Small intestinal neuroendocrine tumours

Disease models, tumour development, and remedy

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Cover illustration: ‘Irradiated.’ by Tobias Hofving.

The GOT1 cell line after staining with anti-SSTR2 and DAPI. Image captured using a 63× oil-immersion objective lens with Zeiss LSM 700 confocal microscope.
‘I’m a scientist and I know what constitutes proof. But the reason I call myself by my childhood name is to remind myself that a scientist must also absolutely be like a child. If he sees a thing, he must say that he sees it, whether it was what he thought he was going to see or not. See first, think later, then test. But always see first. Otherwise you will only see what you were expecting. Most scientists forget that.’

As soberly stated by ‘Wonko’ in The Hitchhiker’s Guide to the Galaxy, written by Douglas Adams, after finding a pack of toothpicks that finally convinced him that that the world at large was insane.
Small intestinal neuroendocrine tumours (SINETs) are malignant neoplasms which at the time of diagnosis often present with distant metastasis. The field of SINET research faces several challenges. There is a lack of preclinical models for studying SINETs, and it is unclear how well currently available models actually recapitulate the tumour disease. The genetic changes that underlie SINET tumour development are largely unknown and, lastly, curative therapy is rarely achieved. Novel therapies, such as the recently FDA-approved $^{177}$Lu-octreotate therapy and up-and-coming immunotherapies need to be further investigated to deliver better response rates for SINET patients.

In our first two papers (papers I and II), we sought to evaluate frequently used and readily available gastroenteropancreatic neuroendocrine tumour (GEPNET) cell lines as models of neuroendocrine tumour disease. We investigated the characteristics of these cell lines in terms of their neuroendocrine phenotype, genomic background, and therapeutic sensitivity. While several cell lines exhibited an expected neuroendocrine differentiation and harboured genetic alterations characteristic of the GEPNET disease, three cell lines did not. In fact, it turned out that one of the most frequently used cell lines in the field – KRJ-I, together with the cell lines L-STS and H-STS, were incorrectly identified and instead lymphoblastoid cell lines (EBV-immortalised B-lymphocytes). This might have led to the incorrect use and potentially faulty conclusions in a number of GEPNET studies. Among authentic cell lines, we performed a large-scale inhibitor sensitivity screening and predicted that SINETs would be more sensitive to HDACi compared to pancreatic neuroendocrine tumours (PanNET) and PanNET more sensitive to MEKi compared to SINET. The prediction was supported by subsequent experiments with primary tumour cells. In our third paper (paper III), we evaluated a mechanism by which hemizygous loss of \textit{SMAD4} could lead to SINET initiation and/or progression by acting as a haploinsufficient tumour suppressor. We found that loss of \textit{SMAD4} was associated with a decrease in corresponding mRNA and protein, and that this correlated to patient survival. We also found that the amount of SMAD4 protein in the primary tumour could predict whether the patient presented with distant metastasis. In our last papers (papers IV and V), we investigated the potential for two novel treatment strategies for SINETs. In paper IV we identified an inhibitor, the heat shock protein 90 inhibitor ganetespib, that could synergistically enhance the $^{177}$Lu-octreotate therapy for SINETs. Ganetespib was initially found to sensitisise SINETs to radiation in a large-scale inhibitor synergy screening, and its radiosensitising effect for radionuclide treatment of SINETs was validated both in mouse xenografts and in primary patient tumours. Lastly, in paper V we characterised the SINET
immune microenvironment. Using immunohistochemistry and flow-cytometry we detailed the immune cell composition of the SINET immune microenvironment and could demonstrate the successful isolation and expansion of tumour-infiltrating lymphocytes. We saw that after infiltrating lymphocytes were expanded they could degranulate when challenged with autologous tumour cells.

In conclusion, these studies have provided a thorough characterisation of authentic, and provided important information regarding misidentified, frequently used gastroenteropancreatic cell lines. It has also investigated the role of hemizygous SMAD4 loss in the development of SINETs and demonstrated the potential of two novel therapies for SINETs: $^{177}$Lu-octreotate combined with Hsp90i ganetespib and immunotherapy.

**Keywords:** neuroendocrine tumours, tumour models, SMAD4, $^{177}$Lu-octreotate therapy, immunotherapy

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Denna avhandling syftade till att förbättra kunskapen inom fältet neuroendokrina tunntarmstumörer för att i förlängningen komma dessa patienter till gagn. Neuroendokrina tunntarmstumörer uppkommer som namnet antyder i människans tunntarm och utsöndrar ofta hormoner, vilket kan leda till svåra biverkningar för patienten.

I avhandlingens två första delarbete (delarbete I och II) har vi undersökt hur väl de cellinjer forskningsfältet använder sig av för att studera dessa tumörer verkligen efterliknar tumörsjukdomen. I delarbete I beskriver vi uttrycket av proteiner som beskriver cellernas karaktär, studerar dess genetiska förändringar och deras känslighet för en stor mängd läkemedel. Detta gav värdefull kunskap forskare kan använda sig av när de använder dessa vanliga forskningsverktyg. Dessutom ledde det till avslöjandet att tre väl använda cellinjer varit helt felidentifierade. Detta var viktigt för att förhindra framtida användande av dessa cellinjer som modell för neuroendokrina tumörer och för att ge information om att tidigare studier kan ha kommit fram till fel slutsatser.

Cancer uppkommer då celler genomgår förändringar som gör att de klarar av att expandera och sprida sig i kroppen. Dessa förändringar sker i DNA, från vilket cellens funktioner utgår. Flera olika typer av förändringar i DNA som leder till olika typer av cancer har identifierats och det finns flera exempel på läkemedel som utnyttjar kunskapen om dessa exakta förändringar. I fallet neuroendokrina tunntarmstumörer är det i många och mycket okänt vilka förändringar som ligger bakom dess uppkomst och utveckling. I det tredje delarbetet (delarbete III) undersöker vi förekomsten av en förändring, förlust av genen $SMAD4$, i tumörernas DNA och utvärderar huruvida det är troligt att denna förändring kan ligga bakom tumörernas framfart. Vi fann dels att denna förändring är mycket vanligt förekommande i tumörsjukdomen och att förlust av genen $SMAD4$ är kopplat både till en minskad mängd protein och till kliniska parametrar, så som patientöverlevnad och huruvida tumörerna sprider sig i kroppen.

Nyligen blev ett nytt läkemedel, $^{177}$Lu-oktreotat, godkänt för behandling av dessa annars svårbehandlade tumörer. Behandlingen tycks ha bättre effekt än tidigare tillgängliga läkemedel, men botar mycket sällan patienterna helt och är därför i behov av förbättring. En
vanlig taktik för att förbättra ett läkemedels effekt utan att behöva öka
dosen med potentiellt förödande bieffe kter som följd är att kombinera
det med ett annat läkemedel. I det fjärde delarbetet (delarbete IV)
identifierar vi ett läkemedel, ganetespib, som kraftigt förstärker ¹⁷⁷Lu-
octreotates behandlingseffekt och vi demonstrerar detta i ett flertal
olika prekliniska modeller.

För att cancer ska uppkomma behöver de maligna cellerna förvärva
inte bara förändringar som exempelvis leder till snabba celldelning
och egenskapen att sprida sig, men även att undvika vårt
immunförsvar. Endast celler som på ett eller annat sätt lyckas
undkomma immunförsvaret kan utvecklas till cancer. Detta utnyttjas
just nu i flera av de nya framgångsrika immunterapier som tagits fram
där immunförsvarvet på olika sätt triggas till att attackera cancerceller. I
det femte delarbetet (delarbete V) utvärderar vi dels vad det finns för
typer av immunceller inne i tumörena och dels huruvida det går att
isolera, expandera och framförallt – återaktivera – dessa immunceller.
Vi fann att det var möjligt att isolera immunceller från tumörena,
expandera dessa och såg att när vi återförde dem till tumörcellerna
reagerade immuncellerna på samma sätt som när de försöker döda
celler. Slutsatsen i delarbete V blev därför att vi anser att det finns
potential för att utveckla immunbaserad behandling för dessa tumörer.
LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Hofving T**, Arvidsson Y, Almobarak B, Inge L, Pfragner R, Persson M, Stenman G, Kristiansson E, Johanson V, Nilsson O. The neuroendocrine phenotype, genomic profile and therapeutic sensitivity of GEPNET cell lines. *Endocrine-Related Cancer, 2018;25(3):367-380*

II. **Hofving T***, Karlsson J*, Nilsson O**, Nilsson JA**. H-STS, L-STS, and KRJ-I are not authentic GEPNET cell lines. *Nature Genetics; accepted for publication*

III. **Hofving T**, Elias E, Inge L, Altiparmak G, Rehammar A, Kristiansson E, Nilsson O*, Arvidsson Y*. **SMAD4** haploinsufficiency in small intestinal neuroendocrine tumours. *Manuscript*

IV. **Hofving T**, Sandblom V, Arvidsson Y, Shubbar E, Altiparmak G, Swanpalmer J, Almobarak B, Elf AK, Johanson V, Elias E, Kristiansson E, Forssell-Aronsson E, Nilsson O. **177Lu-octreotate therapy for neuroendocrine tumours is enhanced by HSP90 inhibition. Endocrine-Related Cancer; 2019;26(4):437-449**

V. **Hofving T**, Liang F, Karlsson J, Yrlid U, Nilsson JA, Nilsson O*, Nilsson LM*. The microenvironment of small intestinal neuroendocrine tumours contains lymphocytes capable of recognition and activation after expansion. *Manuscript*

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Additional publications not part of this thesis:

i) Panova M, Boström J, Hofving T, Areskoug T, Eriksson A, Mehlig B, Mäkinen T, André C, Johannesson K. Extreme female promiscuity in a non-social species. *PLoS One* 2010; 5(3):e9640.

ii) Andersson E, Arvidsson Y, Swärd C, Hofving T, Wängberg B, Kristiansson E, Nilsson O. Expression profiling of small intestinal neuroendocrine tumors identifies subgroups with clinical relevance, prognostic markers and therapeutic targets. *Mod. Pathol* 2016; 29(6): 616-29.

