Loss of DAXX/ATRX expression and alternative lengthening of telomeres in insulinomas and neuroendocrine tumours of the small intestine

Inaugural-Dissertation
zur Erlangung des Doktorgrades

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vorgelegt von
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### Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJCC/UICC/CAP</td>
<td>American Joint Commission on Cancer/Union of International Cancer Control/College of American Pathologists</td>
</tr>
<tr>
<td>APB</td>
<td>ALT associated PML-NB</td>
</tr>
<tr>
<td>ALT</td>
<td>Alternative lengthening of telomeres</td>
</tr>
<tr>
<td>ASVS</td>
<td>Arterial stimulation venous sampling</td>
</tr>
<tr>
<td>ATRX</td>
<td>Alpha thalassemia/mental retardation syndrome X-linked</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative genomic hybridisation</td>
</tr>
<tr>
<td>CIN</td>
<td>Chromosome instability</td>
</tr>
<tr>
<td>CNA</td>
<td>Copy number alterations</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CUP</td>
<td>Cancer of unknown primary</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>DAXX</td>
<td>Death domain-associated protein 6</td>
</tr>
<tr>
<td>DDR</td>
<td>DNA damage response</td>
</tr>
<tr>
<td>EC</td>
<td>Enterochromaffin cell</td>
</tr>
<tr>
<td>ENETS</td>
<td>European Neuroendocrine Tumor Society</td>
</tr>
<tr>
<td>EUS</td>
<td>Endoscopic ultrasonography</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HE</td>
<td>Haematoxylin &amp; eosin</td>
</tr>
<tr>
<td>HPF</td>
<td>High-power field</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IRS</td>
<td>Immunoreactive Remmele Score</td>
</tr>
<tr>
<td>MANEC</td>
<td>Mixed adenoneuroendocrine carcinoma</td>
</tr>
<tr>
<td>MEN1</td>
<td>Multiple endocrine neoplasia, type 1</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>n.a.</td>
<td>Not assessable</td>
</tr>
<tr>
<td>NE</td>
<td>Neuroendocrine</td>
</tr>
<tr>
<td>NEC</td>
<td>Neuroendocrine carcinoma</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine tumour</td>
</tr>
<tr>
<td>NF1</td>
<td>Neurofibromatosis type 1</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron-specific enolase</td>
</tr>
<tr>
<td>PANEC</td>
<td>Pancreatic neuroendocrine carcinoma</td>
</tr>
<tr>
<td>PANET</td>
<td>Pancreatic neuroendocrine tumour</td>
</tr>
<tr>
<td>PAS</td>
<td>Periodic acid-Schiff staining</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PML-NB</td>
<td>Promyeloc leukaemia nuclear bodies</td>
</tr>
<tr>
<td>PP</td>
<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>SiNET</td>
<td>Neuroendocrine tumour of the small intestine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SNV</td>
<td>Single nucleotide variant</td>
</tr>
<tr>
<td>SRS</td>
<td>Somatostatin receptor scintigraphy</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>SwItch/Sucrose NonFermenTable</td>
</tr>
<tr>
<td>TMA</td>
<td>Tissue microarray</td>
</tr>
<tr>
<td>TMM</td>
<td>Telomere maintenance mechanism</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNM</td>
<td>Tumour (T), lymph node metastasis (N), distant metastasis (M) – classification system of malignant tumours</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasonography</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
1. Introduction

1.1 Neuroendocrine tumour (NET) - neuroendocrine carcinoma (NEC)

Neuroendocrine tumours (NETs) arise from neuroendocrine cells. These cells have neuroendocrine differentiation characterised by positivity for immunohistochemical markers such as synaptophysin and chromogranin and by secreting proteins such as normal endocrine cells [2]. Neuroendocrine cells (NE cells) are located in the gastrointestinal tract (GI tract), the lung, the urogenital tract, the cardiovascular system, the thyroid gland (C cells), the parathyroid gland, the adrenal medullary, the skin (Merkel cells), paraganglia, the hypothalamus, the pituitary gland and the pineal gland. In general, NETs are rare neoplasms with an incidence of 2-4 per 100,000 population [3], which is increasing at a rate of 3-10% per year, depending on the subtype [4]. One reason for this increase could be improved diagnostic methods. The distribution between the sexes is balanced [1]. The mean age of the first diagnosis is 56 years; the range is between 14 and 93 years [1]. NETs represent 0.5% of all malignant tumours [3]. Most NETs are found in the pancreas (34.2%) and the small intestine (25.8%) [1]. Tumours vary considerably in size, growth rate and biological behaviour. NETs can cause clinical symptoms due to the secretion of functional hormones, such as hypoglycaemia in patients with insulinoma [5]. Another example is the carcinoid syndrome with flush, diarrhoea and cardiac symptoms in patients with a serotonin-producing tumour arising from enterochromaffin (EC) cells of the gastrointestinal tract [6]. Functional NETs are called functional because of their clinical presentation, not because of positive immunohistochemical staining. About 60% of NETs are non-functional [1, 7] and are diagnosed when local symptoms of the primary tumour or its metastasis occur, or they are found accidentally. Lymph node metastases are often detected; at time of diagnosis, 50% of patients already have distant metastases, usually located in the liver [8, 9]. The time of diagnosis, the initial TNM classification and the grade of proliferation are relevant for the prognosis [10-12]. The probability of a five-year survival time of a NET with distant metastasis is about 50-75% [13, 14]. Over the
last fifty years, the classification of NETs varied considerably until the WHO classification (Table 1) and the TNM classification of ENETS (see Chapters 2.1 and 3.1) became standard in 2010. Well-differentiated neuroendocrine tumours are classified as neuroendocrine tumours (NETs) and not as carcinomas (NECs), even if they exhibit angioinvasion or metastasis. Neuroendocrine carcinomas (NECs) are diagnosed when they are poorly differentiated and the mitosis rate or the Ki-67 index is higher than 20%. Other features of NEC can be the presence of necrosis; recently, the importance of immunohistochemical staining with CK19 and c-kit has been under investigation. [15].

