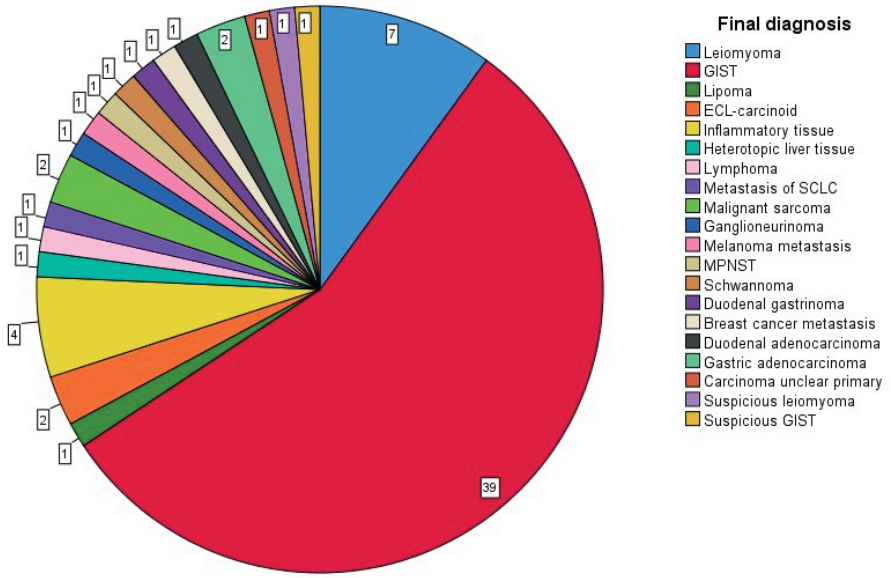

***Figure 12**. The spectrum (n) of diagnostic entities presenting as subepithelial lesions in Paper II. ECL-carcinoids = Enterochromaffin-like cell carcinoids. SCLC=Small cell lung cancer. MPNST=Malignant peripheral nerve sheet tumor.*

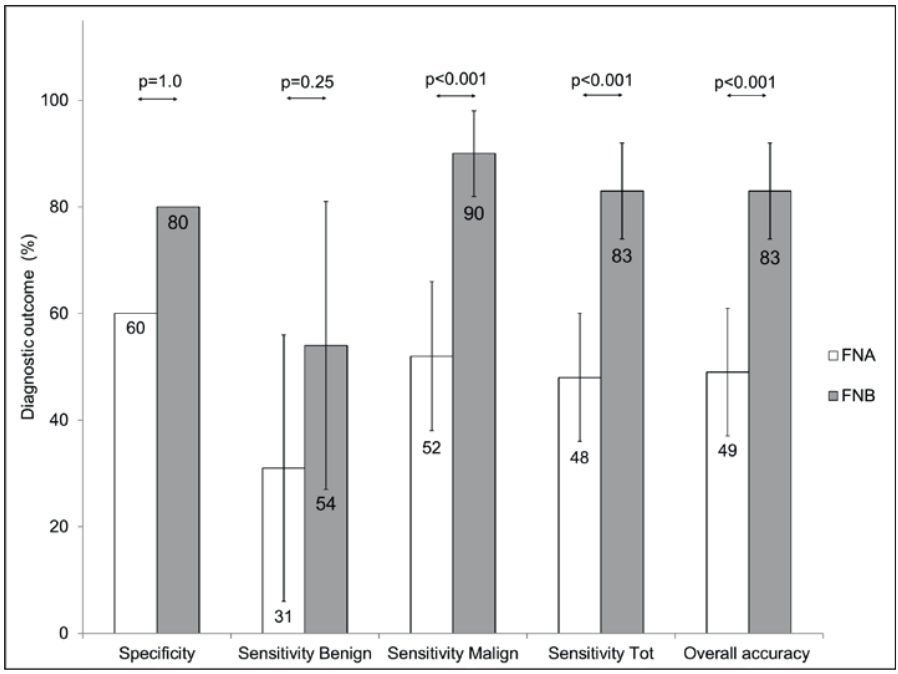

Figure 13*. The diagnostic outcomes (%) of sampling of subepithelial lesions (n=70). EUS-FNA (bars in white) and EUS-FNB (bars in grey). The error bars equal the 95% CI. The diagnostic sensitivity for benign neoplasms is represented by bars second to the far left, for malignant neoplasms by bars in the center, and for the whole group of neoplasms by bars second to the far right.*

4.1.3 Sampling of GISTs (Paper III)

A total of 44 study patients (m/f: 19/25; median age: 68) with GISTs were subjected to dual needle sampling EUS-FNA+EUS-FNB (n=38) or single needle EUS-FNB (n=6). EUS-FNB had a diagnostic sensitivity for GIST of 43/44 (98 %) and was superior to EUS-FNA in dual needle sampling procedures 37/38 (97%) *vs* 22/38 (58%), p<0.001. The diagnostic sensitivity of EUS-FNB was also superior compared with the sensitivity of EUS-FNA performed in the *baseline cohort* 2006–2011*,* 43/44 (98%) *vs* 8/16 (50%), p<0.001. There was no technical failure recorded neither using EUS-FNB nor EUS-FNA.

4.2 The patient safety of EUS-FNB (Paper I-III)

The performance of reverse bevel EUS-FNB was found to be safe and associated with few adverse events both in solid pancreatic lesions and in subepithelial lesions.

Regarding SPLs, there was one recorded event among a total of 74 EUS-FNB-procedures performed (adverse event rate: 1.4%). A 67-year-old man developed a necrotizing pancreatitis after transduodenal, single-needle EUS-FNB of a lesion located in the periampullary region. The procedure was performed during the pilot phase of the study. The patient stayed for 4.5 months in hospital. This incident motivated the exclusion of ampullary lesions from study inclusion.

Regarding SELs, there was one recorded event among a total of 86 EUS-FNB-procedures performed (adverse event rate: 1.2%). A 68-year old man had a post-FNB bleeding in a highly vascularized, 30 mm gastric GIST. As a result the patient developed melena the night after the procedure with need for erythrocyte transfusion. The bleeding was stopped the day after by gastroscopy and local injection of epinephrine.

As a complimentary finding, the intense dual needle sampling approach (FNA+FNB) during the *study cohort* was not associated with an increase of the adverse event rate as compared with the single needle EUS-FNA-procedures of the *base-line cohort* [SPL: 0/68 (0%) *vs* 0/102 (0%), p=1.0; SEL: 1/70 (1.4%) *vs* 0/59 (0%), p=1.0].

4.3 The clinical impact of EUS-FNB (Paper II)

EUS-FNB performed in the *study cohort* of subepithelial lesions (2012–2015) resulted in the reduced performance of an additional diagnostic procedure compared with the *base-line cohort* (2006–2011), **Table 3**. There were also fewer unwarranted resections performed in the *study cohort* compared with the *base-line cohort*, 3/48 (6%) *vs* 12/35 (34%), p=0.001.