iii) Elf AK, Bernhardt P, Hofving T, Arvidsson Y, Forssell-Aronsson E, Wängberg B, Nilsson O, Johanson V. NAMPT inhibitor GMX1778 Enhances the Efficacy of $^{177}$Lu-DOTATATE Treatment of Neuroendocrine Tumors. *J Nucl Med.* 2017; 58(2): 288-292.
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### ABBREVIATIONS

| Abbreviation | Definition |
|--------------|------------|
| 17-DMAG      | 17-dimethylamino ethylamino-17-demethoxygeldanamycin |
| 3D           | three-dimensional space |
| 5-HIAA       | 5-hydroxyindoleacetic acid |
| ABL          | abelson murine leukemia viral oncogene homolog 1 |
| ACT          | adoptive cell transfer |
| ATCC         | American Type Culture Collection |
| ATP          | adenosine triphosphate |
| BCR          | breakpoint cluster region protein |
| BMP          | bone morphogenetic protein |
| BMPR1A       | bone morphogenetic protein receptor 1A |
| CAR          | chimeric antigen receptor |
| CCD          | charge-coupled device |
| CD           | cluster of differentiation |
| CDKN1B       | cyclin-dependent kinase inhibitor 1B |
| CDX          | cell line-derived xenograft |
| CGH          | comparative genomic hybridisation |
| CT           | computed tomography |
| CTLA4        | cytotoxic T lymphocyte antigen 4 |
| DAPI         | 4',6-diamidino-2-phenylindole |
| DcR3         | decoy receptor 3 |
| DMSO         | dimethyl sulfoxide |
| DNA          | deoxyribonucleic acid |
| E.U.         | European Union |
| EBV          | Epstein-Barr virus |
| EC           | enterochromaffin |
| EGF          | epidermal growth factor |
| EGFR         | epidermal growth factor receptor |
| ELISA        | enzyme-linked immunosorbent assay |
| EMT          | epithelial to mesenchymal transition |
| ENETS        | European Neuroendocrine Tumor Society |
| FBS          | foetal bovine serum |
| FDA          | Food and Drug Administration |
| FDR          | false discovery rate |
| Abbreviation  | Full Form                                                                 |
|--------------|---------------------------------------------------------------------------|
| FISH         | fluorescence in situ hybridisation                                       |
| GEEM         | genetically engineered mouse model                                       |
| GEPNET       | gastroenteropancreatic neuroendocrine tumour                             |
| GI           | gastrointestinal                                                         |
| HDAC         | histone deacetylase                                                      |
| HER2         | human epidermal growth factor receptor 2                                 |
| HIP          | hsp70 interacting protein                                                 |
| HLA          | human leukocyte antigen                                                   |
| HMGA2        | high-mobility group AT-hook 2                                             |
| HOP          | hsp70-hsp90 organizing protein                                            |
| HPF          | high-power field                                                          |
| HSP          | heat-shock protein                                                        |
| IL-2         | interleukin 2                                                             |
| INF          | interferon                                                                |
| JCRB         | Japanese Collection of Research Bioresources Cell Bank                    |
| JPS          | juvenile polyposis syndrome                                               |
| LAR          | long-acting repeatable                                                    |
| LGR5         | leucine-rich repeat-containing G-protein coupled receptor 5               |
| LSM          | laser scanning microscopy                                                 |
| MDM2         | mouse double minute 2                                                     |
| MEK          | MAPK ERK kinase                                                           |
| MH2          | mad homology domain 2                                                     |
| MHC          | major histocompatibility complex                                           |
| miRNA        | microRNA                                                                  |
| mRNA         | messenger RNA                                                             |
| mTOR         | mammalian target of rapamycin                                             |
| NEC          | neuroendocrine carcinoma                                                  |
| NET          | neuroendocrine tumour                                                     |
| NSE          | neuron-specific enolase                                                   |
| PanNET       | pancreatic neuroendocrine tumour                                          |
| PCR          | polymerase chain reaction                                                 |
| PD-1         | programmed cell death protein 1                                           |
| PDGFR        | platelet-derived growth factor receptor                                   |
| PD-L1        | programmed death-ligand 1                                                |
| PDX          | patient-derived xenograft                                                 |
| Abbreviation | Definition |
|--------------|------------|
| PRRT         | peptide receptor radionuclide therapy |
| PTEN         | phosphatase and tensin homolog |
| PγST         | polyoma small T antigen |
| RB           | retinoblastoma protein |
| RIP          | rat insulin promotor |
| RNA          | ribonucleic acid |
| RPMI         | Roswell Park Memorial Institute |
| SCNV         | somatic copy-number variation |
| SEER         | Surveillance, Epidemiology, and End Results |
| SINET        | small intestinal neuroendocrine tumour |
| SSTR         | somatostatin receptor |
| STR          | short tandem repeat |
| SV40         | simian vacuolating virus 40 |
| Tag          | T antigen |
| TCGA         | The Cancer Genome Atlas |
| TCR          | T cell receptor |
| TFF3         | trefoil factor 3 |
| TGFβ         | transforming growth factor β |
| TGFβR        | transforming growth factor β receptor |
| TIL          | tumour-infiltrating lymphocyte |
| TMA          | tissue microarray |
| TNM          | tumour-node-metastasis |
| TP53         | tumour protein p53 |
| U.S.         | United States |
| VEGFR        | vascular endothelial growth factor receptor |
| WHO          | World Health Organization |
INTRODUCTION

We are all a matter of cells. From the simplest nematode to the human being, cells make up the living material, tied together in the utmost complex networks. Key is communication. In the early embryonic development and in the fully developed human alike, the exchange of precise and accurate information is a necessity to ensure that all the processes of the body are in concert. And every bit as important as the interplay in-between cells is the communication taking place within the cells. Cancer can develop first when these fine-tuned and tightly regulated intra and inter-cell signalling pathways are disrupted, and once this happen, tragedy often follows. Close to 10 million people are estimated to die globally from the disease in 2018 (1), but there is hope.

Over the past decades, advancements in the field of cancer research have led to significant improvements of patient survival after receiving a cancer diagnosis. New therapies are continuously emerging, and more and more patients are cured. Successful therapies have in common that they kill tumour cells while sparing untransformed cells from harm. One way to discover such therapies is through the use of preclinical experimental models of cancer. These models are crucial for the continued development of cancer therapies and it is thus vital that these models as accurately as possible mirror the biological aspects being investigated. This is not always the case, and unless we have a clear understanding of how the models recapitulate different biological aspects of the disease it can be of hindrance to the field and to the development of novel therapies.

Another attractive approach to discover novel therapies is through an increased understanding of the underlying mechanisms of tumour development. There are several examples of therapies that have been developed specifically against genetic changes with fundamental functions in tumour development, such as fusion proteins (e.g. imatinib for BCR-ABL), gene amplification (e.g. trastuzumab for HER2+ breast cancer) and activated proteins/pathways (e.g. vemurafenib/trametinib for BRAF-mutated melanoma).
Alternatively, currently already available therapies can also be improved. Research of $^{177}$Lu-octreotate therapy for SINETs has resulted in that the therapy is now approved in the U.S. and E.U. for the treatment of somatostatin receptor type 2-positive gastroenteropancreatic tumours, but still with low curative rates. One attractive approach of improving such a therapy is through a combination with another therapy, preferably with synergistic interaction.

Lastly, we can also look beyond the tumour and change our focus to its surroundings. In the tumour microenvironment we find a wide diversity of cells, including immune cells. These immune cells would normally function to attack anything foreign to the body, including malignant tumour cells. In fact, it is believed that all cancer in one way or another need to develop mechanisms to actively avoid the detection of immune cells. The recent success of immune therapies has put emphasis on the very promising task of reactivating the immune system to target cancer.

In this thesis we have addressed all of these aspects within the scope of small intestinal neuroendocrine tumours (SINETs). We have looked at which models are available and how well they recapitulate various aspects of the tumour disease, at the molecular mechanisms underlying SINET tumour development, how to improve the $^{177}$Lu-octreotate therapy, and finally, looked at the potential for immune therapy for these tumours.
Small intestinal neuroendocrine tumours

Tumours arising from the neuroendocrine cells of the body are collectively termed neuroendocrine tumours (NETs). Small intestinal NETs (SINETs) are believed to arise from the serotonin-secreting enterochromaffin cells of the small intestinal mucosa.

The neuroendocrine system

The neuroendocrine system consists of cells that share characteristics of both the nervous and endocrine systems. Neuroendocrine cells typically receive signalling input in the form of neurotransmitters from nerve cells or neurosecretory cells, which is termed neuroendocrine integration. This serves to regulate synthesis, storage and ultimately secretion of hormones and peptides. These neuroendocrine cells are often located in glands and exist throughout the body, including the brain (hypothalamus, pituitary gland, pineal gland), kidneys (adrenal glands), ovaries, pancreas, testes, thyroid (thyroid, parathyroid), and the gastrointestinal tract. Effects of hormones and peptides span a wide range of physiological mechanisms, such as the stimulation or inhibition of cell growth, activation or inhibition of immune response, and regulation of the metabolism.

In the gastrointestinal tract, endocrine cells – termed enteroendocrine cells – are not gathered in a gland but are rather scattered throughout the mucosa and as such an example of a diffuse endocrine system, with anatomical connections to neurons (2). In fact, it has been argued that the gut is the largest endocrine organ in the body in terms of the amount of hormone-producing cells (3,4). The whole intestinal mucosa can even be regarded as a large sensory organ with complex interactions between neurons, endocrine cells, and the immune system leading to stimulus-adequate responses such as the modulation of motility, perfusion, and tissue defence (5).

Hormones in the gastrointestinal tract are secreted by many different types of enteroendocrine cells (6). Traditionally, they are classified according to what hormone they secrete (7) and while some hormones are produced in the entire intestine – such as serotonin – others are produced at a particular location.
Although constituting less than 1% of the total intestinal epithelia, the most abundant enteroendocrine cell is the enterochromaffin (EC) cell, a cell type that was first proposed to have endocrine capability by Feyrter in 1938 (8). The EC cell can detect irritants, metabolites, and catecholamines (9). Just like other primary sensory cells, EC cells are electrically excitable and express functional voltage-gated sodium and calcium channels (9). Its activation leads to serotonin-release, which is the source of >90% of all serotonin produced in the human body (9).

**Epidemiology**

One of the larger studies, from the United States Surveillance, Epidemiology, and End Results (SEER) data base, reports an age-adjusted incidence for SINETs of 0.86/100,000 for patients during years 2000-2004 (10). Reported data from other countries contain similar numbers with slight variations, e.g. Sweden (1.33/100,000), Norway (1.01/100,000), Netherlands (0.47/100,000), Japan (0.33/100,000) and England (0.78/100,000) (11-15). Common for many studies are that they report an increasing incidence over time (10,11,14,16,17). This reported increase is slightly higher in the United States compared to other countries, but whether this is a true difference is unknown. It has been suggested that the overall observed increase is mainly due to improved detection methods (18), better knowledge about the molecular and cell biological aspects and clearer histopathological characterisation (19). It seems like far from all tumours are ever diagnosed, as suggested by a post-mortem study which observed SINETs in as much as 0.93/100 patients (20). Some studies show a slight male preponderance in reported numbers (15,21,22).

**Clinical presentation**

As it is common that patients are affected by nonspecific abdominal pain, most SINETs are discovered during surgery for these conditions. Alternatively, for cases with distant disease where the tumour produces hormones that can escape hepatic inactivation (23), SINETs can be suspected on the basis of symptoms of the carcinoid syndrome (24). This syndrome is caused by hormones such as serotonin and tachykinins and can lead to, among other things, diarrhoea (73%), flushing (65%), carcinoid heart disease (21%), and asthma-like episodes (8%) (25). Incidental discoveries such as during a CT scan performed in another clinical context are rare (19).
Nonspecific abdominal pain symptoms can be due to various reasons, including dysmotility, obstruction, intermittent mesenteric ischemia, and secretory diarrhoea (19). Other less specific symptoms include nausea, vomiting, jaundice and even gastrointestinal bleeding (19).