![Distribution of localisation of primary NETs, modified [1]. CUP = cancer of unknown primary.](image)

The pathogenesis of NETs is not yet fully understood, but there are a few syndromes known that are associated with NETs: Von Hippel-Lindau (VHL), tuberous sclerosis complex (TSC1), neurofibromatosis type 1 (NF1) and multiple endocrine neoplasia type 1 (MEN1). In these cases, the VHL or MEN1 gene is mutated or there are mutations in the neurofibromatosis gene locus [15].
<table>
<thead>
<tr>
<th>WHO classification 2010</th>
<th>Neuroendocrine tumour</th>
<th>Neuroendocrine tumour</th>
<th>Neuroendocrine carcinoma (NEC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade</td>
<td>G1</td>
<td>G2</td>
<td>G3</td>
</tr>
<tr>
<td>Ki-67(%)</td>
<td>&lt;2%</td>
<td>2-20%</td>
<td>&gt;20%</td>
</tr>
<tr>
<td>Histological differentiation</td>
<td>well</td>
<td>well</td>
<td>poor</td>
</tr>
<tr>
<td>Mitosis</td>
<td>&lt;2/10 HPF</td>
<td>2-20/10 HPF</td>
<td>&gt;20/10 HPF</td>
</tr>
</tbody>
</table>

Table 1: WHO classification 2010 of neuroendocrine neoplasms. WHO = World Health Organization, HPF = high-power field [16]

1.2 Insulinomas

1.2.1 Pancreatic neuroendocrine tumours and insulinomas - Definition

Pancreatic neuroendocrine tumours (PANETs) can be classified in several ways: by grade (see Table 1), by size and by functional status. The classification of PANETs by functional status is described below (Tables 2 and 3). In addition, there are two different staging classifications of PANETs: AJCC/UICC/CAP (American Joint Commission on Cancer/Union of International Cancer Control/College of American Pathologists) and ENETS (European Neuroendocrine Tumor Society). The ENETS classification was more significant in correlation with the risk of death [17, 18]. It is state of the art to apply both (Table 4).

Insulinomas are well-differentiated neuroendocrine tumours arising in the β cells of the Langerhans islets of the pancreas that produce insulin. About 10% of all insulinomas exhibit malignant behaviour [19-23]. The median survival of patients with metastases in the liver, bone or lymph node is < 2 years [24].
### Classification of pancreatic neuroendocrine tumours (PANETs)

<table>
<thead>
<tr>
<th>Endocrine micro adenoma</th>
<th>Well-differentiated PANETs</th>
<th>Poorly differentiated PANETs</th>
<th>Mixed endocrine carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Functional</td>
<td>Non-functional PANET</td>
<td>Mixed ductal-endocrine carcinoma</td>
</tr>
<tr>
<td></td>
<td>Insulinoma</td>
<td></td>
<td>Mixed acinar-endocrine carcinoma</td>
</tr>
<tr>
<td></td>
<td>Glucagonoma</td>
<td></td>
<td>Mixed acinar endocrine ductal carcinoma</td>
</tr>
<tr>
<td></td>
<td>Somatostatinoma</td>
<td>Small cell carcinoma</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Large cell carcinoma</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gastrinoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>VIPoma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PP cell PANET</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

#### Table 2: Classification of pancreatic neuroendocrine tumours (PANETs). [25]

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Cell type</th>
<th>Syndrome</th>
<th>Clinical findings</th>
<th>Percentage of functional PANETs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagonoma</td>
<td>α cell</td>
<td>Glucagonoma syndrome</td>
<td>Rash, stomatitis, diabetes, weight loss</td>
<td>8-13% [26]</td>
</tr>
<tr>
<td>Somatostatinoma</td>
<td>δ cell</td>
<td>Somatostatinoma syndrome</td>
<td>Diabetes hypochlorhydria, cholelithiasis</td>
<td>2% [27]</td>
</tr>
<tr>
<td>Insulinoma</td>
<td>β cell</td>
<td>Insulinoma syndrome</td>
<td>Hypoglycaemia</td>
<td>42% [5]</td>
</tr>
<tr>
<td>Gastrinoma</td>
<td>G cell</td>
<td>Zollinger-Ellison syndrome</td>
<td>Peptic ulcers, diarrhoea</td>
<td>Second common functional PANET</td>
</tr>
<tr>
<td>VIPoma</td>
<td>Unknown</td>
<td>Verner-Morrison syndrome</td>
<td>Watery diarrhoea, hypokalaemia, achlorhydria</td>
<td>10% [28]</td>
</tr>
<tr>
<td>PP cell PANET</td>
<td>PP cell</td>
<td>None</td>
<td>None (ev. elevated levels of PP)</td>
<td></td>
</tr>
</tbody>
</table>

#### Table 3: Types of functional PANETs. Insulinomas represent the most common functional PANETs [5].
<table>
<thead>
<tr>
<th>T</th>
<th>AJCC/UICC/CAP TNM</th>
<th>ENETS TNM</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Confined to pancreas, &lt;2 cm</td>
<td>Confined to pancreas, &lt;2 cm</td>
</tr>
<tr>
<td>T2</td>
<td>Confined to pancreas, &gt;2 cm</td>
<td>Confined to pancreas, 2-4 cm</td>
</tr>
<tr>
<td>T3</td>
<td>Peripancreatic spread, without infiltration of the coeliac trunk or the A. mesenteria superior</td>
<td>Confined to pancreas, &gt; 4 cm or invasion of the duodenum or bile duct</td>
</tr>
<tr>
<td>T4</td>
<td>Peripancreatic spread, with infiltration of the coeliac trunk or the A. mesenteria superior</td>
<td>Invasion of adjacent organs or major vessels</td>
</tr>
</tbody>
</table>

Table 4: Staging classifications of pancreatic neuroendocrine tumours (PANETs)

Historically speaking, the first adenoma of pancreatic islets was reported by Nicholls in 1902; the first insulinoma was described in 1927 in Mayo Clinic. The first enucleating was performed in 1931 in St. Louis, Missouri [29]. In 1935, Whipple and Franz described the clinical symptoms of this tumour. [30]

1.2.2 Epidemiology

Insulinomas are most common among functional PANETs, accounting for 42% [5]. The incidence of insulinomas is four per million cases a year, and is increasing [30, 31]. One reason for this could be that better diagnostic methods exist, which are able to find even the smallest tumour. The distribution between sexes is balanced at a ratio of 1:1.4 (M: F). Other studies yield a slightly higher occurrence in men [23]. Most insulinomas occur in patients at the age of 40-60 years (mean 46 years), but an occurrence between 20-40 is also common [23, 32].

1.2.3 Aetiology

A recent review of 6,222 cases reported that 94% of all insulinomas are sporadic [23]. Familial insulinomas are mainly due to autosomal dominant mutations in the MEN1 (multiple endocrine neoplasia type 1) gene. This gene operates as a tumour suppressor gene; mutations lead to the inactivation of its function, and tumours in the pancreas,
parathyroid gland (more than 90%), duodenum (50-85%) and pituitary gland can occur. It is rare to find additional tumours in other regions such as lung, thymus, adrenal gland and thyroid in these patients [33-36]. However, insulinomas are often multiple in patients with MEN1 syndrome. Another disease involving multiple insulinomas is insulinomatosis, with the occurrence of synchronous and metachronous insulinomas [37]. Neurofibromatosis type 1 (NF1), the von Hippel-Lindau disease (VHL) and tuberous sclerosis complex (TSC) known as familial diseases with NETs are uncommon for insulinomas [38-43].