Table 3 *Additional procedures post-EUS of subepithelial lesions*

	Study cohort	Base-line cohort	p-value
Diagnostic procedures post-EUS			
All lesions (n)	83	73	
No additional diagnostic procedure, n (%)	71 (86)	34 (47)	
Additional diagnostic procedure, n (%)	**12 (14)**	**39 (53)**	**<0.001**
Diagnostic surgical resection or biopsy	*5*	*19*	
Repeated EUS	*3*	*13*	
Repeated EUS and diagnostic resection	*-*	*3*	
PET-CT	*-*	*2*	
Transabdominal sampling	*1*	*1*	
Diagnostic endoscopic EMR[a]	*1*	*1*	
Broncoscopy	*1*	*-*	
Repeated endoscopy forceps biopsy	*1*	*-*	
Malignant lesions (n)	63	38	
No additional diagnostic procedure, n (%)	57 (90)	21 (55)	
Additional diagnostic procedure, n (%)	**6 (10)**	**17 (45)**	**<0.001**
Diagnostic surgical resection or biopsy	*2*	9	
Repeated EUS	*2*	4	
Repeated EUS and diagnostic resection	*-*	1	
PET-CT	*-*	2	
Transabdominal sampling	*1*	1	
Diagnostic endoscopic EMR	*-*	*-*	
Broncoscopy	*-*	*-*	
Repeated endoscopy forceps biopsy	*1*	*-*	

[a]) EMR=endoscopic mucosal resection

4.4 The sequencing in FNB-tissue of GIST (Paper III)

The FNB-tissue acquired from the 44 GIST-patients in *Paper III* was adequate for successful Sanger sequencing of *KIT* and *PDGFRA* in 43/44 (98%) of the cases, **Figure 14** and Table 3 in *Paper III*. Among the resected patients (n=27), there was full congruence (100%) comparing the mutations detected in the FNB-tissue and the mutations detected in the corresponding resection specimens. No secondary mutations in *KIT* or *PDGFRA* were detected in the resection specimens.

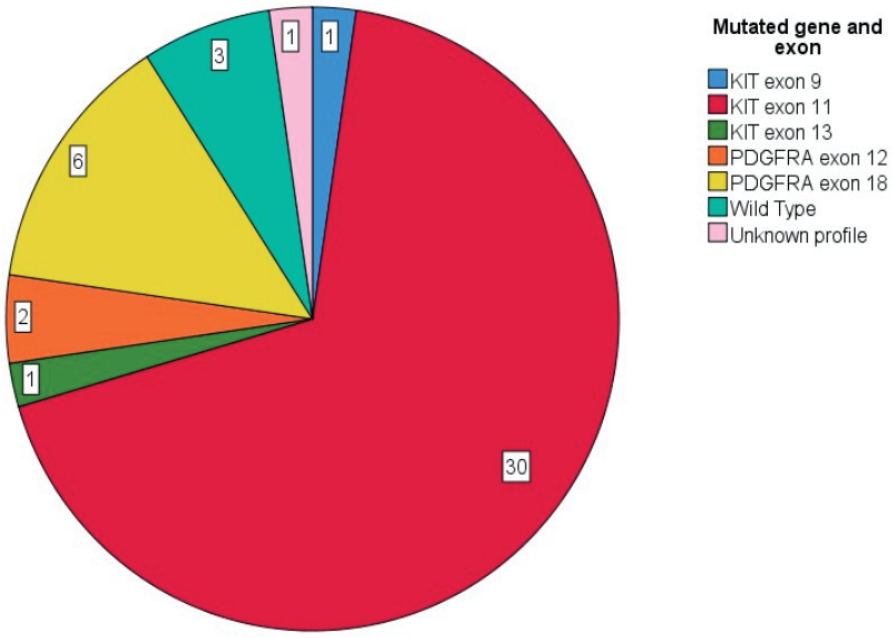

***Figure 14**. The spectrum (n) of mutated genes and exons (KIT and PDGFRA) detected in the EUS-FNB-tissue of the GISTs included in Paper III (n=44).*

4.5 The Ki-67-index in FNB-tissue of GIST (Paper III)

In the patients subjected to dual needle sampling (n=38), the EUS-FNB-tissue was more often adequate for the evaluation of the Ki-67-index compared with the EUS-FNA-aspirate (35/38, 92%, *vs* 15/38, 40%, p<0.001). The Ki-67-index of the FNB-tissue could be calculated in all cases subjected to surgical resection 27/27 (100%).

In the patients subjected to up-front surgical resection without down-sizing imatinib (n=12), the Ki-67_{EUS} did not significantly differ from the Ki-67_{SURG}: Ki-67_{DIFF} = -0.30 (95% CI: -0.62 to 0.57, p=0.64), **Figure 15**. There was no significant reduction of the Ki-67-index [median Ki-67_{RED}= 10.7% (95% CI: -22.3% to 26.5%, p=0.70)].

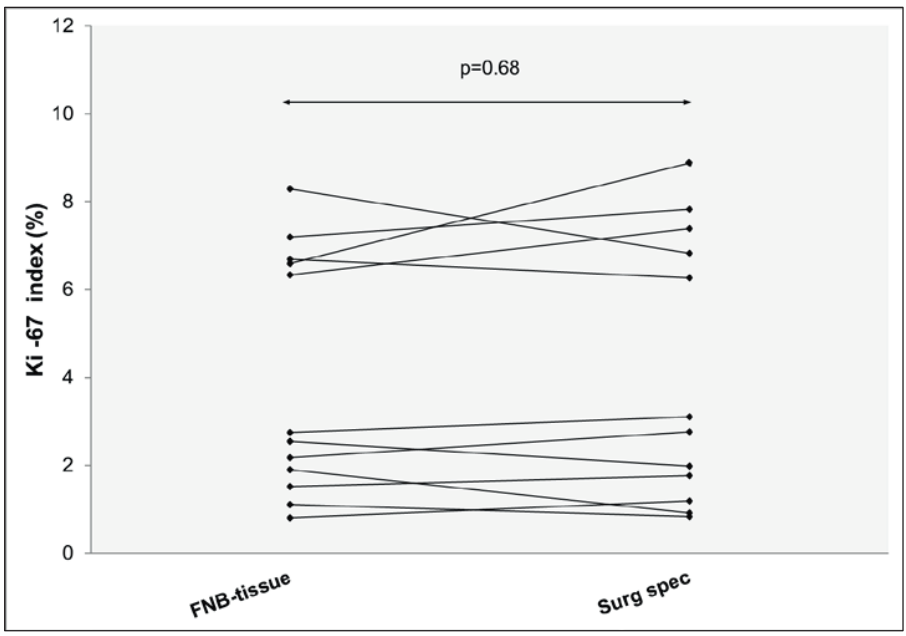

Figure 15. *The Ki-67-index (%) assessed in the EUS-FNB-tissue and in the corresponding surgical specimen in each of the patients (n=12) who underwent resection without preceding down-sizing imatinib therapy (Paper III).*