Figure 1. Resected part of the small intestine of a patient that underwent surgery at Sahlgrenska University Hospital, Gothenburg. Small intestinal neuroendocrine tumours are indicated by numbers. 1-6: Multiple synchronous primary tumours, 7-8: lymph node metastasis. Image courtesy: Erik Elias/Gülay Altiparmak

The gold standard for confirming an SINET diagnosis is by histopathological analysis (26). Tissues are fixed in formalin and embedded in paraffin and analyses typically include conventional morphological analysis, immunohistochemistry to confirm the neuroendocrine phenotype, and evaluation of the Ki67 index. The morphology is examined on haematoxylin & eosin stained sections and the neuroendocrine phenotype is confirmed by staining for a number of markers, including cytokeratins, synaptophysin (marker of small synaptic-like vesicles (27)), chromogranin A (large dense-core vesicles (28)), and serotonin.
At the time of diagnosis, SINETs have often metastasised and frequently display regional disease and distant metastasis. In the late SEER data set, the numbers are 41% and 30% respectively (10). Most frequent site for distant metastasis is the liver (89%), followed by mesentery (19%), and bone (11%) (29). Interestingly, about a quarter of all patients present with multiple synchronous primary tumours (30) (Figure 1). It has been speculated that this is connected to familial cases of SINET (31).

Classification, staging and grading

In 1980, the first presented WHO classification of GEPNETs used the term ‘carcinoid’ to describe most gastrointestinal NETs, with exception for pancreatic islet cell tumours and small cell carcinoma. The classification has since been revised, and in the latest revision tumours are now classified as either well-differentiated NETs (grade 1 and 2) or poorly-differentiated neuroendocrine carcinomas (grade 3) (NECs) (32). Neuroendocrine carcinomas and neuroendocrine tumours differ in several aspects. In terms of genomic background, grade 3 carcinomas frequently harbour $\text{TP53}$ and $\text{RB}$ mutations, which are very rarely found in grade 1 and 2 tumours (33). $\text{TP53}$ mutations have been shown to alter tumour cell biology and lead to a worse prognosis for patients with neuroendocrine tumours (34). Although WHO classification guidelines were updated in 2017 for pancreatic neuroendocrine tumours (PanNETs) to now distinguish grade 3 PanNETs and grade 3 pancreatic NECs, this separation is not yet applied for SINETs and small intestinal NECs.

Tumour grading is based on Ki67 index and mitotic count. Grade 1 tumours are defined as having <2 mitoses per 10 high-power fields (HPF) and/or a Ki67 index of $\leq$2. Grade 2 tumours are defined as having a mitotic count of 2-20 per 10 HPF and/or 3-20% Ki67 index. Finally grade 3 tumours have a mitotic count $>$20 per 10 HPF and/or $>$20% Ki67 index. The TNM (tumour-node-metastasis) system is used to specify disease stage (35). Disease stages I, IIA, IIB, and IIIA correspond to localised disease with variations in tumour invasion (T1-T4). Stage IIIB describes any tumour with regional lymph node metastasis (N1; regional disease) and stage IV is used to describe tumours with any distant metastasis (M1; metastatic disease).
Survival and prognosis

Compared to other cancers that commonly arise in the small intestine, e.g. lymphomas, adenocarcinomas, and sarcomas, SINETs have a better survival (22). The 5-year overall survival in the United States SEER database is 68.1% (36). The disease-specific survival, which is naturally higher, has also been investigated in smaller cohorts. Two European (German and Swedish) studies have found the 5-year and 10-year disease-specific survival to be 88.9%/69.2% and 75.0%/63.4% respectively (37,38).

SINET prognostication is usually based on grading and staging, which described in the WHO classification stated in the previous section. Ki67 is more accurate than mitotic count (39) and correlates to patient survival and progression-free survival (29,40). Studies using the current Ki67 cut-offs could observe a statistical difference in 5-year survival between grade 1/2 and grade 3 tumours, and between disease stages I, IIX, IIIX (localised and regional disease) and disease stage IV (metastatic disease) (37,41). Correlation between ethnicity and prognosis has not been shown (10).

The commonly clinically used diagnostic biomarkers 5-HIAA and chromogranin A has not convincingly shown a reliable prognostic potential. There are however other emerging biomarkers that have shown such potential, but there is a need to validate these in prospective trials. Emerging biomarkers with prognostic potential include: serum NSE, pancreastatin, DcR3, TFF3, neurokinin A, neuroendocrine-associated transcripts in serum, and circulating tumour cells (42-44).
Experimental models of SINET disease

Preclinical cancer research utilises a wide range of experimental models to study cancer disease. Models differ in properties that govern how well they reflect various aspects of the tumour disease and so in their applicability to different research questions. These models have helped researchers make ground-breaking discoveries leading to new innovative medicines, but they are also problematic seen to how many pharmaceuticals that are discovered in preclinical models that ultimately fail in clinical trials due to factors such as lack of treatment response or adverse effects (45). Therefore it is of great importance to understand and validate the models being used (46). Below we examine some of these models, which based on experimental setting can be divided into three broad categories: in vitro models, ex vivo models, and in vivo models (Figure 2).

**In vitro models**

In vitro (Latin, approx.: ‘in glass’) models in cancer research usually refers the use of cell lines. Patient tumour-derived cell lines as models of tumour disease have been widely used in cancer research for studying the molecular mechanisms of tumours and their response to therapy. However, cell lines do not perfectly recapitulate the tumour disease and in terms of genomic

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Figure 2. Preclinical models are often subdivided into in vitro, ex vivo, and in vivo models. CDX, cell line-derived xenograft; PDX, patient-derived xenograft; GEM, genetically engineered mouse.
alterations, protein expression, and therapeutic sensitivity, they can differ substantially (47-51).

It has turned out that GEPNET cell lines are very hard to establish. This has been attributed to their low proliferative rate and to the limited amount of donor tissue available (52). Throughout the years, only a few cell lines have been established from human SINETs (Table 1). Unfortunately, the authenticity of several of these cell lines has since been questioned.

Although results are still occasionally published using the CNDT2 cell line, its authenticity has been challenged by several researchers (53,54). In response to the criticism, short tandem repeat (STR) analysis to match the cell line with the NET that was thought to be the source of the cell line was performed, but the STR profiles did not match (53). We also here show in paper I and II that the cell lines KRJ-I, L-STS, and H-STS do not consist of SINET cells, but rather Epstein Barr-virus (EBV)-immortalised B-lymphocytes, and are thus so-called lymphoblastoid cell lines (55). This we based on the lack of a neuroendocrine phenotype, high expression of B cell markers, and a presence of EBV. In paper II we also show that the KRJ-I cell line, based on RNA-sequencing data, most closely resembles diffuse large B-cell lymphoma. KRJ-I, established from a hepatic SINET metastasis (56), is one of the most frequently published SINET cell line. L-STS and H-STS were established together with P-STS from the same SINET patient. P-STS was established from the primary tumour, L-STS from a lymph node metastasis, and H-STS from a hepatic metastasis (57).

Remaining are only two authentic non-transfected SINET cell lines, GOT1 and P-STS. GOT1, first published in 2001 (58), has because of its high expression of somatostatin receptor subtype 2 (SSTR2) mainly been used as a model for peptide receptor radionuclide therapy (59-63). P-STS, contrary to L-STS and H-STS, display both epithelial and neuroendocrine differentiation and is therefore presumed to be authentic. It is however worth noting that it was established from the terminal ileum of a grade 3 tumour, making it essentially not a model of SINET disease but rather a model of small intestinal neuroendocrine carcinomas (64). A molecular characterisation of the P-STS cell line has been published and the cell line has been used to study hormone secretion (65,66).
Table 1. Cell lines established from SINETs as stated in the original publications.

| Cell line | Published (ref) | Established from | Tumour grade | Cell type† |
|-----------|-----------------|-----------------|--------------|------------|
| STC-1     | 1990 (67)       | Intestine of a RIP1Tag2/RIP2-PγST1-transgenic mouse | N.A. | NET       |
| KRJ-I*    | 1996 (56)       | Human primary small intestinal neuroendocrine tumour | N.A. | B-cell   |
| GOT1      | 2001 (58)       | Hepatic metastasis of a human small intestinal neuroendocrine tumour | Grade 1 | NET |
| CNDT2*    | 2007 (54)       | Hepatic metastasis of a human small intestinal neuroendocrine tumour | ‘Low-grade’ | Unknown |
| HC45      | 2007 (68)       | SV40 T antigen-transfected human tumour cells from a hepatic metastasis | N.A. | NET       |
| P-STS     | 2009 (64)       | Human primary small intestinal neuroendocrine tumour | Grade 3 | NEC       |
| L-STS*    | 2009 (64)       | Lymph node metastasis of a human small intestinal neuroendocrine tumour | Grade 3 | B-cells |
| H-STS*    | 2009 (64)       | Hepatic metastasis of a human small intestinal neuroendocrine tumour | Grade 3 | B-cells |

*The authenticity of these cell lines has been challenged. †As stated in original publication or demonstrated in subsequent studies.

Abbreviations: N.A., Not available; NET, neuroendocrine tumour; NEC, neuroendocrine carcinoma; PγST, polyoma small T antigen; RIP, rat insulin promotor; SV40, simian vacuolating virus 40; Tag, T antigen.

The two most frequently published pancreatic NET (PanNET) cell lines are QGP-1 and BON1. QGP-1 was established from a human pancreatic somatostatin-producing islet cell carcinoma (69,70) and BON1 was established from the lymph node metastasis of a PanNET patient (71). The QGP-1 and BON1 cell lines have been previously characterised in terms of exome-sequencing and copy-number alterations (72,73). In addition to these cell lines, there are two other human tumour-derived PanNET cell lines: the CM cell line (74) and the more recently established NT-3 cell line (75), both
from insulin-secreting tumours. The CM cell line has however been criticised for seemingly lacking insulin secretion (76).

There also exists multiple PanNET cell lines established from mouse and rat, most of which came about before the publication of human tumour-derived cell lines. They do not only derive from another species, but were also established in ways that do not necessarily represent naturally occurring tumorigenesis. The following cell lines were derived by transgenic SV40 T antigen-expressing mice: MIN6, βTC, NIT-1 (insulinomas; insulin promoter-driven) (77-79), TGP61 (PanNET; elastase promoter-driven) (80), and Alpha TC (glucagonoma; preproglucagon promoter-driven) (81). The RIN and INS-1 insulinoma cell lines were derived from x-ray irradiated NEDH rats (82,83). Mu Islet E6/E7 (mouse) and HIT (Syrian hamster) were established from transduced pancreatic islets cells (84).

**Ex vivo models**

Ex vivo (Latin, approx.: ‘outside the organism’) models are due to their limited availability not as frequently used in cancer research as immortalised cell lines but have the large benefit of not having been in culture for a longer time period. This means they have not nearly in the same extent gone through the same selection and adaptation process to cell culture conditions, which in many aspects do not reflect growth conditions in the human body. Two commonly studied ex vivo model types are primary cell cultures and organoids.

Primary cell culture is the initial cultivation of cells derived from a tissue. Typically the process of establishing a primary culture is to obtain a tissue biopsy and produce single-cell suspensions by various disassociation techniques. In cancer research they have been used to study many aspects of tumour biology, such as therapeutic sensitivity and imaging (85). SINET primary cell cultures have been used to evaluate the therapeutic sensitivity of patient tumours cells to various pharmaceuticals and to study the SINET hypoxic response (86,87).