Little has been reported about the risk factors for developing insulinomas. An Italian study of 17 patients with insulinomas reported that a family history of cancer and alcohol abuse are risk factors [44]. A Chinese study [45] included 196 patients with insulinomas and compared them with a control group (benign or malignant tumours, autoimmune or genetic diseases were excluded) of 233 patients who underwent surgery. 12% of the study group had MEN-1-related insulinomas. This study also showed that in sporadic insulinomas a family history of cancers, especially of PANETs, is a risk factor. They found no correlation between alcohol consumption and the occurrence of insulinomas, consistent with the results of a number of studies from the USA [46, 47]. A high body mass index (BMI) and cigarette smoking are not associated with insulinomas. Recent studies explore molecular pathological changes, mentioned in Chapter 1.2.8.

1.2.4 Clinical presentation

Patients with insulinomas suffer early from hypoglycaemia and the resulting catecholamine response, rather than from local complications, because the tumour is usually very small [5]. There is no correlation between the size of the tumour and the severity of symptoms [5, 48]. Hypoglycaemia causes symptoms such as diplopia, blurred vision, confusion and fatigue. It can also lead to unconsciousness and seizures. Hypoglycaemia can also mimic cardiovascular diseases or movement disorders such as hemiplegia or hemiballismus [49, 50]. The effects of catecholamine response are hunger, weakness, perspiration, palpitations, sweating, anxiety and nausea.
In conclusion, insulinoma causes the so-called “Whipple trias”, including hypoglycaemia symptoms, low blood glucose levels (< 3.0 mmol/l or < 55 mg/dl) and improvement by administering glucose.

In rare cases, the tumour can grow to 10 cm in diameter [22] and cause local problems such as abdominal pain, weight loss and, if the tumour constricts the choledochus duct, jaundice.

1.2.5 Diagnosis

As mentioned above, post-prandial low blood glucose levels are a reason to suspect an insulinoma. Plasma insulin and prosinsulin are elevated, particularly after a period of fasting [5]. The gold standard is to measure the plasma glucose, insulin, C-peptide and proinsulin over a 72-hour fasting period. 99% of all insulinomas can be detected using this diagnostic method [51]. In order to localise the tumour, non-invasive diagnostic methods are used, including transabdominal ultrasonography (US), computed tomography (CT) and magnetic resonance imaging (MRI). The sensitivity of transabdominal US is poor, at 9%-64% [30]. The sensitivity of CT and MRI is 33%-64% and 40%-90% [52, 53]. When the tumour grows extra-pancreatic, the sensitivity and specificity of MRI is superior to that of CT [54].

Figure 2: CT imaging of an insulinoma located in the pancreas [55]. Since insulinomas are hyper vascular tumours, a greater enhancement in the arterial and capillary phase in contrast to the normal pancreas parenchyma is visualised [54]
CT is currently accepted as first line investigation. It visualises the exact localisation of the tumour and its relationship to neighbouring structures. It also shows the presence of metastasis [53]. Insulinomas are known as hyper vascular tumours, so in CT the tumour shows a greater enhancement in the arterial and capillary phase than the surrounding tissue [54]. In MRI, insulinomas are detected as low signal intensity on T1-weighted images and high signal intensity on T2-weighted images [56]. A highly sensitive (94%-100%) [57, 58] invasive diagnostic method is angiography combined with arterial stimulation venous sampling (ASVS) to exactly localise the tumour prior to surgery. Invasive diagnostic methods such as endoscopic ultrasonography (EUS) and ASVS are useful for precisely localising the tumour prior to surgery. In EUS, insulinomas are visualised as hypo-echoic rounded mass with distinct margins. Some insulinomas may be missed in this method because they are isoechoic or because of other artificial reasons that occur in a special collective of patients [59]. Detection rates of 86.6%-
92.3% are reported [52, 60]. EUS-guided fine-needle aspiration (FNA) is becoming increasingly popular in the preoperative diagnosis of insulinomas. Another diagnostic method, specific for NETs, is the $^{68}$Ga-DOTATATE PET, which uses a radioactively labelled somatostatin analoga. The $^{68}$Ga-DOTATATE PET was reported to detect NETs as small as 6 mm [61]. According to recent ENETS guidelines, it is recommended for localising the primary of metastatic NETs [62]. In some cases, surgical exploration, intraoperative ultrasound and manual palpation of the pancreas by an experienced surgeon are required [63-65].

1.2.6 Gross findings

Insulinomas are small red to brown, soft, well-circumscribed homogenous tumours without a capsule. They can occur in any part of the pancreas. They are found extra-pancreatic extremely rarely, usually in the duodenal wall [66]. They normally measure between 0.5-1.0 cm; 25% are larger than 2 cm. [48]. Cases involving a size of 10 cm or more have also been described [22]. Bigger tumours are reported to be more likely to be malignant. The majority (about 90%) are solitary [23]; multiple insulinomas are documented in MEN1 syndrome and insulinomatosis. They are multiple extremely rarely, in sporadic cases.

Figure 4: Neuroendocrine tumour of the pancreas shown as a yellowish well-circumscribed nodule with a diameter of 4 mm.
1.2.7 Histological findings

Insulinomas grow in a solid/nested, trabecular, micro glandular or acinar pattern. They do not have a capsule, although larger ones may have a pseudo capsule. Stromal hyalinisation or amyloid deposits may be present, and calcifications or psammoma bodies can also be seen. The monomorphous cells have a polygonal shape. A typical nuclear feature is the “salt and pepper”-like chromatin pattern. The criterion for malignant behaviour is the detection of lymph node or distant metastases. Morphologically, there is no difference between benign and malignant insulinomas.

Immunohistochemistry of PANETs: In general, more than 95% of all PANETs are at least positive for one endocrine marker, such as chromogranin A and synaptophysin, or CD56 [67]. Chromogranin A is part of the membrane of large dense-core secretory vesicles; its function is to complexate peptide hormones. Synaptophysin is known as part of small synaptic vesicles, which are found in all NE cells. CD 56 is a synonym for the neural cell adhesion molecule (NCAM), and is expressed on the surface of NE cells and other cells such as neurons, glia, skeletal muscle and natural killer cells. CD 56 plays inter alia an important role in cell adhesion.

MIB1/Ki-67 staining is a fundamental method of demonstrating the rate of proliferation, which is important when grading the tumour (see Table 1, Chapter 1.1). Some peptides can be positive in PANETs, such as glucagon, gastrin, insulin, pancreatic polypeptide and somatostatin. There is often a correlation between the functional presentation of the tumour and immunohistochemistry results.

Immunohistochemistry of insulinomas: Insulinomas should stain positive for insulin and proinsulin. The reason why insulinomas may not stain positive for insulin could be that they secrete the peptide but there is no accumulation, so it cannot be detected by immunohistochemistry staining. Another reason could be that the peptide is changed and is negative for staining, but still has its functional activity. About 50% of insulinomas are positive for other peptides such as glucagon, gastrin, pancreatic polypeptide and somatostatin [32].
Figure 5: HE, 200x, insulinoma; monomorphic cells with eosinophilic cytoplasm, disposed in a trabecular and pseudo glandular pattern.