4.6 The evaluation of down-sizing imatinib efficacy by the Ki-67-index reduction (Paper III)

Fifteen patients were resected and treated with preoperative down-sizing imatinib. In the patients carrying mutations indicating imatinib sensitivity [n=10: *KIT* exon 11 (n=9); *PDGFRA* exon 12 (n=1)], the tumor proliferation rate was found significantly higher in the pretreatment FNB-tissue compared with the corresponding resection specimen: $Ki\text{-}67_{DIFF}$ = 2.3 (95% CI: 0.67 to 5.37, p=0.005), **Figure 16**. There was also a significant reduction of the Ki-67-index: $Ki\text{-}67_{RED}$ = -91.5% (95% CI: -82.4% to -96.0%, p=0.005). The resected tumors carrying mutations suggestive of primary resistance (n=5) showed no obvious difference in the Ki-67-index, *Paper III*.

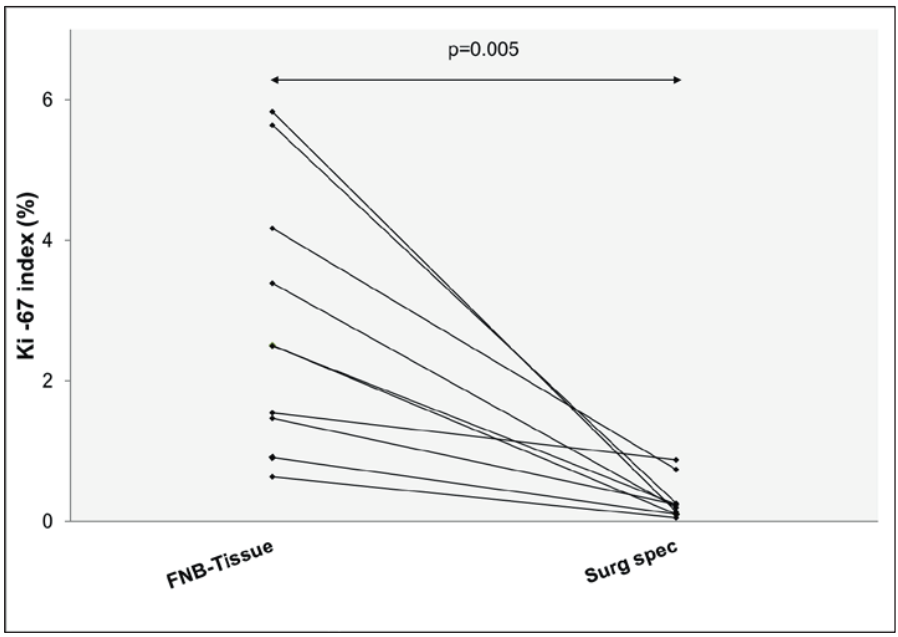

***Figure 16**. The Ki-67-index (%) assessed in the EUS-FNB-tissue and in the corresponding surgical specimen in each of the patients (n=10) who underwent resection after down-sizing imatinib therapy. In all ten tumors a mutation was detected indicating full sensitivity to imatinib [KIT exon 11 (n=9); PDGFRA exon 12 (n=1)].*

4.7 Guiding down-sizing therapy by pretreatment sequencing of GIST-tissue (Paper IV)

Immediate, pretreatment sequencing of GIST-tissue (EUS-FNB n=46; TUS-NB n=7; Forceps n=6) was successfully carried out in 57/59 (97%) patients of the study cohort (ISC: 2014–2017). Consequently, the number of patients receiving a correct down-sizing regimen was significantly higher in this cohort (ISC) compared with the reference cohort (RC: 2006–2013), 57/59 (97%) *vs* 33/47 (70%), p<0.001, **Table 4**.

	Correct Ther	**Incorrect Ther**	**P-value**
ISC	57	2	<0.001
RC	33	14	

	Standard IMA [a]	**High IMA**	**Alternative TKI** [b]	**No TKI therapy**
ISC				
KIT exon 11[c]	40			**1**
KIT exon 13 (p.K642E)	1		1	
PDGFRA exon 12	2			
KIT exon 9	**1**	1		
Wild type			2	2
KIT exon 11 (p.L576P)				1
KIT exon 17 (p.Y823D)				1
PDGFRA exon 18 (p.D842V)				6
RC				
KIT exon 11[c]	31			
KIT exon 13 (p.K642E)	2			
PDGFRA exon 12	-			
KIT exon 9	**2**			
Wild type	**3**			
KIT exon 11 (p.L576P)	**2**			
KIT exon 17 (p.Y823D)	-			
PDGFRA exon 18 (p.D842V)	**7**			

Table 4. **bold text** = case given incorrect therapy or incorrectly abstained from therapy; white background= mutations indication full sensitivity to imatinib; grey background= mutations indication reduced sensitivity or resistance to imatinib; standard: 400 mg; high: 800 mg; [a]) IMA = imatinib; [b]) TKI = tyrosine kinase inhibitor; [c]) all mutations in *KIT* exon 11 except p.L576P

4.8 Pretreatment sequencing and tumor response induced by down-sizing

Down-sizing standard dose imatinib was initiated in 92 patients (ISC: n=45; RC: n=47). In the evaluation by CT-scan (ISC: n=29; RC: n=36), a positive tumor response on CT-scan was more common among the ISC-tumors compared with the RC-tumors, 19/29 (66%) *vs* 14/36 (39%), p=0.046. The ISC-tumors had also a higher level of tumor size reduction on CT-scan and a higher level of Ki-67-index reduction, **Figure 17**.