Recently the practise of 3D culturing has led to the development of a new ex vivo model. Taking tissue cells, embryonic stem cells, or induced pluripotent stem cells and growing them in a 3D matrix under the right stimulatory conditions can lead to self-organising organotypic structures called
organoids. In this manner for example LGR5+ intestinal stem cells can grow into highly polarised epithelial structures with both proliferative crypts and differentiated villus compartments (88). Organoids have however rarely, if ever, been used in SINET research. However, Bellono et al. recently studied the biology of untransformed EC cells in cultured intestinal organoids, showing the potential for using this research model for studying SINET development (9).

**In vivo models**

‘In vivo’ (Latin, approx.: ‘inside the organism’) models have contributed largely to science. Using organisms such as the *Drosophila* fly or the house mouse, *Mus musculus*, have allowed researchers to conduct research not otherwise feasible. The model used should be carefully evaluated with respect to the research question at hand and to avoid any unnecessary suffering. For SINETs, the model of choice (with some exceptions mentioned below) has been *Mus musculus*. This animal model has several benefits, including the relative ease of housing, that it can be standardised by inbreeding, and that their genome well resembles that of the human. In fact, more than 99% of mouse genes are homologous to human (89).

While the mouse as mentioned has been most commonly used as a study model for NETs, certain rodents which more or less spontaneously develop NETs, like the *Praomys (Mastomys) natalensis*, have also been used to study NETs. These do however not well mirror SINET or PanNET disease but rather gastric NET disease (90). Additionally, serotonin release has been studied in a model were SINETs were transplanted in the anterior eye chamber of cyclosporine-treated rats (91,92).

SINET cell line-derived xenografts (CDX) have been used mainly to study therapeutic sensitivity of PRRT or experimental therapies. CDXs are however still hampered by the many adaptations required for immortalised cell lines to be established. An alternative to CDX models are patient-derived xenografts (PDXs) established directly from patient tumours. A study by Berglind et al. demonstrated that gene expression differ substantially between CDX and PDX models, and argues that this at least partly is due to that cell lines experience ‘pseudo-hypoxia’ when grown in vivo (93). PDXs have also been shown to be useful for predicting therapeutic sensitivity (94-97).
like cell lines, PDX models seem difficult to establish for NETs. Yang et al. attempted to establish PDXs from 106 NETs, including 38 SINETs, but only managed to serially passage a single PDX from a rare gallbladder NET (98).

Genetically engineered mouse models (GEMs) are another alternative, used widely in cancer research (99). This could provide important information about aspects about tumour development. However, no SINET GEMs have been reported, likely at least partly due to the lack of identified driver mutations of SINET disease.
Cancer genetics

The human genome consists of roughly three billion nucleotide pairs, together making up the nucleic DNA. The nucleotides consist of guanine, cytosine, thymine, and adenosine, commonly represented by the letters ‘G’, ‘C’, ‘T’, and ‘A’. To give a hint of how extensive the code for DNA is: this thesis, from front to back page is roughly 300,000 letters long. If one were to print the code for DNA it would require about 10,000 of these books, producing a 100 meter tall pile. This vast genetic material is most commonly distributed onto twenty-two pairs of homologous chromosomes, and 2 sex chromosomes, in total dividing the human genome onto forty-six chromosomal units. DNA both governs the sequence of transcribed RNA by templates called genes and provides the platform for the regulation of when and how much RNA should be transcribed from each gene. The majority of the produced RNA is then translated into functioning proteins which executes most biological processes in the cell.

In the untransformed cell the proteins that should be present under given conditions, homeostasis, is tightly controlled. It is when alterations occur in the DNA that this fine-tuned regulation, and/or the function of proteins is altered. Damage to the DNA is commonly caused by chemical agents or radiation. These genotoxic agents can derive from external exposures or internal biological processes. However, not all damage or errors in the DNA lead to harm. In fact, when alterations to the DNA occur, may it be through a genotoxic agent or by a naturally occurring mistake, it is commonly repaired by the cells’ native DNA repair mechanisms. Furthermore, even if the repair by any reason fails, most mutations have no effect on the cell’s phenotype, so called passenger mutations. It is only when the alteration leads to a change in the coding sequence resulting in an amino-acid change, so-called non-synonymous mutations, a phenotypic effect first occurs.

Genetic aberrations in small intestinal neuroendocrine tumours

Genetic aberrations can be divided into the following types, based on the nature of the genetic consequence: point mutations and indels, copy number alterations and gene fusions. For SINETs, characterisation of substitutions
and indels, and in some degree gene fusions, has mainly been addressed in two publications (100,101) and copy-number alterations in a larger number of studies.

Commonly, genomic sequencing studies aim towards identifying cancer drivers, alterations that lead to the initiation or progression of cancer. These can be identified simply by frequent recurrence, which indicate disease-specific influence, but should also subsequently be validated in cancer models. Compared to many tumour types, SINETs are genetically stable tumours. In a standardised normal-matched sequencing study by Lawrence et al. the somatic nonsynonymous mutational frequency of carcinoids was 0.65 per Mb, more than 10-fold lower than that of cutaneous melanomas, squamous cell carcinomas and adenocarcinomas of the lung (102). Perhaps this is why the first larger exome-sequencing study aimed at identifying substitutions and indels, performed by Banck et al. in 2013, on forty-eight SINETs failed to identify any recurrently mutated genes (101). Later the same year Francis et al. published exome-sequencing of another fifty-five SINETs. This resulted in the discovery of the so far only gene to be identified as recurrently mutated in SINETs, CDKN1B. CDKN1B was found to have heterozygous frameshift mutations in 14/180 (8%) SINETs.

As mentioned, several studies have looked at copy-number alterations in SINETs, including the ones published by Banck et al. and Francis et al. Most of them are based on the comparative genomic hybridisation (CGH) technique, but analysis using microsatellite markers and whole-exome sequencing also occurs (100,101,103-112). The most common somatic copy-number variation (SCNV) is loss of one copy of chromosome 18, which occurs in more than 60% of all tumours. It is also in some tumours the only SCNV reported. Other commonly reported losses, albeit in substantially lower frequencies, include 3p, 9p, 11q, and 16q. Gains are usually of whole chromosomes, including chromosomes 4, 5, 7, 10, 14, and 20 (Figure 3).
Figure 3. Copy-number analysis of two representative SINETs biopsies. Tumour A harboured only loss of chromosome 18, while tumour B instead harbour multiple gains on chromosomes 4, 5, 7, 10, 14, 17, and 20.

Haploinsufficiency

Most humans have 22 pairs of homologous chromosome pairs and two sex chromosomes altogether making up forty-six chromosomes. Since we have homologous chromosomal pairs, the vast majority of all genes are represented by two homologues copies – one on each chromosome. In 1971, Alfred G. Knudson JR presented data that showed that a gene mutation causing retinoblastoma (a gene defined in 1986 and now known as \( RB \) (113)) needed two mutations, one in each allele of the gene, to give rise to the disease. This has been termed the ‘Knudson hypothesis’, or the ‘two-hit hypothesis’(114), and it is today believed that most tumour suppressors are indeed inherited in a recessive manner and in essence follow the two-hit hypothesis. However, many examples of genes that deviate from this hypothesis have been discovered, with prominent examples being e.g. \( PTEN \) (115) and \( TP53 \) (116). A loss-of-function in just one of the alleles of these genes is sufficient to cause a change in the tumour cells’ phenotype and can lead to disease initiation or progression. There are two main mechanisms as to why this happens: either the mutated protein interact with the wild-type protein and inhibit the function of the same, so-called dominant negative mutation. Or, the gene product produced from the one remaining functioning gene is not sufficient to withhold cell homeostasis, which is termed haploinsufficiency. The concept that the number of genes can affect the cell phenotype is called gene dosage. In fact, also the opposite is true, that an addition of genes, such as in the amplification of oncogenes \( MYCN \) (117) and \( EGFR \) (118) or in the gain of whole chromosomes, as in germline trisomy 21, causing Down syndrome, can cause robust phenotypic changes. In the case of
Down syndrome, the phenotype is complicated by the vast amount of genes affected by an increased gene dosage. There are however other congenital disorders at the other side of the spectrum, caused by smaller chromosomal losses or loss or loss-of-function in a single gene that are slightly less complex to decipher. Dozens of human developmental syndromes are caused by hemizygous chromosomal loss (119). Although their effect is debatably less studied than other alterations, the concept of gene dosage can be very important in cancers, which often harbour multiple gains and losses of large chunks of DNA.
Hsp90 and the heat-shock response

A normal cell is often subjected to stress. May it be from reactive agents, pH, temperature, or radiation, stress poses a threat to the cell homeostasis and all of the above mentioned factors can either directly or indirectly lead to considerable harm. It was when, according to Ferruccio Ritossa, a colleague of his had turned up the heat of the incubator containing his *Drosophila melanogaster* flies that he noticed chromosomal puffs indicative of localised and extensive gene transcription (120,121). This was the first reported observation of what came to be termed the heat-shock response. It is now known that key to this response is the upregulation of heat-shock proteins, notably Hsp90, and that it in addition to heat protect against many types of stress.

While bacteria only have one Hsp90 gene that encodes cytosolic proteins, budding yeast and humans have two: *HSP90α* and *HSP90β* (122). Throughout this book, unless otherwise stated, we use ‘Hsp90’ to address proteins from both these paralogues. They differ in that Hsp90β is constitutively expressed in the cell and that Hsp90α is induced by stress (123,124). In fact, in non-stressed cells Hsp90 comprise as much as 1–2% of the total cellular protein content. When subjected to stress, Hsp90 can increase to more than two-fold. In addition to the two mentioned genes, humans have genes encoding Hsp90 homologues also expressed in the mitochondria (125) and the endoplasmic reticulum (126).

Being a chaperone protein, Hsp90 functions by assisting newly translated proteins during the polypeptide-chain synthesis to fold correctly, translocating proteins across membranes, exerting protein quality control in the endoplasmic reticulum, and assisting proteasome-mediated degradation (127). Failure of these functions can lead to protein misfolding and aggregation. Unlike many other chaperones, Hsp90 is however not required for biogenesis of most proteins, but is instead important to govern the conformation of key signalling transducers. Chaperones generally do not covalently modify their substrates; they rather interact with them in an ATP-dependent cyclical fashion (128). This is also true for the heat-shock response (Figure 4).
The cancer cell is under significant stress and this in turn make keeping aberrant protein interactions and misfolding yet more challenging (129). Thus, it is perhaps not surprising to find expression of heat shock proteins upregulated in several types of human cancers, both solid and haematological (130-133). Hsp90 clients are involved in many types of cell signalling associated with the promotion of cancer, including proliferation (134-137), immortalisation (138), impaired apoptosis (139), angiogenesis (140), and invasion/metastasis (141). Hsp90 can as such function both as a potentiator by assisting oncoproteins and as a capacitor by allowing tumours to tolerate external and internal stress (142).