Figure 6: HE, 630x, insulinoma; typical salt and pepper chromatin pattern.
Figure 7: Chromogranin staining, 200x. Chromogranin staining shows a focally strong positivity.

Figure 8: Ki-67, 200x. Ki-67/MIB1 staining shows a positivity of 3%.
Figure 9: Synaptophysin, 200x. The tumour cells are strongly positive for synaptophysin.

Figure 10: Insulin, 200x. Insulin staining is positive.
1.2.8 Molecular pathology

**MEN1/VHL:** Familial PANETs such as MEN1 syndrome and VHL syndrome are associated with mutations in the **MEN1** and in the **VHL** gene [68, 69], as mentioned above (see Chapter 1.2.3). In about 40% of sporadic insulinomas [70], mutations in the **MEN1** gene or loss of heterozygosity in the **MEN1** gene are reported, which are associated with a better prognosis [71-74]. Pancreatic neuroendocrine carcinomas (PANECs) are commonly not associated with alterations in the **MEN1** gene. Deletions in the **VHL** gene are also documented in sporadic cases [42].

**DAXX/ATRX:** Recent studies (see Table 7, Chapter 1.7) explore mutations in the **DAXX** and **ATRX** gene. A loss of expression in immunohistochemical staining is reported in 45% of cases of **DAXX** and **ATRX** mutations [75].

**mTOR-Pathway:** Mutations in **PI3K**, a member of this pathway, are reported in 1.4% of cases [70, 76, 77]. Mutations in the **PTEN** gene are more common in 10-29% of cases [70, 76, 77]. The **TSC2** (tuberous sclerosis gene, encoding for the protein tuberin) gene, another member of this pathway, was mutated in 8.8% of PANETs [70, 76, 77]. No mutations of **mTOR** in PANETs are found [77, 78]. In conclusion, 16% of all cases exhibit mutations in this pathway [76, 77], which is already a target for therapy in advanced neuroendocrine tumours (see Chapter 1.2.9) [79].

Numerous chromosomal aberrations were detected in a study cohort of 37 primary PANETs and 11 metastasis with copy number gains in Chromosome 06p22.2-p22.1 (27.1%), 17p13.1 (20.8%) 7p21.3-p21.2 (18.8%), and 9q34.11 (18.8%). Genomic loss was observed at 8q24.3 (6.3%). Copy number alterations (CNAs) were heterogeneous between the metastasis and primary tumour, and slightly increased in metastasis [80]. Mutations detected in the ductal pancreatic adenocarcinoma such as **KRAS**, **TGF-β**, **CDKN2A** or **TP53** are not usually seen in PANETs, and vice versa [81-84]

For insulinomas in particular, little is reported about their molecular pathology.
1.2.9 Therapy and prognosis

In most cases, a resection of the tumour cures the patient. Enucleation of the tumour is usually carried out (56%). About 32% of patients undergo a distal pancreatectomy; in 3% a Whipple procedure is performed. Fistula represent a worrisome surgical complication. Recurrence of the tumour is reported in 7% of cases [23]. About 10% of all insulinomas exhibit malignant behaviour with lymph node metastases, local (peritoneum) and distant metastases. Most of these tumours measure more than 2 cm [85]. In those cases, systemic therapy is indicated. The treatment is identical to that for other PANETs, besides treatment for hormone-related symptoms. In addition to dietary recommendations, octreotide and diazoxide are administered to control hypoglycaemia symptoms. Octreotide is administered after a positive octreotide scan for expression of somatostatin receptor [86, 87]. Diazoxide is a potassium channel opener that inhibits the secretion of insulin [88]. The side effects of diazoxide are oedema and hirsutism; recurrence of the symptoms is also common [89]. Local palliative treatment for patients with liver metastases who are not candidates for surgery includes selective bland embolisation, chemoembolisation and radio embolisation [90, 91]. For metastatic insulinomas, systemic cytotoxic therapy and systemic targeted therapy are available. Systemic cytotoxic treatments are streptozocin, 5-FU, platinum and temozolomide. Tumour respond rates for streptozocin plus doxorubicin or 5-FU range from 16-69% [92-94]. Targeted therapies are based on the following pathways: PI3K/Akt/mTOR pathway and angiogenesis in insulinomas. Although mutations of MEN1 are most common, there is no targeted therapy as yet. There is also no special therapy for DAXX/ATRX-mutated PANETs. As mentioned above, these mutations are correlated with the ALT pathway. The development of efficient ALT inhibitors could establish new targeted therapies such as targeted therapy for telomerase inhibitors, which already exist.

Targeted therapies

**Pi3k/Akt/mTOR pathway:** The function of mTOR (mammalian target of rapamycin) is the regulation of cell metabolism, proliferation, survival and coordination of protein translation [95]. 410 patients with advanced PANETs were included in a placebo-
controlled phase III study (RADIANT-3). Compared to the placebo group, patients who were treated with the mTOR inhibitor everolimus showed a significantly prolonged survival time (11 vs. 4.6 months) [79]. Combining everolimus with other drugs, vertical inhibition or targeting another pathway, are under investigation.

**Angiogenesis:** Like other PANETs, insulinomas are well-vascularised neoplasms. There are contradictory results about the correlation between expression of PDGF (platelet-derived growth factor), FGF (fibroblast growth factor) or VEGF (vascular endothelial growth factor) and the biological behaviour of insulinomas. However, it is a fact that these proteins are expressed in insulinomas and/or the surrounding endothelia [77]. Consequently, the inhibition of neoangiogenesis is a treatment option in insulinomas. The application of sunitinib, a tyrosin kinase inhibitor which is approved in treatment of insulinomas, resulted in a longer survival time of 10.2 vs. 5.4 months in a placebo-controlled phase II study [96]. Sorafenib is another agent that inhibits VEGFR2, PDGFR, FGFR1 and RAF [97, 98]. Another drug, which demonstrates a partial response of 18.9%, is an inhibitor of VEGFR1, PDGFR and c-kit, called pazopanib. The progression-free survival was 9.5 months [99]. An antibody against VEGF is bevacizumab, which is approved in the treatment of several tumours such as colorectal cancer, breast cancer, lung cancer and tumours of the urogenital tract. This drug is combined with others such as temozolomide or octreotide in phase II studies with a partial response (86% and 18%, respectively). The progression-free survival was 14.3 months and 15 months [100, 101]. However, bevacizumab is not approved in the treatment of insulinomas.