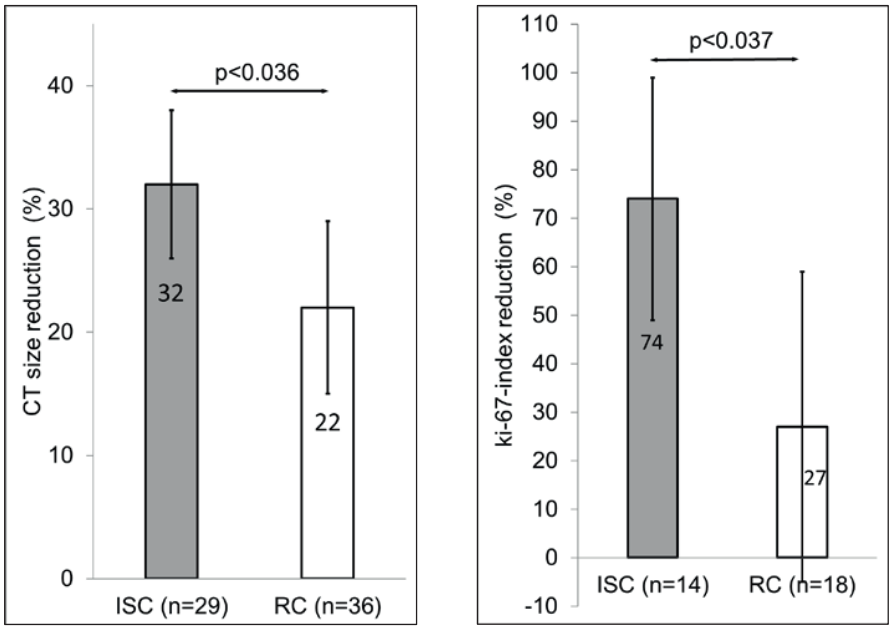

Figure 17*. Two bar charts demonstrating the tumor response induced by down-sizing, standard dose imatinib in patients of the interventional, study cohort (ISC: grey bars, 2014-2017) and the historical reference cohort (RC: white bars, 2006-2013).* ***Left****: The bars represent the tumor size reduction (%) comparing the pretreatment, base-line CT scan with the evaluation CT scan.* ***Right****: The bars represent the Ki-67-index reduction (%) comparing the Ki-67-index of the pretreatment tissue with the Ki-67-index of the corresponding surgical specimens. The error bars equal the 95% CI.*

By ^{18}FDG-PET, a positive tumor response was recorded in 19/20 (95%) of the ISC-tumors and in 19/25 (76%) of the RC-tumors, p=0.11.

5 DISCUSSION

This thesis included four prospective studies. The study on subepithelial lesions showed that the acquisition of pretreatment tumor tissue guided by endosonography has an important diagnostic value and clinical impact. By providing a stable diagnostic ground before therapy, this study challenges the current, suboptimal management of these lesions with unwarranted resections.

Also the study performed on solid pancreatic lesions had a unique design including dual needle sampling of each lesion. No obvious benefit of EUS-guided tissue acquisition was found. Nevertheless, the biopsy approach combined with routine fine-needle aspiration for cytology seemed advantageous in pancreatic neoplasms other than adenocarcinoma. This is new knowledge provided in a group of lesions not frequently studied before.

In the last two studies including exclusively GISTs, the acquired tissue was highly accurate for the diagnosis as such but also, and importantly, for the pretreatment mutational analysis leading to a facilitated decision-making in down-sizing therapy. As compared with current literature, these studies provide the most accurate and profound characterization of GISTs prior to treatment. The presented ability to extract extensive information in pretreatment GISTs will have a probable impact on future guidelines of the field.

The clinical implementation of the presented work-up concept could hopefully help in personalizing the care of patients with suspected pancreatic and gastrointestinal neoplasms, especially GIST. Below the results will be put into context and discussed with respect to the current literature.

5.1 Interpretation of the accuracy in EUS-diagnostics

There is a crucial aspect to keep in mind when interpreting the results of this thesis. Two separate procedures including several factors influence the final diagnostic accuracy of endosonography-guided sampling: *a)* The acquisition of the sample (the *sample adequacy*), in which the needle design is one factor among many others and *b)* The processing and assessment of the acquired sample, **Figure 18**.

As an example, the experience of the performing endosonographer, who needs to find and assess the target lesion, must not be underestimated[140,141]. Another example is small tumor size, which can decrease the chance of sampling success[142]. In the end, poor quality of any individual factor risks to finally result in a non-diagnostic (cyto)pathology report. Consequently, improved quality in the acquisition of the sample does not necessarily compensate for low cytopathology competence, and vice versa. Hence, when the accuracy of EUS-FNB (and EUS-FNA) is mentioned in the following paragraphs of this discussion, a lot more than the needle design and the sampling maneuver is of importance.

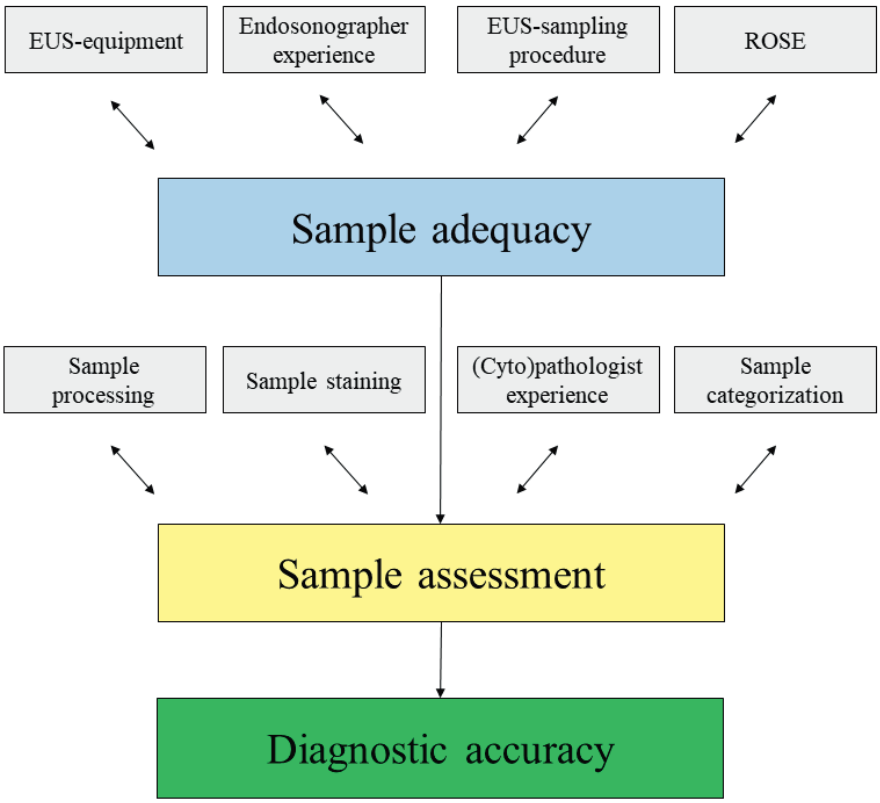

***Figure 18**. A flow chart symbolizing the complexity of obtaining a correct and conclusive diagnosis by EUS-guided sampling. Multiple factors (boxes in grey) influence both the adequacy of the sample and the assessment of the acquired sample, which both in the end affect the diagnostic accuracy.*

5.2 Internal validity and confounding factors

Internal validity measures the extent to which the studied independent variable, i.e. the intervention, actually causes the recorded value of the dependent variable, i.e. the study outcome. In the worst case scenario, a high proportion of the assumed causality is instead induced by a completely different (known or unknown) variable, which leads to a low internal validity. Such a variable is called a *confounder*.