**Figure 4.** The ATP-dependent cyclic action of the heat-shock response. The cycle starts (1) with Hsp70 and Hsp40 binding to the client protein. This complex is stabilised by HIP (2). Hsp90 can bind into the complex with the help of HOP (3), which stabilises the interaction between Hsp90 and Hsp70. ATP is loaded onto Hsp90 (4), an action that could be blocked by Hsp90 inhibitors. The addition of ATP can also be accompanied by immunophilins, co-chaperones, partner proteins, and p23. At the same time Hsp70, Hsp40, HIP, and HOP dissociates from the complex. ATP is hydrolysed (5) in order for Hsp90 to carry out its conformational action.
Hsp90 was thus thought to be a good target for cancer therapy. Initially, naturally occurring substances geldanamycin and radicicol were used to inhibit Hsp90 activity. They however turned out to be unstable and toxic, but inspired the development of first-generation Hsp90 inhibitors. This in turn led to the development of the geldanamycin analogue 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG, alvespimycin), which was water-soluble, had higher potency, and improved bioavailability compared to previous inhibitors (143). It later became the first Hsp90 inhibitor to enter clinical trials. However, adverse clinical effects forced researchers to look for new compounds. Instead, synthetic small molecule inhibitors were developed to target Hsp90, commonly known as second-generation Hsp90 inhibitors. Most Hsp90 inhibitors, with few exceptions, functions by binding and blocking the N-terminal ATP-binding domain of the Hsp90 protein. Ganetespib (STA-9090) is an example of a non-geldanamycin, second-generation Hsp90 inhibitor that binds to the ATP binding pocket of the amino (N) domain and thereby prevents ATP hydrolysis and chaperone function. Ganetespib has shown effect, albeit overall limited, as a monotherapy and in combination with other therapies, in several solid tumour diseases (144-148). These trials have also demonstrated that ganetespib, in contrast to first-generation Hsp90 inhibitors, has improved solubility and reduced risk of cardiac, ocular, and liver toxicities.
SMAD4 and TGFβ-signalling

Transforming growth factor β (TGFβ) is a regulatory cytokine involved in a multitude of biological processes (149). TGFβ-signalling is also well-known to play dual roles in cancer progression (149). While its tumour-suppressing effect is a hurdle transforming cells must bypass, it also promotes cell invasion, immune regulation, and microenvironment modulation that cancer cells can benefit from. Cancer has been shown to circumvent the inhibiting effects TGFβ-signalling in several ways. Biallelic inactivation of TGFBRII are recurrently found in colon, gastric, biliary, pulmonary, ovarian, oesophageal, and head and neck carcinomas (150). TGFBRI mutations are less prevalent but exist in a minority of patients in several cancer types. RSmads are also found inactivated in cancer, but in much lesser degree. For example, recurrent SMAD2 mutations have been found in colorectal cancers (151). The gene for SMAD4, on which the TGFβ canonical signalling converges (Figure 5), is most frequently mutated in cancer, and in a particular high frequency in pancreatic carcinoma and colorectal cancers with microsatellite instability.

Interestingly, SMAD4 seems to play an important part in the GI tract in relation to cancer. Among the five tumour types in The Cancer Genome Atlas (TCGA) with highest frequency of SMAD4 mutations with one exception are adenocarcinomas in the gastrointestinal (GI) tract: pancreas (23%), rectum (20%), colon (14%), and stomach (9%). In addition, SMAD4 has been suggested to have a critical role in the tumourigenesis of small intestinal adenocarcinomas (152). A published analysis of TCGA shows that hotspot mutations in TGFβ pathway members are highly overrepresented in GI cancers (153). Heterozygous inactivation of the SMAD4 gene in humans frequently leads to the familial juvenile polyposis syndrome (JPS) (154). The syndrome predisposes the carriers to GI hamartomatous polyps and GI cancer. SMAD4 accounts for about 15% of all JPS cases and the majority of the SMAD4 germ line mutations are located in the MH2 domain which participates in RSmad-SMAD4 complex formation (homo- and hetero-oligomerization) (155). Compared to mutations in BMPRIA (account for 25% of cases), patients with SMAD4 are more likely to present with massive gastric polyposis (156).
Figure 5. TGFβ canonical signalling. A ligand brings receptors of type I and II together. These can either belong to the TGFβ or bone morphogenetic protein (BMP) families. Upon binding, the type II receptor phosphorylates the type I receptor which becomes active and propagates the signal by phosphorylating receptor substrate Smad transcription factors (R-SMADs). Receptors of the TGFβ family phosphorylate and thereby activate the RSmads SMAD2 and SMAD3, while receptors of the BMP family phosphorylate SMAD1, SMAD5, and SMAD8. The R-SMADs, either SMAD2/3 or SMAD1/5/8, binds to SMAD4 and translocate into the cell nucleus where it associates with additional DNA-binding cofactors and induce gene transcription (157). The complex can in addition recruit other coactivators, corepressors, and chromatin remodelling factors which can further modulate gene transcription.
Treatment of small intestinal neuroendocrine tumours

There is a general lack of efficient and curative therapies for SINETs. The palliative and somewhat tumour growth-inhibiting somatostatin analogues are standard care for most patients. For localised disease surgery is a viable option, but for disseminated disease there is currently no curative treatments available. Below follows a brief review of common treatment options for SINETs, including the newly recommended $^{177}$Lu-octreotate therapy (158).

Current treatment options

Traditionally radical surgical resection has been the only hope for curing SINETs. Primary SINETs are usually relatively small and easily removed, but also very frequently present together with lymph node metastasis (86% in the SEER data base (159)). In about 5% of patients also miliary seeding in the intra-abdominal cavity is observed (160). Distant metastases are also commonly occurring, posing a much larger challenge for surgery. Localised and regional tumours are often removed by surgical resection. There is an absence of internationally standardised surgical procedures, but when performing surgery of lymph node metastasis it is recommended to remove at least 8 nodes (158). In the cases where growth of the primary tumour and involvement of mesenteric disease, often together with fibrosis, complete resection can be more challenging, but can still be achieved in up to 80% of cases (161-163). As previously mentioned, distant metastasis are frequent and by far most commonly found in the liver. The distribution of neuroendocrine liver metastasis can be classified into three types: type 1 (single metastasis of any size), type 2 (isolated bulk with smaller deposits), and type 3 (disseminated metastatic spread) (164). While radical surgery for type 1 liver metastasis seems to be associated with improved outcome, radical surgery for type 2 and type 3 is more controversial. In addition, surgery to remove hepatic metastasis is in general not performed on poorly-differentiated (G3) tumours, which are associated with much greater risk of metastasis (165).

Somatostatin analogues, such as octreotide and lanreotide, are used to treat symptoms related to hormone hypersecretion. Somatostatin analogues however not only inhibit hormone release, but can also lead to increased time
to tumour progression (166,167). For in particular somatostatin receptor negative or refractory tumours, INF-α2b, which has shown improved progression-free survival for SINETs (168), can be administered (169).

Everolimus and sunitinib are two targeted therapies that are approved for the treatment of advanced neuroendocrine tumours. Everolimus, an inhibitor of the mTOR pathway, which controls functions such as cellular proliferation, metabolism, protein synthesis, and autophagy, has shown a significant improved progression-free survival for advanced progressive gastrointestinal neuroendocrine tumours (170). This despite an overall lack of activating mutations in the mTOR pathway in SINETs (100,101). Sunitinib malate is instead an inhibitor of tyrosine kinases, including vascular endothelial growth factor receptors (VEGFR), platelet-derived growth factor receptors (PDGFR), CD117 (KIT), and RET, and although it improves progression-free survival for patients with pancreatic neuroendocrine tumours (171), its efficacy is yet to be demonstrated for SINETs.

Systemic chemotherapy is recommended by European Neuroendocrine Tumor Society (ENETS) treatment guidelines only for grade 3 NETs (or advanced PNETs) (172). For high-grade NETs, chemotherapy involving platinum-based substances is recommended, such as the combination of cisplatin and etoposide.

177Lu-octreotate therapy

Peptide receptor radionuclide therapy (PRRT) is a treatment modality that uses a therapeutic radionuclide conjugated to a targeting vector. PRRT can be used both as a potentially curative therapy and for palliation. It thus can be viewed as a way to combine radiation therapy with systemic administration and tumour selectivity. Both the properties of the radionuclide, which can emit different types of particles and electrons (173), and the targeting vector, determines the success of the radionuclide therapy.

A recently FDA-approved PRRT is the 177Lu-octreotate therapy, which has been granted approval for the treatment of somatostatin receptor subtype 2 (SSTR2)-positive GEPNETs (174). 177Lu-octreotate therapy consists of the radionuclide 177Lu conjugated to the somatostatin analogue octreotate, which can bind to somatostatin receptors and provide tumour-selective irradiation (Figure 6). 177Lu, the radionuclide, mainly emits β particles, but also gamma
radiation, and its emission can cause double-strand breaks in the cell (175). It has a half-life of 6.7 days and a tissue penetration of about 2 mm. Together with the conjugated somatostatin analogue octreotate, $^{177}\text{Lu}$-octreotate therapy mainly adheres to human somatostatin receptor subtype 2, but also shows measurable affinity for subtypes 4 and 5 (176,177).

Several trials using $^{177}\text{Lu}$-octreotate therapy for GEPNETs have been reported (178-187), but comparisons have been complicated by varying selection criteria, treatment regimens, and outcome measures. These studies in addition rarely include a control group, further complicating conclusions regarding efficacy. There has been retrospective and phase II studies with $^{177}\text{Lu}$-octreotate that have shown a median progression-free survival of over 30 months in patients with advanced SINETs with documented tumour progression or uncontrolled carcinoid symptoms (183,187). This was enough to initiate the first randomised controlled trial, the cross-institutional phase III trial NETTER-1 (185). In this trial patients were treated with 4 cycles of 7.4 MBq $^{177}\text{Lu}$-octreotate every 8 weeks plus long-acting repeatable (LAR) octreotide and compared to patients treated only with high-dose LAR octreotide. In total 229 patients with octreoscan-positive tumours were enrolled. At month 20 the progression-free survival was 65.2% vs. 10.8% and
the response rate was 18% vs. 3%. There has also been shown an overall improvement in quality of life in NET patients treated with $^{177}$Lu-octreotate (188,189). On the basis of this trial, $^{177}$Lu-octreotate therapy was FDA-approved for treating SSTR2-positive GEPNETs.

While $^{177}$Lu-octreotate in previous studies have shown similar efficacy to $^{90}$Y-DOTATOC, it has also shown a better toxicity profile – especially related to haematological adverse effects. Haematological adverse effects are although still a prevalent side effects of $^{177}$Lu-octreotate therapy. Overall however, the most common adverse effects are nausea and abdominal discomfort. More serious adverse effects include renal toxicity and the already mentioned haematological toxicity (190,191). Renal toxicity is believed to be caused by the renal excretion of $^{177}$Lu-octreotate and can be somewhat mitigated by renal-blocking amino acid infusions.
Cancer and the immune system

In order for cancer to thrive, the immune system is a hurdle that needs to be overcome. Immune cells are primed to detect and eliminate any cells that do not look domestic. Indeed, most tumour cells express antigens that can mediate recognition by host CD8+ cells and applying immune evasive mechanisms is therefore a prerequisite. Tumour cells have been shown to evade the immune system in several ways, by both tumour-intrinsic and tumour-extrinsic mechanisms. Tumour cell-intrinsic mechanisms can include loss of major histocompatibility complex (MHC) class I proteins, inhibition of the antigen processing machinery, loss of tumour-associated antigens, or expression of inhibitory proteins. Tumour cell-extrinsic factors include the modulation of the microenvironment to recruit immune-suppressive cells (such as regulatory T cells), inactivation of immune receptors and secretion of immune suppressive cytokines. Novel therapeutic strategies have focused on overturning these evasive mechanisms. The recently successful check-point inhibitors are focusing on abrogating the immune receptor proteins expressed by the tumour cells, but there are more ways to go.