**Prognosis**

Patients with benign insulinomas (90%) have an excellent prognosis and can be cured by enucleation of the tumour. The median survival of patients with metastatic insulinomas is three years for G1 graded tumours, and 24 months for G2 graded tumours [102]. Among immunohistochemical markers, CK19 [103] was significantly predictive, while others under investigation like such as CD99, COX2 and p27 [104] failed to be labelled as prognostic markers. Proteins ALDH1A1, VDAC1 and TPD52 recently proved to be biological markers [105].
1.3 Neuroendocrine tumours of the small intestine

1.3.1 Definition

Neuroendocrine tumours of the small intestine (siNETs) are well-differentiated tumours of neuroendocrine origin. They are the second most common neuroendocrine neoplasms (26%) [1]. They are graded as G1, with a proliferation index of <2%; G2 has a proliferation index between 2% and 20%. According to WHO 2010, neuroendocrine neoplasms with a poor differentiation and a proliferation rate higher than 20% are called neuroendocrine carcinomas. These can be differentiated into small and large cell NEC (see Chapter 1.1., Table 1). NECs are virtually never seen in the small intestine. Other subtypes of neuroendocrine neoplasms of the small intestine are: mixed adenoneuroendocrine carcinoma (MANEC), enterochromaffin cell (EC) serotonin-producing NET, L cell glucagon-like peptide-producing, PP/PYY-producing NET, and the following, which usually appear in the duodenum: somatostatin-producing NET, gangliocytic paraganglioma and gastrinoma.

<table>
<thead>
<tr>
<th>Classification of neuroendocrine neoplasms of the small intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neuroendocrine tumour (NET)</strong></td>
</tr>
<tr>
<td><strong>Neuroendocrine carcinoma (NEC)</strong></td>
</tr>
<tr>
<td><strong>Mixed adenoneuroendocrine carcinoma (MANEC)</strong></td>
</tr>
<tr>
<td><strong>EC cell serotonin-producing NET</strong></td>
</tr>
<tr>
<td><strong>L cell, glucagon-like peptide-producing and PP/PYY-producing NETs</strong></td>
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<tr>
<td><strong>Somatostatin-producing NET</strong></td>
</tr>
<tr>
<td><strong>Gangliocytic paraganglioma</strong></td>
</tr>
<tr>
<td><strong>Gastrinoma</strong></td>
</tr>
</tbody>
</table>

Table 5: WHO classification of neuroendocrine neoplasms of the small intestine (2010).
AJCC/UICC/CAP TNM

T1  Infiltration of the lamina propria, <1 cm
T2  Infiltration of the muscularis propria, >1 cm
T3  Infiltration of the subserosa*
T4  Infiltration of the serosa or other structures

Table 6: Staging classifications of neuroendocrine tumours of the small intestine. *
Tumours of the duodenum: infiltration of the retroperitoneal space or the pancreas.

1.3.2 Epidemiology

The incidence of NETs in the jejunum and ileum is 0.3-1.1 per 100,000 population [106, 107]. At first diagnosis, patients are between 50 and 70 years old. The distribution between sexes is balanced [7, 107].

1.3.3 Aetiology

NETs of the small intestine are sporadic neoplasms, although a few familial occurrences are reported. The genetics in these cases remain unclear [107]. Common hereditary diseases such as MEN1, VHL and NF1 are not documented in siNETs. There are no known risk factors, but an increased risk with a family history of NETs is suggested [14]. An occurrence with other tumours, especially colorectal carcinomas, is documented [108, 109]. Genetic alterations in siNETs are discussed in Chapter 1.3.8.

1.3.4 Clinical presentation

SiNETs are diagnosed accidently, or because of clinical symptoms of hormone-producing tumours, or because the primary or the distant metastasis causes local
symptoms. Local clinical presentations such as abdominal pain, subileus and ischemia may occur. In these cases, local metastases are already present, which induce desmoplasia of the stroma and lead to motility disorders of the intestine [110]. Many siNETs are functional neoplasms that produce hormones such as serotonin, bradykinin, tachykinin or substance P[38]. The so-called carcinoid syndrome, first described by Thorson and Hedinger, arises when there are already metastases in the liver, because otherwise the substances mentioned above would be degraded in the liver. The symptoms are flush (90%), diarrhoea (80%), constriction of the bronchus (10%) and abdominal pain [111]. 15-20% of patients with liver metastasis suffer from the carcinoid syndrome [107]. Carcinoid heart disease occurs in patients with elevated circulating blood serotonin levels, and leads to fibrotic changes in heart valves and further to cardiac dysfunction [112-114].

1.3.5 Diagnosis

The hormones mentioned above and inactive proteins produced by NETs are considered as tumour markers. Although chromogranin A is a sensitive marker, it is less specific and therefore not recommended as a screening test [115]. However, because it is not metabolised in the liver the level of Chromogranin correlates with the tumour level, and is therefore a good marker of surveillance [107, 116]. 5-HIAA, a metabolic substance derived from serotonin, is measured in a 24-hour urinary excretion in patients with suspected carcinoid syndrome [117]. Measuring serotonin in blood samples was not specific, but a recent study yielded a 89% sensitivity and 97% specificity for plasma serotonin in those patients [118]. Other substances such as neuron-specific enolase (NSE), substance P, neurokinin A and pancreastatin failed as tumour markers [112, 119, 120]. CT, MRI scans and somatostatin receptor scintigraphy (SRS, OctreoScan) are the diagnostic methods used to localise the tumour. In the CT scan, the tumour exhibits an enhancement during the arterial phases, because it is a well-vascularised tumour [121]. Metastases often produce desmoplastic fibrosis, which can be detected by CT scans or, since it is reported to be more sensitive, by MRI [122]. SRS is not only important as a diagnostic method, but is also relevant for peptide receptor radiotherapy to predict response to treatment [123, 124]. $^{68}$Ga-DOTATATE PET yielded a 81% sensitivity and
a 90% specificity for localising the primary of metastatic NETs and is recommended by ENETS [61]

1.3.6 Gross findings

In 70% of cases, NETs of the small intestine are located in the ileum, usually in the distal part. In 2% of cases, the primary is located in a Meckel diverticula [42, 125]. The diameter is <1 cm in 13% of cases, and <2 cm in 47% [125]. 2% of neoplasms, which are smaller than 1 cm, exhibit metastatic lymph nodes. When the diameter exceeds 2 cm, the likelihood of finding metastatic lymph nodes is 100% [126]. Metastases can also be located in the liver, peritoneum and bone marrow. 20-40% of siNETs are multiple [127]. Grossly, they are well-circumscribed yellowish nodules without a capsule.