In the performance of the interventional study of *Paper I-III* (2012–2015) we had the ambition to obtain a high level of internal validity. We tried to minimize the number of potential confounders and the variability of all the factors influencing the final diagnostic accuracy, **Figure 18**. As an example, only endosonographers with sufficient experience were responsible for the inclusion of patients and the performance of EUS. Moreover, each lesion was sampled with both techniques – EUS-FNB and EUS-FNA. The sampling maneuver itself, including the applied needle suction and the needle movement, was also kept intact throughout the study. We used the identical study pathologist and cytopathologist during the complete study time (2012–2015). They were also responsible for the assessment of the vast majority of the EUS-samples during the base-line period (2006–2011). Finally, there were very few patients lost from follow-up, which also strengthens the internal validity.

Nevertheless, it can be practically challenging, or even impossible, to accomplish complete conformity among potential confounders. Therefore, a number of such factors were separately tested with respect to the diagnostic accuracy (Supplementary Table, *Paper I*, and Table 3, *Paper II*).

A weak point with respect to the internal validity was that the diagnosis in every single patient was not confirmed by surgical resection; histopathology was available in 40 % (*Paper I*) and in 69 % (*Paper II*). However, the scenario of having surgical specimens as the reference standard in all cases is probably unattainable in a cohort including many patients with severe malignancy.

As a final remark, we decided to use only the reverse bevel FNB-needle throughout this research. FNB-needles with an alternative design might be as appropriate but they were not evaluated in this project.

5.3 EUS-FNB in solid pancreatic lesions (Paper I)

The diagnostic capacity of EUS-FNB was found comparable to EUS-FNA in the sampling of solid pancreatic lesions. We had hypothesized that the acquisition of whole tissue by EUS-FNB would be favorable (as compared with the aspiration of cells), especially when targeting tumors with the need for diagnostic immunostaining such as PNETs.

It may be that the construction of the FNB-needle used in this thesis (reverse bevel) is not the optimal one for sampling of solid pancreatic lesions. In a recent study by Nayar and colleagues[143] the accuracy of reverse bevel EUS-FNB was found similar as in our study (~74 %), while the accuracy of another FNB-needle with an alternative design (opposing bevel) was found significantly higher (~92 %). Needles of different sizes (22 G/25 G/20 G) were used in the study and the majority of lesions were pancreatic adenocarcinomas.

In some publications, including mostly PDACs, the sensitivity of EUS-FNA has been reported as high as 90 %[144]. However, one must keep in mind that immunostaining is not indispensable in PDACs[145], which makes this high number almost unachievable in cohorts with a high distribution of neoplasms requiring immunostaining. The few studies reporting on populations exclusively consisting of PNETs suggest an accuracy of EUS-FNA around 80 %[146], which is comparable to the findings of *Paper I*. Regarding metastatic lesions, most reports have been retrospective without mandatory immunohistochemistry[21,22].

In our institution, the surgeons not routinely refer patients with SPLs for EUS. Therefore, the cohort of lesions studied in *Paper I* included a high number of PNETs and metastases. Hypothetically, this matter could have had a negative impact on the accuracy. Still, the accuracy of EUS-FNB was comparable to the result presented in the study by Nayar mentioned above[143]. The sensitivity of EUS-FNA was also in line with other publications[107]. These comparisons suggest that the dual needle sampling approach was not obviously disadvantageous for the outcome of sampling.

We believe that *Paper I* contribute with new knowledge regarding the value of dual needle sampling as such. The approach is sparsely studied. Some groups have performed dual needle sampling, but without reporting the accuracy of

the combination of EUS-FNA and EUS-FNB[147]. To the best of our knowledge, only one study has analyzed, and found some benefit, by combining a 22 gauge FNB-needle with a 25 gauge open-tip FNA in the same SPL[148].

Whatever the reason for the recorded high accuracy of dual needle sampling, the approach is costly and increases the procedural time. Therefore, it should be considered only in selected cases having a high probability of harboring neoplasms other than PDAC or in centers without the access to ROSE.

5.4 EUS-FNB in subepithelial lesions (Paper II)

In line with the pre-study hypothesis, the EUS-FNB approach was found significantly superior to EUS-FNA in providing a conclusive diagnosis of subepithelial lesions. This large, prospective study fills an empty gap in the available EUS-literature since few studies evaluating EUS-FNB have been published [149] and only two exclusively on SELs[150,151].

In the first one by Kim and colleagues[151], 22 patients were randomized to EUS-FNB (n=12) or to EUS-FNA without ROSE (n=10). Lesions <20 mm were excluded. The diagnostic accuracy of EUS-FNA was recorded 20% and the accuracy of EUS-FNB was recorded 75%. The low number of patients in this study limits the value of comparison with our results. The accuracy of EUS-FNA was also remarkably low. In the second one, a recent retrospective study by El Chafic and collaborators[150], 106 SELs were included and punctured with EUS-FNA (n=91) or EUS-FNB (n=15). The needle size varied but was in most cases 22 gauge. The accuracy of EUS-FNA with ROSE was similar to our study (53%), while the accuracy of EUS-FNB including conclusive immunostaining was 87 %. Thus, these results were well in line with what was found in *Paper II*, even though the few EUS-FNB procedures performed make a direct comparison somewhat difficult.

In *Paper II*, the superiority of EUS-FNB was clear in the subgroup of malignant neoplastic lesions such as GIST and leiomyosarcoma. The lack of statistical significance among the non-malignant cases was possibly due to the low number of study patients having a benign neoplastic lesion (n=13), such as a leiomyoma, or a non-neoplastic lesion (n=5), such as heterotopic tissue.

Catch or no catch? *A brown pelican (Pelecanus occidentalis) with a powerful beak is hunting fish in the Mexican gulf outside Estero Island, Southeast USA. Photo by the author.*

When testing for potential confounders, the lack of ROSE and a number of needle-passes below three showed a tendency to impact (reduce) the sensitivity of EUS-FNA (Table 3, *Paper III*). Therefore, a comparison EUS-FNB *vs* EUS-FNA was performed in a subgroup excluding these cases. The diagnostic sensitivity of EUS-FNB was still found to be superior to that of EUS-FNA, 28/30 (93%) *vs* 20/30 (66%), p=0.01.

The sampling order (EUS-FNA first and EUS-FNB second; or vice versa) was not found to have an impact on the sensitivity of the respective technique (Table 3, *Paper III*). This finding strengthens the validity of the presented accuracy and sensitivity both regarding EUS-FNA and EUS-FNB.

5.5 The patient safety of EUS-FNB

Physicians should never do more harm than good. Therefore, the patient safety in all diagnostic and therapeutic procedures is of high priority.