The therapy that first attracted large attention to check point inhibition was the inhibitor ipilimumab, a monoclonal antibody directed against cytotoxic T lymphocyte antigen 4 (CTLA4), which was approved in 2011 and was the first therapy to show an overall survival advantage in metastatic melanoma (192). CTLA4 inhibition has now been largely taken over by inhibitors against PD-1 and PD-L1, which show a better toxicity profile. A large amount of clinical trials have paved the way to the FDA-approval PD-1/PD-L1 inhibitors for a large variety of cancers (193). To date, five PD-1/PD-L1 inhibitors have been FDA-approved for the treatment of cancer (194). However there are also other interesting immunotherapies designed to enhance the immune system against cancer. These include tumour-directed monoclonal antibodies, oncolytic viruses, cancer vaccines, and T-cell-focused therapies. Tumour-directed monoclonal antibodies are designed to target tumour-specific antigens, stay on the surface and activate antibody/complement-dependent cytotoxicity, oncolytic viruses can selectively infect and kill cells that express specific proteins, and cancer vaccines can work by immunising patient to tumour-associate antigens.
Other immune therapies have focused on T cells, including the manufacturing of chimeric antigen receptor (CAR) T cells, which recognises a specified tumour-antigen and are activated in an MHC-independent manner and T cell receptor (TCR) gene-modified T cell therapy, which works by modifying the TCR to detect specific tumour antigens presented by HLA proteins. Adoptive cell transfer (ACT) is another T-cell focused immunotherapy, it refers to the stimulation and expansion in vitro of endogenous or allogeneic immune effector cells for patient administration (Figure 4). For ACT to work, IL-2, a signalling cytokine that stimulates immune cells, is often co-administered to ensure the viability and function of infused cells. ACT has achieved a 20% complete response lasting longer than 3 years in stage IV melanoma (195).

Figure 7. Schematic of the adoptive cell transfer therapy. (1) The tumour is excised from the patient, (2) plated as single cells, and (3) tumour-infiltrating T cells are selectively expanded by IL-2 stimulation. (4) An assay for tumour recognition can be performed and (5) functional clones selected and expanded. (6) Expanded T cells are reinfused into the patient.
With limited clinical experience, investigation for the role of the immune therapy in SINETs have in light of the success of check-point inhibitors recently mainly focused on characterising the expression of programmed death-ligand 1 (PD-L1) and programmed cell death protein 1 (PD-1) by immunohistochemistry. The positivity of these proteins in SINET biopsies has varied, with reported PD-L1 positivity ranging between 0-39% and PD-L2 positivity between 0-82% (196-198). The most notable difference has been that of comparing well-differentiated (grade 1 and 2) and poorly-differentiated (grade 3) tumours as PD-L1 expression has been observed to be significantly higher in grade 3 GEPNETs (199).
AIMS

All papers within the scope of this thesis aimed towards expanding the knowledge of small intestinal neuroendocrine tumours to give instruments for discovery and implementation of clinical therapies that benefit patients affected by this tumour disease.

Specifically, the aims of the papers were:

**Paper I and II:** To characterise and evaluate frequently used gastroenteropancreatic cell lines in aspects relevant for studying neuroendocrine tumour disease.

**Paper III:** To shed light on the genetic mechanisms underlying the initiation and/or progression of small intestinal neuroendocrine tumours.

**Paper IV:** To identify and validate a novel combination therapy to potentiate the efficacy of the $^{177}$Lu-octreotate therapy for small intestinal neuroendocrine tumours.

**Paper V:** To evaluate the potential for immunotherapy in small intestinal neuroendocrine tumours.
METHODOLOGY

In the following sections some selected key materials and methods are detailed.

Material

Material used in the papers included in vitro models, ex vivo models, in vivo models, and patient samples.

**Cell culture (Papers I, IV, and V)**

All cell lines and primary cells were grown in specified media compositions and were kept at 37°C in a humidified incubator with an atmosphere of 5% CO₂ (Table 2). All cell lines were subject to short tandem repeat (STR) analysis at a DANAK/ILAC DS/EN ISO 15189:2008 accredited laboratory (IdentiCell, Department of Molecular Medicine at Aarhus University Hospital, Denmark) and were regularly tested for *Mycoplasma* species by PCR (200) at a Swedac SS-EN ISO 15189 accredited laboratory (Bacteriological laboratory, Sahlgrenska University Hospital, Gothenburg, Sweden). Primary cells were generated from tumour tissue collected at surgery by cutting the tumour tissue into small pieces and digested them in 50 mL RPMI 1640 containing 2mg/ml collagenase I (Sigma) and 24μg/ml DNAse (Sigma) for 1–3 h at 37°C. All primary cultures were used for experiments at first passage.

**Tissue microarray (Papers I, III, IV, and V)**

A tissue microarray (TMA) was constructed using biopsies from patients who underwent surgery for SINETs at Sahlgrenska University Hospital from 1986 to 2013. Formalin-fixed and paraffin-embedded tumour tissue from this cohort was originally retrieved from the archives of the Department of Clinical Pathology and Genetics, Sahlgrenska University Hospital, Gothenburg. The diagnosis was confirmed by reviewing haematoxylin and eosin-stained sections and immunohistochemical stainings. Sufficient tumour material for construction of tissue microarray was available from 846 tumours from 412 patients. 1.0 mm core biopsies were obtained from each tumour. Eight recipient blocks were created and each block contained a total
of 121 core biopsies. Each block also included normal tissue from gut, small intestine, and large intestine. When available, core biopsies were taken from primary tumour, lymph node metastases, liver metastases, and other distant metastases. The quality of the constructed tissue microarray was evaluated on hematoxylin and eosin-stained sections and on immunohistochemical stainings for chromogranin A, synaptophysin, serotonin, and Ki67. We obtained approval from the Regional Ethical Review Board in Gothenburg, Sweden, for the use of clinical materials for research purpose.

**Tumour xenografts (Papers IV and V)**

Tumour xenografts were studied in two different papers (paper IV and V). In paper IV, in vivo experiments were based on cell-line derived xenografts, specifically from the GOT1 cell line. GOT1 tissue was transplanted subcutaneously to BALB/c nude mice (Janvier Labs) and growing tumours were measured twice weekly with slide calipers. In study V, we instead opted for establishing patient-derived xenografts in NOG mice. For this purpose we tried both different ways of pre-processing patient tumour tissue and different transplantation approaches. Tumour tissue was either collected directly from surgery or thawed from cryofrozen material before transplantation. Transplantation was done either subcutaneously or through orthologous liver injections.

For all experiments water and autoclaved food were available *ad libitum* and the well-being of the mice continuously looked after. Mice were sacrificed at the end of experiment by intraperitoneal injection of 60 mg/mL pentobarbital (Pentobarbitalnatrium vet., Apotek Produktion & Laboratorier), followed by cardiac puncture. We obtained approval from Regional Ethical Review Board in Gothenburg, Sweden, for all animal procedures.
Table 2. In vitro models used within the scope of this thesis, the cell media they were kept in, and from where they were acquired.

| Identification | Cell media* | Source |
|----------------|-------------|--------|
| Primary cells  | RPMI-1640 supplemented with 4% FBS, 200 IU/mL penicillin and 200 µg/mL streptomycin | Generated in lab |
| GOT1           | RPMI-1640 supplemented with 10% FBS, 5 µg/mL insulin and 5 µg/mL transferrin | Established in lab |
| KRI-I          | M199:Ham’s F12 (1:1) supplemented with 10% FBS | Prof. R. Pfragner |
| P-STS          | M199:Ham’s F12 (1:1) supplemented with 10% FBS | Prof. R. Pfragner |
| L-STS          | M199:Ham’s F12 (1:1) supplemented with 10% FBS | Prof. R. Pfragner |
| H-STS          | M199:Ham’s F12 (1:1) supplemented with 10% FBS | Prof. R. Pfragner |
| QGP-1          | RPMI-1640 supplemented with 10% FBS | JCRB |
| BON1           | DMEM:Ham’s F12 (1:1) with 10% FBS | Prof. B Wiedenmann |
| MCF10A         | DMEM:Ham’s F12 (1:1) supplemented with 5% horse serum, 10 µg/mL insulin, 20 ng/mL EGF, 0.5 µg/mL hydrocortisone and 0.1 µg/mL cholera toxin | ATCC |
| BJ             | Eagle’s minimum essential medium supplemented with 10% FBS | ATCC |
| HUV-EC-C       | F12K medium supplemented with 10% FBS, 100 µg/mL heparin and 50 µg/mL endothelial cell growth supplement | ATCC |

* All media in addition contained 200 IU/mL penicillin and 200 µg/mL streptomycin.

**Abbreviations:** ATCC, American Type Culture Collection, EGF, epidermal growth factor; FBS, foetal bovine serum; JCRB, Japanese Collection of Research Bioresources Cell Bank; RPMI, Roswell Park Memorial Institute
Selected methods

The results of the papers presented in this thesis were generated by more than twenty defined methods (Table 3). For details of each methodology, please refer to the specified papers. Below a few selected key methods are detailed.

**Immunohistochemistry (Papers I, III, IV, and V)**

Immunohistochemistry was performed on different types of material, including cell lines, primary cell cultures, CDXs, PDXs, patient tumour tissue, and TMAs. All material was fixed by 4% buffered formaldehyde or methanol and then embedded in paraffin. Sections (3–4 μm) from paraffin blocks were placed on glass slides and treated in Dako PT-Link using EnVision™ FLEX Target Retrieval Solution (high pH). A wide selection of antibodies was used and information about antigen, clone, and manufacturer is specified in the material and methods section of individual papers. Immunohistochemical staining was performed in a Dako Autostainer Link using EnVision™ FLEX according to the manufacturer’s instructions (DakoCytomation). For most stainings, EnVision™ FLEX+ (LINKER) rabbit or mouse was used. Positive and negative controls were included in each run.