Figure 11: Submucosal NET, shown as a well-circumscribed yellowish nodule, which is not infiltrating the tunica muscularis. M = mucosa, musc = Tunica muscularis.
1.3.7 Histological findings

Histologically, the tumour exhibits an insular, solid, and glandular-cribriform pattern. If present, the muscle invasive part has a retiform pattern. The cells are round and monomorphic. The chromatin shows the typically salt and pepper aspect; mitotic activity is rarely seen. The cells are negative for PAS (periodic acid-Schiff staining). Necrosis and apoptosis are not common. Angioinvasion, lymphangiosis and invasion of nerves can be detected. Bordering mesenteric vessels can be obliterated due to obliterating intima fibrosis, which leads to ischemia [107]. Immunohistochemically, the tumour stains positive for synaptophysin and chromogranin A. CD56 is also a marker for NETs, but is less specific [107]. MIB1/ Ki-67 is an obligatory proliferation marker in pathological diagnosis. The Ki-67 index provides information about grading and prognosis [107]. Immunohistochemical positivity of the primary for somatostatin receptors is important for surveillance control and finding distant metastases in somatostatin receptor scintigraphy (SRS), especially when no presurgical SRS was performed. In fact, there is an over 90% correlation between immunohistochemistry and SRS-based results [123, 124].

Figure 12: HE, 25x. Well-circumscribed lesion with a nested and cribriform pattern.
Figure 13: HE, 100x. The tumour exhibits a nested as well as cribriform pattern. The mucosa is not affected.

Figure 14: The tumour is strongly positive for synaptophysin and chromogranin A. A: 200x synaptophysin, B: 200x chromogranin A
Figure 15: Ki-67, 200x. Since the proliferation index is lower than 2%, it is graded as G1 NET.

1.3.8 Molecular pathology

SiNETs are described as genetically stable cancers by a study of 48 siNETs, revealing an average of 0.1 SNVs per 10^6 nucleotides. Mutations relevant for targeted therapies were found in the following genes: SRC, SMAD family genes, AURKA, EGFR, HSP90 and PDGFR [128]. Further somatic mutations in the cyclin-dependent kinase inhibitor (CDKN1B) gene were found. This gene encodes for the protein p27, which operates as a tumour suppressor [129]. In 69% of ileac neuroendocrine tumours, allelic loss of Chromosome 18 was found [130, 131]. Research conducted by our study group involves genes located on Chromosome 18 such as DCC, Smad2, Smad4/DPC and Maspin/SerpinB5. Immunohistochemically, there was no loss of expression of the protein products of these genes. Smad4 was expressed in 97% of the tumours (J. Brix, Master thesis). In addition, an exome sequencing of five siNETs was performed, and more than 590 single nucleotide variations (SNV) were found. Mutations in genes located on Chromosome 18 (CABYR, NFATC1, PIEZO2, PIK3C3, LAMA3) and other genes such as ERBB2, ERCC4 and MSH6 were re-sequenced by Sanger sequencing.
However, it transpired that none of these targets play a major role in the pathogenesis of siNETs (L. Stoß, Master thesis).

1.3.9 Therapy and prognosis

Depending on the localisation of the primary, a right hemicolectomy, a small bowel resection or, in some cases when the tumour is close to the ampulla of Vater, Whipple surgery is performed [132]. At an early stage, surgical resection is a curative therapy; in advanced disease, surgical resection of primary and/or distant metastases can be performed to reduce clinical symptoms and complications such as bleeding and ileus [133]. Somatostatin analogues such as octreotide and lanreotide are used to reduce carcinoid symptoms; in 24-57%, a stabilisation of tumour growth is reported [134]. As mentioned above, somatostatin receptors on tumour cells are useful for peptide receptor radiotherapy, which is why SRS is performed. Depending on the findings, radioactive marked somatostatin is administered. A 23% response among patients with carcinoid syndrome was revealed [135]. Trials with interferon α were performed, since interferons have an effect on the stimulation of T-cells, the inhibition of angiogenesis and the induction of cell cycle arrest. However, several studies give no definitive conclusion about the overall survival, and no optimal doses of treatment were established [136]. In contrast to PANETs, everolimus, an mTOR inhibitor extensively studied in NETs, is not approved by the FDA for the treatment of siNETs. Everolimus resulted in a progression-free survival of 16.4 vs. 11.3 months compared to the placebo group; this result fell short of statistical significance [137]. Several studies have been performed with the inhibition of VEGF. Results suggest that the antibody VEGF bevacizumab in combination with other agents (mTOR inhibitors and interferons) is effective in advanced disease [138]. However bevacizumab is not approved in treatment of neuroendocrine tumours. For NECs, chemotherapeutical drugs such as etoposide and cisplatin are administered, which show a good response (70%) in the first 8-10 months [139].
1.4 DAXX (death domain-associated protein 6)

*DAXX*, death domain-associated protein 6, is also known as *BING2* and *DAP6*. The *DAXX* gene is located on 6p21.3 and consists of eight exons. DAXX protein is found in the nucleus as well as in the cytoplasm. It plays an important role in apoptosis. Three isoforms generated by alternative splicing are known.

**DAXX plays an important role in the Fas/JNK pathway:** When Fas-receptor (=APO1, CD95), a TNF (tumour necrosis factor) receptor, is stimulated, DAXX is activated. This induces the c-JUN-N-terminal kinase pathway by activating the JNK kinase kinase ASK1 (apoptosis signal-regulation kinase1). JNK then activates HIPK2 (home domain interacting protein kinase2), which transfers DAXX from the nucleus into the cytoplasm and reactivates ASK1 [140]. Bcl2 is able to block DAXX and prohibit apoptosis.

Another mechanism that activates the JNK pathway is when TGF-β (transforming growth factor) is stimulated, which activates HIPK2 and phosphorylates the DAXX protein.

Stress stimuli such as glucose reduction, UV radiation and oxidative stress are also capable of inducing the JNK pathway by activating DAXX [141]. In immortal cells such as HeLa cells, both pathways the DAXX and the FADD-induced cell death pathways have to be blocked to prevent Fas-induced apoptosis [142]. Overexpression of DAXX induces the activation of JNK and multiplies the Fas pathway to apoptosis [142]. In addition, DAXX is able to suppress transcription factors such as p53, p73 and NF-κB.

**DAXX in the nucleus:** DAXX also acts as a component of promyelocytic leukaemia nuclear body (PML-NB) in the interphase. Histone H3.3 is targeted to PML-NBs in a DAXX-dependent fashion, instead of being localised at senescence-associated heterochromatin foci (SAHF). An overexpression of DAXX leads to an enhancement of the targeting of H3.3 [143].
Figure 16: The role played by DAXX in apoptosis by interacting with JNK, after binding with FAS. Bcl2 is able to block DAXX.

### 1.5 ATRX (alpha thalassemia/mental retardation syndrome X-linked)

ATRX is a member of the SWI/SNF (SwItch/Sucrose NonFermentable) helicase family; its main function is to remodel the chromatin structure [144]. The gene is located on Chromosome Xq13.3. The ATRX protein consists of 2,492 amino acids; the following regions can be distinguished: DNA-binding domain, a large exon, a polyglutamic acid stretch, the seven conserved helicase motifs found in DNA-stimulated ATPase and DNA helicase and a glutamine-rich domain [145, 146]. A mutation in the ATRX gene on the locus Xq21.1 in men can cause the alpha thalassemia mental retardation X-linked syndrome. These patients have particular facial features with hypertelorism, a snub nose and an everted lower lip. They are mentally retarded, the genitals are often malformed, and the testes fail to descend. The ATRX protein is associated with EZH2, which plays a major role in neuronal development [144]. Further, VAV1-EZH2-ATRX is known as a complex, which plays an important role in haematopoiesis. [144].