The performance of reverse bevel EUS-FNB was found to be safe and related to a low adverse event rate in SPLs (1.4 %) and in SELs (1.2 %). These findings and numbers are comparable to the results of a few studies on SPL[121,152] and on SELs[121,151]. Tumor bleeding post sampling could have been a potential concern since, in general, both PNETs and GISTs are highly vascularized.

No additional risk for events was observed due to the dual needle sampling approach as such. According to the available literature, this specific issue has not been addressed before[148].

Nevertheless, EUS-FNB is obviously not performed completely without risk in the pancreatic head since one patient in *Paper I* had a severe pancreatitis post-EUS. Therefore, ampullary lesions were excluded from dual needle sampling in *Paper I*. This means that the presented adverse event rate might not be representative for the lesions localized in this defined part of the pancreatic head.

Certainly, the study patients were not actively contacted by phone to inquire for symptoms or problems after EUS. On the other hand, the development of adverse symptoms, as defined in this thesis, implies that the affected patients would have been in need of a hospital visit. Any such visit was screened for in the national medical file system (*NPÖ*).

Finally, and as with the diagnostic accuracy, the occurrence of adverse events in EUS-procedures is related to more than one factor. Hence, it can be difficult to conclude if it is the actual needle or the maneuver that provokes the adverse event, not least in a dual needle study.

Probably not a patient friendly bite. *A cottonmouth viper (Agkistrodon piscivorus) is targeting its prey in the high grass of the Corkscrew swamp sanctuary, Southwest Florida. Photo by Klara Hedenström.*

5.6 Clinical impact of EUS-FNB in subepithelial lesions (Paper II)

The results of *Paper II* showed that the shift from routine EUS-FNA (2006–2011) to EUS-FNB (2012–2015) resulted in a significant clinical impact demonstrated by the reduced need for additional diagnostic procedures post-EUS. Likely, the complete benefit was thanks to the acquisition of FNB-tissue since EUS-FNA was non-diagnostic in all the study cases where EUS-FNB was also non-diagnostic.

Paper II contributes with new valuable information. There are few works analyzing the clinical impact of EUS-guided tissue acquisition. The impact of the old generation biopsy needle (tru-cut) on clinical decision making was found moderate in an article published in 2011[153]. Since the tru-cut needle had a high rate of technical failures, this study is not the ideal one for comparison. In a retrospective article published in 2015, the authors found that the resection of a gastric leiomyoma could be avoided in 16/18 (89 %) patients thanks to a

high recorded accuracy of the reverse bevel EUS-FNB[154]. No extensive analysis of the clinical impact in all study subjects was however performed.

In *Paper II*, the rate of benign lesions being sampled was somewhat higher in the base-line cohort (2006–2011) compared with the study cohort (2012–2015). That might have had some influence on the detected difference between the cohorts. To compensate for that disparity in the distribution of lesions, the need for additional procedures was analyzed and found comparable in the subgroup of malignant lesions only.

There is an important relevance of measuring the clinical impact. Additional diagnostic procedures post-EUS prolong the time to a conclusive diagnosis and risk to have a negative impact on patient well-being. Unnecessary follow-up is waste of money and resources. Unwarranted surgery is quite obviously related to patient morbidity and a diagnostic laparotomy seems obsolete in modern healthcare.

5.7 Mutational analysis of FNB-tissue (Paper III)

The FNB-tissue acquired from GIST was highly accurate for sequencing. This finding is significant since the preoperative work-up of GIST is not complete until the DNA-sequence of *KIT* and *PDGFRA* has been determined.

The unveiling of mutations in *KIT* and *PDGFRA* has a so called *theranostic* dual implication[72,155]. First, the therapy of GISTs is hugely impacted by the mutation profile of individual tumors. Importantly, the efficacy of tyrosine kinase inhibitor therapy can be firmly predicted only by knowing the status of *KIT* and *PDGFRA*[44]. Not less important from a surgical point of view, the risk for tumor rupture during resection is strongly related to mutations in *KIT* exon 11[156]. Second, the prognosis of a patient affected by a GIST will be precise and reliable only by the inclusion of the mutation profile[79,157].

The feasibility of sequencing using EUS-FNA-samples has been explored only in two small, retrospective case-series[158,159] and in a minor retrospective cohort of twenty patients[155]. This thesis (*Paper III/IV*) is the first publication to present a prospective and systematic sequencing of GISTs.

The aim of *Paper III* was to investigate the feasibility of mutational analysis using tissue harvested by EUS-guidance. The finding that EUS-FNB actually provides the tissue needed for accurate sequencing in most cases was strengthened by the fact the mutation profile was re-analyzed and confirmed in the resection specimens of all resected patients (n=27).

In *Paper III*, Sanger sequencing was the method used. The amount of tumor cells required in Sanger analysis is higher than in NGS, which is currently the primary method used (*Paper IV*). Consequently, in 2018 it can expected that almost any yield of FNB-tissue will be adequate for successful sequencing.

5.8 The Ki-67-index of GISTs in FNB-tissue (Paper III)

The Ki-67-index determined in pretreatment GIST-tissue corresponded well with the Ki-67-index assessed in the corresponding surgical specimens of patients not treated with down-sizing therapy.

Certainly, the Ki-67-index is not included in the validated NIH risk score, which instead uses the mitotic index (MI) for the determination of the tumor proliferation rate[57]. Nevertheless, the assessment of the pretreatment Ki-67-index is not futile, since the tumor proliferation rate is more important than tumor size with respect to the prognostic risk[72].

Other groups have investigated the Ki-67-index as an alternative method to MI but only in surgical specimens of GIST. The level of the Ki-67-index was shown to strongly correlate with the prognosis[37,60,62]. In the study by Nilsson and collaborators[37], the maximum Ki-67-index was proven to be an independent risk indicator in 251 patients with GIST. Given a fixed tumor size of 5 cm, each percentage point increase in the maximum Ki-67-index was associated with a 5 % increased risk of dying. A Ki-67-based GIST risk score (GRS) was constructed (GRS = maximum tumor diameter (cm) + maximum Ki-67-index). A GRS > 7 was found to be associated with an increased risk of dying within five years. The results of *Paper III* suggests that the GRS-score could be applied also in pretreatment EUS-FNB-tissue.

In an interesting study, Kemmerling and colleagues assessed both the Ki-67-index and the MI in 154 specimens of documented GISTs[62]. They showed that the proliferation rate measured by the Ki-67-index actually predicted the

recurrence and metastasis rate of GIST better than the MI. Moreover, a linear regression model was elaborated for the reliable prediction of the MI based on the Ki-67-index (MI = f (x) = 0.084x – 6.328), where x is the Ki-67-index expressed as the number of Ki-67 positive nuclei per mm^2. Using this equation, the authors found that a Ki-67-index of 135 positive nuclei/ mm^2 equaled an MI of 5. Thereby this Ki-67-index could be used as the surrogate cut-off value for a high mitotic index, which means increased risk. A similar conversion model and equation could probably be elaborated for EUS-FNB-tissue. The publication by Kemmerling *et al* stresses that the Ki-67-index assessed in pretreatment tissue justifies attention as a promising and clinically useful risk indicator in GIST.