**Fluorescence in situ hybridisation (Paper III)**

Fluorescence in situ hybridisation (FISH) was performed on 4 µm paraffin sections from the TMA. Pre-processing of paraffin sections, hybridisation to the probe, post-hybridisation washing and fluorescence detection were performed according to manufacturer’s instructions (Abnova). Tumours were examined using an Axioplan 2i epifluorescence microscope (Zeiss, Oberkochen, Germany) equipped with a 6 megapixel CCD camera (CV-M4+CL, JAI) controlled by Isis 5.5.9 imaging software (MetaSystems Group Inc, Waltham, MA, USA). Within each section, normal regions/stromal elements served as the internal control to assess quality of hybridisation. Cases were scored at 100× magnification, counting at least three distinct areas and at least 30 discrete nuclei.
Table 3. Compilation of methods used in the papers included in this thesis.

| Methods                              | Used in paper(s) |
|--------------------------------------|------------------|
| ArrayCGH                             | I and III        |
| Cell block generation (HOLOGIC)      | I                |
| Confocal laser scanning microscopy   | I                |
| Degranulation assay                  | V                |
| DNA extraction                       | I, II, III, and V|
| ELISA                                | I and V          |
| External beam irradiation            | IV               |
| Fluorescence in situ hybridisation   | III              |
| Generation of primary cell cultures  | I, IV, and V     |
| Immune cell phenotyping              | V                |
| Immunohistochemistry                 | I, III, IV, and V|
| Isolation and expansion of TILs       | V                |
| Ki67-index quantification            | I and IV         |
| Mycoplasma detection                 | I and IV         |
| Real-time PCR                        | I                |
| RNA extraction                       | I and II         |
| RNAseq                               | II               |
| Short tandem repeat analysis         | I                |
| Synergy screening                    | IV               |
| Therapeutic sensitivity screening    | I                |
| Transcriptomic classification        | II               |
| Whole-exome sequencing               | I, III, and V    |
| Viability assay                      | I and IV         |
| Viral sequence detection             | II               |

Abbreviations: CGH, comparative genomic hybridisation; ELISA, enzyme-linked immunosorbent assay; TILs, tumour-infiltrating lymphocytes; PCR, polymerase chain reaction.

Inhibitor screening (Papers I and IV)

The screening library consisted of 1224 compounds (Inhibitor library, no. L1100; Selleckchem). Inhibitors were subjected to a maximum of five freeze-thaw cycles. From frozen stocks, cells were expanded 2 to 5 passages before being used in experiments. Seeding density was adjusted for each cell line so
that control cells were approximately 70–80% confluent at treatment endpoint in 100 µL cell medium/well in black solid-bottom 96-well plates. The plates were incubated at 37°C to allow for cell attachment. Each treatment plate included 8 internal control wells with DMSO, and each experiment included an additional plate with 96 DMSO control wells. Additionally, each experiment contained one cell-free control plate for background subtraction. For screenings in both paper I and IV, the end-concentration in the wells was 1µM. Cell viability was estimated using a fluorescence-based assay to measure the reducing capacity of metabolically active cells (alamarBlue, DAL1100; Life Technologies). The plates were read using a 96-well fluorescence plate reader (Victor³ multilabel reader, ex. 560 nm/em. 640 nm).

**Generation of tumour infiltrating lymphocytes (Paper V)**

Patient tumour tissue samples were obtained from patients undergoing surgery for SINET disease at Sahlgrenska University Hospital, Gothenburg, Sweden. Tumour tissue obtained directly from surgery were cut into 1-2 mm² pieces and placed into separate wells in a 24 well-plate (Sarstedt) with 2 ml of culture medium (90% RPMI 1640 (Invitrogen), 10% heat inactivated Human AB serum (HS, Sigma-Aldrich), 6000 IU/ml recombinant human IL-2 (Peprotech) and gentamicin (Invitrogen). TILs were isolated from each fragment as previously described (201-203), before cryopreservation. TILs were expanded according to previously described procedures (203). In brief it was performed as follows: Irradiated (40 Gy) allogeneic feeder cells (5×10⁶), 30 ng/ml anti-, antibody (Miltenyi; OKT3), 5 ml culture medium, 5 ml REP medium (AIM-V, Invitrogen) supplemented with 10% HS and 6000 IU/ml IL-2) and isolated TILs (5×10⁴) were mixed in a 25-cm² tissue culture flask. Flasks were incubated upright at 37°C in 5% CO₂. On day 5, half of the medium was replaced. On day 7 and every day thereafter, cells were split into further flasks with additional medium as needed to maintain cell densities around 1–2×10⁶ cells/ml. On day 10–14, cells were harvested and cryopreserved. We obtained approval from Regional Ethical Review Board in Gothenburg, Sweden, for the use of clinical materials for research purposes.
RESULTS AND DISCUSSION

The characteristics of GEPNET cell lines (paper I)

Experimental models of neuroendocrine tumour disease are scarce, and no comprehensive characterisation of existing gastro-entero-pancreatic neuroendocrine tumour (GEPNET) cell lines has previously been reported. In this study, we aimed to define the molecular characteristics and therapeutic sensitivity of these cell lines. We therefore performed immunophenotyping, copy-number profiling, whole-exome sequencing, and a large-scale inhibitor screening of seven GEPNET cell lines.

![Figure 8: Immunohistochemical staining of four authentic GEPNET cell lines. Shown are two of the many investigated protein markers. All four cell lines expressed endocrine differentiation marker synaptophysin (SYP) and expressed varying degree of therapeutically relevant somatostatin receptor subtype 2 (SSTR2).](image)

The gold standard of diagnosing a cancer disease is by histopathological examination, including immunohistochemical staining of biomarkers. To validate the diagnosis of frequently used GEPNET cell lines, we performed immunophenotyping investigating commonly used markers for GEPNET diagnostics (Figure 8). These normally include neuroendocrine markers
synaptophysin (small synaptic-like vesicles (27)) and chromogranin A (large dense-core vesicles (28)) (26). To ensure an epithelial phenotype cytokeratin is also often investigated. Intriguingly, the diagnosis could not be confirmed for cell lines KRJ-I, L-STS, and H-STS, which we further address in the next results section. Remaining cell lines all expressed synaptophysin and pan-cytokeratins strongly but varied in their expression of other neuroendocrine markers, potentially indicative of partly lost neuroendocrine phenotypes.

Genomic background influences both prognosis and therapeutic sensitivity of tumour cells. There are for example mutations both confirmed to lead to a worse patient prognosis and mutations that are directly targeted by pharmaceuticals. If we are to study such aspects of cancer biology, we thus need to know which genetic characteristics our models harbour, and importantly, if they recapitulate the disease afflicted upon the patients. For these reasons we studied both somatic copy number alterations as well as genetic mutations using arrayCGH and whole-exome sequencing.

The copy number profiling revealed both common alterations, but also changes that are rarely detected in patient tumours. SINETs most frequently harbour loss of chromosome 18. Because of this chromosome 18 has been the subject of extensive investigation to identify inactivated tumour suppressors localised on the chromosome. Interestingly, the GOT1 cell line harboured 1.6 Mb segmental loss on 18q involving 7 genes, including SMAD4. While the SINET cell lines had a predominance of chromosomal losses, the PanNET cell lines had higher frequency chromosomal gains. Notably, BON1 harboured homozygous loss of the well-known tumour suppressors CDKN2A and CDKN2B and QGP-1 was the only cell line that harboured chromosomal amplifications, including HMGA2 and MDM2, the former often found upregulated in cancer and the latter an established oncogene.

We finished the study looking at the therapeutic sensitivity of the cell lines. This had several purposes: a) As a way of characterising the cell lines, b) to study whether the therapeutic sensitivity of the cell lines could predict the sensitivity of primary tumour cells, and c) to provide leads for potentially interesting inhibitors for GEPNET therapy. To minimise the risk of identifying efficient inhibitors based on cell culture conditions rather than tumour cell characteristics, all results were given comparing SINET and PanNET cell lines to each other. We found that SINET cell lines were more
sensitive to HDACi compared to PanNET cell lines, and that PanNET cell lines were more sensitive to MEKi compared to SINET cell lines. These findings also held true when comparing primary cells generated from SINETs and PanNETs.

In conclusion, we provided a thorough and well-needed characterisation of frequently used GEPNET cell lines. This characterisation included a comprehensive immunophenotyping, copy number alterations, gene mutations, and the therapeutic sensitivity to more than 1224 inhibitors.

**H-STS, L-STS, and KRJ-I are not authentic GEPNET cell lines (papers I and II)**

When characterising the KRJ-I, L-STS, and H-STS SINET cell lines in paper I, we were surprised to find that the cell lines expressed extremely low or undetectable levels of neuroendocrine markers chromogranin A and synaptophysin. This was also the case for all other neuroendocrine, enterochromaffin, and importantly, epithelial markers. Given the lack of even an epithelial phenotype, and the peculiar fact that, contrary to other GEPNET cell lines, they grew as sphere-forming suspension cultures, we postulated that these cell lines may be lymphoblastoid. Lymphoblastoid cell lines are immortalised B-lymphocytes that do not undergo senescence because they are infected and driven by the Epstein-Barr virus (EBV). Indeed, we could confirm the strong expression of lymphoid marker and B-cell marker CD45 and CD20 in all three cell lines. These markers were at the same time undetectable in the other GEPNET cell lines. Furthermore, EBV DNA was found in all three cell lines, which again was not the case for the other GEPNET cell lines.

This provided strong proof that the cell lines we had obtained did in fact not even consist of epithelial tumour cells, but rather immortalised B-cells. Since many publications have been produced using these cell lines, and in particular the KRJ-I cell line, we wanted to see if this was a problem not only in our lab. We therefore confirmed with the lab where the cell lines were established that the cell lines also had a lack of neuroendocrine markers, expressed B-cell markers, and had presence of EBV in early passages of the
cell lines. This implies that any SINET cells present in culture from the start got overgrown early or where never present to start with. To that follows that it is likely that most or all published articles using these cell lines could present inaccurate research findings.

A few months after paper I was published, Alvarez et al. published in *Nature Genetics* an ambitious original research article on GEPNETs (204). The study pooled GEPNET patient samples from 18 institutions, performed RNAseq on these and inhibitor-treated cell lines. In brief, using bioinformatics approaches they drew conclusions on the therapeutic sensitivity of GEPNETs and verified their findings in cell line-derived xenografts. The study contained five main data-presenting figures and the results in all of them except the first figure were based on the use of the KRJ-I and/or H-STS cell lines. Based on our previous findings, we found it likely that Alvarez et al. had used lymphoblastoid cell lines rather than SINET cell lines. Using an approach some of the co-authors previously benchmarked (205), we reanalysed RNAseq data from 51 DMSO-treated cell line samples made publically available from Alvarez et al. study and could conclude that their transcriptomic profile in the TCGA data base most closely resembled that of diffuse large B-cell lymphoma. As a control we RNA-sequenced seven own SINET samples and saw they most closely resembled either pheochromocytoma/paraganglioma or pancreatic adenocarcinoma. We could even, using the VirusFinder 2 algorithm confirm the presence of EBV unique transcripts in the cell line data published by Alvarez et al.. This all strongly indicated that the cells used in the study shared the same B-cell phenotype found in our laboratory.

In conclusion, we have revealed that the previously presumed and frequently in the field used SINET cell lines KRJ-I, L-STS, and H-STS are not authentic. They instead consist of immortalised EBV-infected B-cells, and are thus better described as lymphoblastoid cell lines. This has now been shown in our lab, shown in the lab that established the cell lines, and more recently shown using the RNAseq data from the Alvarez et al. study. We therefore urge that interpretation of data from studies using these cell lines should be conducted with large caution.
SMAD4 haploinsufficiency in SINETs (paper III)

The genomic alterations that lead to tumour initiation and progression are termed driver mutations. Identifying driver mutations is important to shed light on the tumour biology of the cancer disease and could lead to an increased understanding of how the tumour cells could be pharmacologically targeted. Currently not much is known about the molecular background of SINETs. Driver mutations can commonly be detected by their frequent occurrence. In SINETs however, despite whole-exome sequencing of more than one hundred patient tumours, only one recurrently mutated gene has been identified, CDKN1B, and in less than a tenth of all tumours.