Together, **DAXX and ATRX** deposit the histone H3.3 at telomeres. The consequence remains unclear [147-151]. There is evidence that decreased ATRX and H3.3 leads to a destabilisation of the telomeres and an up-regulation of telomere repeat sequences [149].
1.6 Telomeres and alternative lengthening

Telomeres are localised at the end of each chromosome and consist of repetitive sequences (TTAGGG in humans), which are repeated several thousand times. The telomere can fold itself back and build a loop [152]. Another form is a t-circle, which comes out of telomere loop junctions [153]. A shelterin complex binds to the telomere repeat sequences and regulates its function. It comprises the following proteins: POT1, TPP1, TIN2, TRF1, TRF2 and RAP1. The complex prevents the telomeric DNA from being detected as a DNA double-strand break (DSB) [154].

![Fluorescence in situ hybridisation showing the chromosomes in the metaphase with red fluorescent telomeres](image)

Figure 17: Fluorescence in situ hybridisation showing the chromosomes in the metaphase with red fluorescent telomeres [155].

Telomeres are important for the stabilisation of DNA. Every time the cell enters the S-Phase (synthesis) and DNA replication starts, telomeres get shorter because, at the end of the chromosome, there is no 3’ where the primase can bind. This shows how important telomeres are—they protect the sequences lying before them, otherwise important information would get lost. This is called the end-of-replication problem [156]. Due to this end-of-replication problem, telomeres lose some kb (kilo base) with every replication. When telomeres are shorter than 4 kb, a point is reached and cell division is no longer possible. Then DNA damage response factors (DDR) induce either programmed self-destruction (apoptosis) or senescence [157]. This turning point is
called the Hayflick limit. In senescence, cells are still active but unable to replicate. Senescence is also induced by oncogenes such as H-RasV12 when DNA damage is recognised [158, 159]. At the Hayflick limit, if there is also a loss of tumour suppressor genes (such as p53) or if the cell is exposed to radiation or carcinogenic substances or viral oncogenes are activated, then the cell proliferates and the already short telomeres become too short and the cell enters the M2 phase (crisis) [160]. This means that the cell can either go into apoptosis or it activates telomerase, an enzyme that can lengthen the ends of chromosomes with repeat sequences independent of primase. Cells with activated telomerase are now immortal cells, and have the unlimited potential to replicate. Normal cells with already activated telomerase without any damage to the DNA are germ cells and stem cells. Telomerase is a reverse transcriptase enzyme, a conglomerate of protein and RNA, which is able to rebuild sequences at the end of telomeres [161]. The RNA component is complementary to the repeat sequence of the telomere [162].

Figure 18: How normal cells become tumour cells by activating either telomerase or the ALT pathway.
ALT (alternative lengthening of telomeres)

85% of all tumours activate telomerase [163]. Another possibility for a telomere maintenance mechanism (TMM) is the ALT pathway (alternative lengthening of telomeres). Tumours with a mesenchymal and neuroepithelial origin tend to the ALT pathway [164, 165]. It is commonly found in osteosarcoma, soft tissue sarcomas, glioblastoma, medulloblastoma, oligodendroglioma, astrocytoma, ganglioneuroblastoma, bladder small cell carcinoma and non-seminomatous germ cell tumours [165-167]. In ALT-positive tumour cells, extra chromosomal telomeric repeats exist in the nucleus. Another feature is the recombination-mediated lengthening of strands and transfer of telomere repeat sequences between sister chromosomes [168]. The aforementioned t-circles are increased in ALT-positive cells [169]. Associated with ALT, nuclear structures called promyelocytic leukaemia nuclear bodies (PML-NB) are found [170]. In fluorescence in situ hybridisation (FISH) staining, the telomeres of these tumours exhibit a large ultrabright signal and irregular length of telomeres (3 kb to more than 50 kb). In contrast, tumour cells with activated telomerase exhibit a homogeneous bright signal [171] and a similar length of 10 kb.

Figure 19: Normal telomere repeat sequence (A), shelterin complex (B). End of replication problem (C). Two types of telomere lengthening, the activation of telomerase and the recombination mediated ALT pathway (D) [27].
Figure 20: FISH showing chromosomes in the metaphase with ALT. The red fluorescent staining is heterogeneous with ultrabright spots [155].

**PML-NB (promyelocytic leukaemia nuclear body)**

PML-NB is a complex of PML and SP100 protein; it is a mobile structure found in the nucleus. The number of PML-NBs varies from five to 30, depending on the type of cell, the cycle phase, differentiation stage and stimuli [172]. It can be modified by small ubiquitin-related modifiers (SUMO) [173]. Proteins such as DAXX, TDG (thymine-DNA glycosylase), BLM (bloom syndrome protein), CBP (CREB binding protein a co-activator of transcription) and IKKe (inhibitor of nuclear factor kappa-B kinase ε) bind on PML-NBs controlled by SUMO, which has functional consequences [174]. PML functions as a tumour suppressor and is inactivated in acute promyelocytic leukaemia (APL). This is due to fusion of the PML gene with the retinoic acid receptor alpha gene (RARα), and the fusion transcript PML-RARα delocalises PML from the PML-NBs [175]. PML is part of PML-NBs. In contrast to PML-NBs, PML alone is also found in the cytoplasm. Several tumour cells such as hepatocellular carcinomas stain positive for PML in the cytoplasm [176]. Reasons for this include accumulation in the cytoplasm, increased nuclear export or increased production of cytoplasmic isoforms of PML [177]. The consequences of the high proliferation of cytoplasmic PML expression are not yet known. However, it is a fact that cytoplasmic PML can induce the export of
nuclear PML into the cytoplasm and impair its ability to activate p53 [178].
Cytoplasmic isoforms interact with the transforming growth factor (TGF-β) pathway, which is involved in many cellular processes such as proliferation, differentiation and apoptosis [179]. It is not clear whether nuclear isoforms of PML are also able to affect the TGF-β pathway. Another function of cytoplasmic PML is to defend the cell against viral infections by colocalisation of PML and integrase interactor 1 (INI-1) with the retroviral preintegration complex [180]. In ALT-positive tumour cells, PML-NB form a shell around telomeric repeat sequences, called ALT associated PML-NB (APB) [181]. Components of APB are PML-NB (PML and SP100 protein, SUMO) and telomere repeat-associated proteins (TRF1, TRF2, PT1, RAP1) and DDR [170].

![Figure 21: ALT-associated PML-NB (APB). The red coloured structure represents the telomeric repeat sequences, surrounded by PML-NB [155].](image1)

Figure 21: ALT-associated PML-NB (APB). The red coloured structure represents the telomeric repeat sequences, surrounded by PML-NB [155].