Apparently the MI is not the appropriate method for the assessment of tumor proliferation rate in pretreatment tissue acquired by EUS. In our study, the acquired FNB-tissue had a maximal size at microscopy of 38 high-power fields (hpf, 1 hpf = 0.16 mm^2) while a maximum of 11 hpf was counted in a study analyzing twelve EUS-biopsies[154]. A minimum of 50 hpf is required for the adequate calculation of the MI[57].

Potentially, the most valuable application of Ki-67-indexing in pretreatment GIST-tissue would be the estimation of the true tumor proliferation rate in patients intended for preoperative down-sizing therapy. Thus, we also compared the Ki-67-index of pretreatment FNB-tissue with the Ki-67-index of the corresponding surgical specimens in patients subjected to down-sizing imatinib. By performing this comparison, we could demonstrate that the Ki-67-index in surgical specimens is significantly lower than in FNB-tissue; provided a sensitive mutation (Figure 3, *Paper III*). This finding implicates that the calculated NIH risk score will be falsely low in surgical specimens. In addition, these patients cannot be properly evaluated for adjuvant therapy.

The presented method could also serve as a marker for the efficacy of down-sizing imatinib therapy. Since ^{18}FDG-PET is an expensive method[160,161]; the Ki-67-indexing of repeated EUS-biopsies would be an attractive alternative by which the therapy response could be evaluated.

5.9 Pretreatment sequencing and down-sizing imatinib

During the study time frame (2014–2017) of *Paper IV*, immediate, pretreatment sequencing was successful in all but two samples resulting in the incorrect down-sizing management of only 2/59 (3%) patients. Given the obvious risk for ineffective treatment and the significant risk for side-effects, the indication for such a work-up in GIST seems strong.

To the best of our knowledge, the current work is the first study presenting systematic and extensive genomic data of GISTs at an early preoperative stage with clear impact upon the clinical management. The study required the collaboration of multiple departments and the combination of several diagnostic procedures and advanced analyses.

The categorization of different variants of mutations with respect to the expected tumor response (Table 1, Pa*per IV*), and thereby the definition of a correct down-sizing therapy, was based on guidelines and relevant literature of the field[78]. The best therapy strategy remains to be decided for certain variants of mutations in *KIT* and *PDGFRA*. Therefore, studies are ongoing, such as the ALT-GIST trial (NCT02365441), which is evaluating alternating imatinib and regorafenib in advanced GIST.

The level of effect induced by imatinib in primary *KIT* exon 13-mutants (p. K642E) is not firmly elucidated because the variant is uncommon. Stable disease is probably a more common result than partial response according to a review by Bachet *et* al[80]. After consideration, we decided to regard primary *KIT* exon 13-mutants as resistant to imatinib in *Paper III*. This might have been erroneous since some tumors actually do respond. In *Paper IV* we applied a more generous interpretation of the literature and considered primary *KIT* exon 13-mutants as sensitive to standard dose imatinib. If regarding *KIT* exon 13 (p. K642E) as non-sensitive the value of pretreatment sequencing would be actually even higher.

Another complicated example is the *KIT* exon 11 (p. L576P)-mutant. Noujaim and colleagues analyzed tumors of five patients diagnosed with *KIT* exon 11 (p. L576P)-mutated GISTs[162]. They noticed a favorable effect of imatinib with partial response (>30 % size-reduction) in two of these five patients. Therefore,

they proposed that imatinib can still be used as the first-line treatment also in p. L576P-mutants. This conclusion is however contradicted by an *in vitro* study, which found that p. L576P-mutants required a 10-fold higher dose of imatinib concentration to obtain the same response as mutants fully sensitive to imatinib[163]. In the end, we decided to regard *KIT* exon 11 p.(L576P)-mutants as non-sensitive to imatinib.

Whatever may be the optimal categorization of mutations, the pretreatment clarification of the mutation profile supports the responsible clinician with valuable theranostic information. Moreover, early reappraisal of the therapy can considered in cases with uncertain sensitivity to imatinib.

Surgery of a gastrointestinal stromal tumor*. The image shows the tumor of a 62-year old man initially suffering from a 10 cm GIST in the gastric fundus with tumor growth on the diaphragm. Up-front surgical resection was not possible. After pretreatment sequencing detecting a KIT exon 11 V558-deletion, the patient was initiated on 12 months of down-sizing imatinib. The tumor reduced in size and could be resected en bloc with a limited resection of the diaphragm. Photo by the author.*

5.10 Tumor response to down-sizing imatinib

The tumor response induced by down-sizing standard dose imatinib was improved in the group of GISTs subjected to pretreatment mutational analysis during the study time frame (2012–2015). The reason for this was most probably that GIST-mutants with a reduced sensitivity or complete resistance to imatinib were identified before the initiation of therapy. Instead these patients could be offered an alternative therapy, such as sunitinib, or up-front surgery.

A challenging group with respect to pretreatment sequencing is distal, small-intestinal GISTs since this region is not within reach for EUS. In these cases, the acquisition of tissue guided by transabdominal ultrasound could be an alternative and safe method[164]. Small-intestinal GISTs of small size could still be problematic but few of those would however require down-sizing therapy.

The imatinib dose in the setting of down-sizing therapy is not actually standardized or well determined. Imatinib in different dose and duration has been evaluated in some other studies[87,88,165]. Rutkowski *et al* initiated imatinib 400 mg daily in all patients, while Wang and colleagues used imatinib 600 mg daily in all patients. Nonetheless, we applied the term “standard dose” on the dose of imatinib 400 mg daily since this is the dose recommended in the adjuvant setting[72].

There are advantages and shortcomings with all methods used for the evaluation of tumor response induced by imatinib therapy. As some examples, the base-line signal of ^{18}FDG-PET can be negative in GIST[166], the appropriate method for PET-signal measurement is a matter of debate[136], and ^{18}FDG-PET does not predict progression free survival[167]. Tumors actually responding to therapy do not necessarily reduce in size on CT scan but rather in density[168]. This was also the reason why the results of three different methods (CT scan, ^{18}FDG-PET, and the Ki-67-index) was presented. Pretreatment sequencing can probably not replace the evaluation by CT scan. The imatinib effect needs to be monitored in some way and measurement of the tumor shrinkage is important for the planning of surgery. However, pretreatment sequencing has the potential to replace the evaluation with ^{18}FDG-PET; at least in regular *KIT* exon 11-mutants, in which the effect of imatinib is high.