Here we instead turned our attention to copy-number alterations. Several copy-number alterations are recurrent in SINETs and although these are rarely reported homozygous, we speculated that these alterations have an important impact to SINETs. The most frequent genomic alteration in SINETs is loss of chromosome 18. SMAD4, located on chromosome 18, has in genetically engineered mouse models been reported to be haploinsufficient (206,207) and heterozygous germline mutations of SMAD4 can lead to familial juvenile polyposis syndrome – a syndrome that among other things predispose the carrier to gastrointestinal cancers (154).

We therefore decided to investigate the role of hemizygous loss of chromosome 18 and its relation to SMAD4 mRNA and SMAD4 protein. Investigating a for the field very large cohort of SINETs, including more than 846 tumours from 412 patients, we found that hemizygous loss of the SMAD4 was correlated to both an approximately two-fold decrease in corresponding mRNA and lower SMAD4 protein levels. Of note, we observed that a decrease in SMAD4 protein in the primary tumours was associated with a worse patient prognosis and with the occurrence of distant metastasis. In colorectal cancer, SMAD4 mutations have been shown to be cancer promoting in the presence of TGFβ stimulation (208). One possible mechanism for this is through promotion of epithelial to mesenchymal transition (EMT) resulting from accumulation of nuclear-β-catenin following SMAD4 downregulation (209). Interestingly, it has been speculated that SINETs are insensitive to TGFβ growth inhibitory effects (210). We also
studied whether monoallelic inactivation of *Smad4* was alone sufficient to induce endocrine cell hyperplasia in a mouse model, but could not find support for this hypothesis.

In summary, the findings in this study suggest that copy number alterations in SINETs can affect protein expression of tumour-associated genes and could thereby represent a novel mechanism underlying SINET tumour pathogenesis. Further research regarding causal link between copy-number alterations and functional consequences is warranted.

**177Lu-octreotate therapy for SINETs can be potentiated by Hsp90 inhibition (paper IV)**

Following promising results in a phase 3 trial (211), 177Lu-octreotate therapy became FDA-approved in 2018 for patients with gastroenteropancreatic neuroendocrine tumours expressing somatostatin receptors (174). The 177Lu-octreotate therapy is indeed showing better results in clinical trials than other therapies for SINETs and lead to longer progression-free survival, but complete responses are still rare. A common strategy to enhance the efficacy of a therapy without a corresponding increase in severe side effects is through implementing combination therapy (212). Our goal with paper IV was thus to identify a therapy that would potentiate the efficacy of the 177Lu-octreotate therapy.

To identify interesting combinations, we screened the two cell lines GOT1 and P-STS for inhibitors that caused a synergistic radiosensitisation. In total, 1224 inhibitors were investigated. Out of these, 2–3% of the inhibitors showed synergistic interaction with external radiation at the evaluated dose. This is similar level to other large-scale screenings looking to identify synergistic pairs (4–10%) (213-215). By performing an analysis looking at inhibitor class overrepresentations, we saw that inhibitors of Hsp90 were highly overrepresented for the GOT1 cell line (False discovery rate; FDR: 3.2×10^{-11}). Hsp90i were however not overrepresented in the P-STS cell line, which we attribute to significant differences between the cell lines. Notably, while GOT1 was established from a grade 1 well-differentiated
neuroendocrine tumour, P-STS was established from a grade 3 poorly-differentiated carcinoma. P-STS also contains mutations that could affect its response to the combination therapy, including uncommon mutations in TP53, BRCA1, and BRCA2 (55). In fact, previous reports suggest that Hsp90 radiosensitisation occurs through impairing the DNA double strand repair mechanisms (216) and then specifically through the inhibition of BRCA1 and/or BRCA2 (217,218).

Although inhibitors of Hsp90 caused a synergistic radiosensitisation to external radiation in the GOT1 cell line, we did not know if it would have the same effect with $^{177}$Lu-octreotate, which rather emits beta radiation. We thus decided to investigate if ganetespib, an inhibitor of Hsp90, could induce a similar synergistic radiosensitisation with $^{177}$Lu-octreotate therapy to treat GOT1 xenograft tumours in mice. This model system was suitable since the GOT1 cell line, as opposed to other cell lines (55,219), has not lost its SSTR2-expression. The effect of $^{177}$Lu-octreotate, ganetespib, and combination of them both on tumour volume was observed over 14 days under which we observed a potent and significant synergistic effect of the combination.

To shed some light as to how many SINET patients may benefit from this combination, and to further validate the results, we studied the combination in first-passage primary cells prepared from patient tumours collected at surgery. All eight patient tumours investigated were poorly differentiated grade 1 or 2 metastatic SINETs. All individuals’ patient tumours trended towards synergy, and looking at the overall effect, we could again observe a significant synergistic radiosensitisation.

In addition, we investigated a larger cohort containing 761 SINETs from 379 patients, for the expression Hsp90 by immunohistochemistry. We could conclude that Hsp90 is upregulated compared to surrounding tumour stromal cells in more than 90% of all tumours. No association between high/low Hsp90 expression and patient survival could be found in neither the large cohort nor a smaller cohort of 43 SINET patients treated with $^{177}$Lu-octreotate.
In conclusion, we identify ganetespib, an inhibitor of Hsp90, to be able to potentiate the $^{177}$Lu-octreotate therapy by radiosensitising SINET cells, and suggest that this combination should be evaluated in a clinical setting.

The SINET immune microenvironment contains lymphocytes capable of recognition and activation after expansion (paper V)

The recent success of check-point inhibitors has shown the large potential for curing cancer with immunotherapy. The development of such immunotherapies came from the realisation that all tumour cells are required to evade the immune system and that inhibiting their evasive manoeuvres could potentially lead to the body’s own defence system being capable of clearing the tumour cells. Indeed this realisation has since in large been proven right, but still immune therapy is successful in far from all patients and cancer types, and for some cancers – including NETs – both preclinical and clinical experience is still very limited.

In this paper we looked closer at the immune cells present in the SINET microenvironment, to investigate its composition and functionality. We also set out to isolate, expand, and activate these immune cells to recognise and retaliate against the SINET cells. We first presented a thorough characterisation of SINET patient samples using immunohistochemistry and flow-cytometric immunophenotyping. Interestingly, we could see that the amount of in particular CD4+ and CD8+ T lymphocytes varied dramatically between tumour biopsies. We could also see that these immune cells were mainly (>90%) localised in the tumour stroma and in the interphase between tumour stroma and tumour nests. PD-L1 positivity was found in 2/7 tumours and NKp46+ NK-cells were very rare in all tumour samples (<10 cells/full tumour section). In total, most abundant were CD4+ T lymphocytes, followed by CD8+ T lymphocytes and B-cells.

We also isolated tumour-infiltrating lymphocytes (TILs) and expanded them through the same methodology as used for adoptive T cell transfer in the clinic, involving anti-CD3 and IL-2 stimulation (220). This successfully led to the expansion of SINET TILs, and mainly T lymphocytes.
As clinical responses to ACT can be modelled using transplanted patient-derived xenograft (PDX) tumours and autologous T cells in non-obese diabetic/severe combined immune-deficient/common gamma chain knock-out (NOG) with the continuous presence of IL-2 (221), we attempted to establish such a model. No SINET PDX model had before been reported successfully established. In total, by both subcutaneous and orthologous liver transplantation we grafted 38 SINETs from 36 patients to 55 NOG mice. Only one tumour, from a grade 1 liver metastasis, was successfully propagated and grown through two passages. The poor take-rate was consistent with previous reports on establishing NET PDXs (98). Instead we attempted to grow tumour spheres in vitro from two patient tumours (T3 and T4), transfec
t them with luciferase, and inject them into mice. After three months we observed an increase in bioluminescence signal, and are still observing an ongoing increase, indicating tumour cell proliferation. One speculation to the potentially improved take rate of tumour spheres is that sphere culturing excludes the potentially tumour growth inhibiting immune microenvironment.

We also investigated whether the TILs that we isolated and expanded through stimulation could recognise and degranulate when challenged with orthologues tumour cells. Indeed, although in varying degree, all expanded TILs degranulated, and several even more than M33 – TILs from a malignant melanoma patient that have previously been demonstrated to be reactive
against autologous tumour cells in vivo (221). Based on this, we hypothesised that SINET TILs have the potential to recognise tumour cells and that their immunologic inhibition can be overcome by the presence of exogenous interleukin-2 (IL-2), something that has been demonstrated for other tumour types (222,223).

In conclusion, we here present the so far broadest characterisation of the SINET immune microenvironment and show that SINET TILs are capable activation when challenged with autologous tumour cells after TIL expansion.
CONCLUDING REMARKS

Small intestinal neuroendocrine tumours globally afflict many patients every year. The fact that the tumour disease often present with distant metastasis, and that curative therapeutic options for spread disease do not exist, is deeply troubling. It must therefore of outmost priority to develop such therapies.

However, in order to do so in a preclinical setting, we need to have a clear understanding of our tumour models and their weaknesses, and they absolutely need to be authentic. In this thesis we conclude that this is not always the case. Paper I demonstrates features of currently used cell lines that recapitulates the tumour disease, but also those that don’t, and importantly, reveal several completely non-authentic cell lines. The latter finding was subsequently reinforced by the analysis of published RNAseq data in paper II. If we are, based on preclinical research, supposed to find a cure, this must be a priority. Furthermore, while the use of cell lines is a very important tool in cancer research, we must be aware of their restrictions – especially in terms of adaptions made in cell culture. The use of alternatives, such as primary cells, has been limited to only a very few studies. Here we demonstrated the utility of using such primary cells in both paper I and IV. In addition, the availability of in vivo models that do not utilize cell lines has also been concerning. We were therefore happy to present both the first established SINET PDX in paper V, and, although it is still an early finding, a possible strategy for how to improve future PDX take-rates.

An attractive approach of identifying new therapies is by revealing the underlying drivers of the tumour disease. As everything has its starting-point in alterations in the DNA, identification of these could lead to viable therapies. This was the case for pancreatic NETs (sirolimus for mTOR-activated tumours), and has previously happened for many other tumour types. Unfortunately, driver mutations are still largely unknown for SINETs. Based on reoccurrence in exome-sequencing studies, only one potential driver has been identified. In paper III we instead propose a role for recurrent copy-number alterations in SINET tumourigensis and suggest that hemizygous loss of SMAD4 can lead to tumour-promoting effects.
In this thesis we also took a look at both established and ‘up-and-coming’ therapies. $^{177}$Lu-octreotate was in 2018 approved for the treatment of SINETs, but its curative rates are still low. We could in paper IV conclude that the use of Hsp90 inhibitor ganetespib could provide an efficient strategy to potentiate the $^{177}$Lu-octreotate for SINETs. In paper V we instead demonstrated the potential for immunotherapy in that we managed to expand and reactivate SINET TILs. Overall, we believe that our findings have increased our understanding for the SINET tumour disease and taken further on the road towards finding a cure.
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