A major limitation of *Paper IV* worth mentioning is that the outcome of surgery and the recurrence free survival was not analyzed. A larger (multi-centric, randomized) study including a high number of patients, would be required to show an effect on these parameters by down-sizing therapy based on pretreatment sequencing. Even so, the results of *Paper IV* clearly illuminates the advantages of mutation-based, preoperative therapy of GIST.

5.11 The external validity of the results

The conclusions of a single research project should always be interpreted with some caution since the results may not always be generalized. Study design and actions undertaken to prevent confounding factors may increase the degree of internal validity but limit the so called *external validity*, i.e. the ability to accomplish the same results in other centers. Nevertheless, the external validity of the presented results is probably rather high.

All the study patients of this thesis were referred for a clinical EUS and no examination was performed for research purpose only. The number of exclusion criteria was few. Thus, the level of patient selection bias was probably quite low in the papers presented. The patient population studied in this thesis should be fairly representative for a university hospital handling upper GI neoplasms.

The major procedures and processes did not profoundly differ from what is common practice within the field of EUS-based diagnostics. Standard equipment was used and should be available for purchase in most countries where EUS is performed. The FNB-sampling technique applied in this thesis is not dramatically different from routine EUS-FNA.

We used a conservative approach regarding the results of (cyto)pathology and the categorization of samples. A more generous approach would have resulted in a higher accuracy and sensitivity for tumors requiring diagnostic immunostaining. Meanwhile we had access to (cyto)pathology expertise with long experience in the assessment of EUS-samples and that may not be the fact in all centers.

The start-up and organization of a new EUS-facility requires some effort and perfect results cannot be expected momentarily since the quality of all

procedures included in the process need to be reasonably high. Therefore, results comparable to the ones presented in this thesis can only be expected in EUS-centers with an equivalent level of experience.

Adding DNA-sequencing of pretreatment tumor tissue as yet another procedure on top of routine work-up might of course not be feasible in all institutions dealing with EUS. Such management requires the access to an advanced laboratory and skilled geneticists.

5.12 Limitations

Specific limitations in each individual study of this thesis has been discussed above.

A limitation regarding the research project as a whole was that it was performed in a single-center setting. Another general limitation was that the project spanned over a long period of time. It cannot be ruled out that the technical development of imaging and the increasing experience of the collaborators involved in the project might have had some influence upon the results presented.

6 CONCLUSIONS

- In solid pancreatic lesions, the work-up with endosonography-guided fine needle biopsy sampling (EUS-FNB) is patient safe, but not superior to fine needle aspiration (EUS-FNA) for cytology. The performance of both modalities could be considered in lesions suspicious for neuroendocrine tumors or metastases.

- In subepithelial lesions, EUS-FNB is patient safe and has a significantly higher diagnostic accuracy compared with EUS-FNA. EUS-FNB also leads to the reduced need for additional diagnostic procedures after EUS including diagnostic, surgical resections.

- The preoperative diagnostics of GISTs was previously challenging with a poor accuracy. Pretreatment EUS-FNB is highly accurate for the conclusive diagnosis of GISTs already at the preoperative stage.

- In GISTs, the acquired EUS-FNB-tissue is well aimed for fast and accurate pretreatment genetic profiling of *KIT* and *PDGFRA*, which can be used to guide down-sizing therapy and reduce the need for expensive ^{18}FDG-PET.

- In GISTs, the tumor response to down-sizing, standard dose imatinib will be more predictable if pretreatment sequencing is performed to select the appropriate patients.

- In GISTs, the pretreatment EUS-FNB-tissue is adequate for the reliable assessment of the tumor proliferation rate (Ki-67-index), which risks to be falsely low in surgical specimens of patients treated with down-sizing therapy.

7 FUTURE PERSPECTIVES

Further and refined studies are needed to optimize the EUS-diagnostics and improve the care of all patients affected by pancreatic malignancies, mesenchymal tumors, and other types of neoplasms.

Future direction? *Tree tops stretching towards the sky, Big Cypress National Preserve, Southwest Florida. Photo by Klara Hedenström.*

During the study time frame (2012–2015), patients with other lesions such as lymph nodes and adrenal tumors were also enrolled as study subjects and sampled with the dual needle approach. By combining these cases with all the cases of this thesis and all the cases sampled in 2006–2011, we plan to perform an extensive study on the diagnostic accuracy of EUS. This study is planned to be part of a future PhD-project.

Regarding pancreatic neuroendocrine tumors, the prognostic risk[59] and the operability[169] strongly correlate with the tumor proliferation rate (Ki-67-index). Ki-67-staining and systematic measurement of the Ki-67-index in the acquired FNB-tissue and the FNA-samples (*Paper I*) could be the focus of a future project.

As described, the experience of the endosonographer is a factor influencing the quality of EUS-diagnostics. However, it is poorly studied what factors that actually influence the EUS learning process and the technical skills of the endosonographer in training. To address these questions we initiated in 2014 a study (GEUSP – Global assessment of the EUS Performance skills), which is currently running and recruiting study subjects. At present, we have enrolled ten endosonographers together performing a total of 105 EUS-examinations. This project is open for international collaboration.

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APPENDIX

Table *NIH consensus prognostic risk criteria for GIST*

NIH Risk	Tumor size (cm)	Mitotic index (per 50 hpf)
Very low risk	<2	<5
Low risk	2-5	<5
Intermediate risk	<5	6-10
	5-10	<5
High risk	>5	>5
	>10	any mitotic index
	any size	>10

Table *DNA-codons coding for the respective amino acids*

Amino acid	Abbreviation	Letter	Codon (base triplet)
Alanine	Ala	A	GCT, GCC, GCA, GCG
Arginine	Arg	R	CGT, CGC, CGA, CGG, AGA, AGG
Aspargine	Asn	N	AAT, AAC
Aspartic acid	Asp	D	GAT, GAC
Cysteine	Cys	C	TGT, TGC
Glutamine	Gln	Q	CAA, CAG
Glutamic acid	Glu	E	GAA, GAG
Glycine	Gly	G	GGT, GGC, GGA, GGG
Histidine	His	H	CAT, CAC
Isoleucine	Ile	I	ATT, ATC, ATA
Leucine	Leu	L	TTA, TTG, CTT, CTC, CTA, CTG
Lysine	Lys	K	AAA, AAG
Methionine	Met	M	ATG
Phenylalanine	Phe	F	TTT, TTC
Proline	Pro	P	CCT, CCC, CCA, CCG
Serine	Ser	S	TCT, TCC, TCA, TCG, AGT, AGC
Threonine	Thr	T	ACT, ACC, ACA, ACG
Tryptophan	Trp	W	TGG
Tyrosine	Tyr	Y	TAT, TAC
Valine	Val	V	GTT, GTC, GTA, GTG