![Figure 22: A nucleus showing PML protein (green) and telomere repeat binding factor TRF2 (red), which is part of the shelterin complex. The yellow signals (arrows) represent TRF2 and PML which, together, form ALT-associated PML-NBs (AGBs) [155].](image2)

Figure 22: A nucleus showing PML protein (green) and telomere repeat binding factor TRF2 (red), which is part of the shelterin complex. The yellow signals (arrows) represent TRF2 and PML which, together, form ALT-associated PML-NBs (AGBs) [155].
1.7 Recent studies about DAXX/ATRX and ALT phenotype

Jiao et al. were first to screen the exons of ten sporadic PANETs for mutations. The most common mutations were further investigated in 58 additional PANETs. The study group found 157 somatic mutations in 149 genes; the mean number of mutations was 16 per tumour, with a range of eight to 23. The most common mutations were detected in the following genes: *MEN1* (44.1%), *DAXX* (25%), *ATRX* (17.6%), *PTEN* (7.3%), *TSC2* (8.8%) and *PIK3CA* (1.4%). Mutations in the *DAXX* and *ATRX* gene were mutually exclusive. The results were then compared with clinical data. It was found that 100% of all patients with NETs that exhibited mutations in *DAXX/ATRX* genes survived for at least ten years, whereas patients without these mutations died within five years [70]. DAXX and ATRX proteins are known to be important for heterochromatin maintenance at telomeres [148, 151, 182]. Heaphy et al. showed a perfect correlation between the presence of ALT phenotype and immunohistochemical loss of DAXX/ATRX expression in PANETs. In addition, they examined 439 other tumours of other types such as glioblastoma multiforme, medulloblastomas and oligodendrogliomas. They found mutations in the *ATRX* gene that were correlated with ultrabright signals in telomere FISH. DAXX mutations were not detected [75].

Figure 23: ALT phenotype and nuclear loss of DAXX/ATRX expression in PANETs and glioblastoma multiforme. A: Ultrabright signals (arrows) in FISH in a PANET. B: Loss of DAXX expression with positive endothelial cells as internal control (arrowheads) in a PANET. C: Ultrabright signals (arrows) in FISH in a glioblastoma multiforme. D: Loss
of ATRX expression in a glioblastoma multiforme. Positive endothelial cells (arrowheads) served as positive internal control.

Chen et al. obtained a similar result, showing a loss of DAXX/ATRX expression in 41.4% of PANETs. In 80% of rectal NETs, 60% of gastric NETs and 27% of duodenal NETs there was also a loss of expression of DAXX/ATRX. Je et al. revealed that alterations of the ATRX gene are not common in gastric, colorectal or prostate cancers. Hence these findings suggest that loss of DAXX/ATRX is special for NETs [183].

There was no loss of expression in these two stainings in normal pancreatic tissue and ductal adenocarcinomas of the pancreas. In addition, the study group reported that the status of DAXX immunoreactivity was positively associated with the Ki-67 index [184]. A Chinese study included 37 Chinese patients with PANETs, five of which were insulinomas. They identified 133 somatic mutations in eight different genes by using Sanger sequencing with the following frequencies: DAXX/ATRX (54%), KRAS (10.8%), MEN1 (35.1%), mTOR pathway genes PTEN and TSC2 (54%), SMAD4/DPC (2.7%), TP53 (13.5%) and VHL (40.5%). In particular for insulinomas, the following genes were showed to have mutations: ATRX, DAXX, MEN1, PTEN, TSC2 and VHL. The study group correlated the results with clinical data and found that patients with mutations in KRAS and DAXX/ATRX had a shortened overall survival [185]. Marinoni et al. confirmed the importance of DAXX/ATRX mutations in PANETs and the correlation with ALT phenotype. In addition, they found a relationship between these results and CIN (chromosome instability). CIN was defined as the total number of gains and losses of eight or more in conventional comparative genomic hybridisation (CGH) data and 20 or more in array CGH. To date, it is unclear whether CIN induces ALT or whether ALT is part of CIN. Concerning the clinical outcome, the study by Marinoni et al. generated different results to those by Jiao et al. In detail, they reported that ALT-positive tumours exhibited a significantly decreased relapse-free survival as well as a reduced tumour-specific survival. In correlation with DAXX/ATRX loss, a decreased relapse-free survival was found, but not a shortened tumour-specific survival [186]. One reason for this discrepancy could be the different collectives. While Jiao et al. examined tumours that had already metastasised, Marinoni et al. recruited PANETs with a benign behaviour.
Another study concerning liver metastasis of NETs (48% PANETs, 52% NETs of the GI tract) explored the correlation between ALT phenotype and clinical data. Among metastases of gastrointestinal NETs, 4% were ALT-positive; 56% of metastases of PANETs exhibited the ALT phenotype. This study group reported that the ALT pathway is associated with an increased overall survival (132 vs. 71 months) [187].

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Number of PANETs</th>
<th>DAXX mutation/loss of expression</th>
<th>ATRX mutation/loss of expression</th>
<th>Associated with</th>
<th>Clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Jiao [70]</strong></td>
<td>Exome sequencing</td>
<td>68</td>
<td>25%</td>
<td>17.6%</td>
<td></td>
<td>Better outcome</td>
</tr>
<tr>
<td><strong>De wilde [76]</strong></td>
<td>IHC</td>
<td>50 (28 MEN1)</td>
<td>6%</td>
<td>2%</td>
<td>ALT phenotype</td>
<td>Larger in diameter, higher grade</td>
</tr>
<tr>
<td><strong>Heaphy [75]</strong></td>
<td>Sequencing/IHC</td>
<td>39</td>
<td>24%</td>
<td>22%</td>
<td>ALT phenotype</td>
<td>No association</td>
</tr>
<tr>
<td><strong>Chen [184]</strong></td>
<td>IHC</td>
<td>70</td>
<td>25%</td>
<td>15.7%</td>
<td>Ki-67 index</td>
<td>Worse</td>
</tr>
<tr>
<td><strong>Yuan [185]</strong></td>
<td>Sequencing</td>
<td>37</td>
<td>54%</td>
<td>11%</td>
<td></td>
<td>Worse</td>
</tr>
<tr>
<td><strong>Marinoni [186]</strong></td>
<td>IHC/sequencing</td>
<td>92</td>
<td>25%</td>
<td>18%</td>
<td>ALT and CIN</td>
<td>Worse</td>
</tr>
</tbody>
</table>

Table 7: Recent studies reporting DAXX/ATRX mutations/loss of expression in PANETs. ALT = alternative lengthening of telomeres, IHC = immunohistochemistry, MEN1 = multiple endocrine neoplasms type 1, CIN = chromosome instability.
2. Aim of thesis

Neuroendocrine tumours are rare neoplasms with an incidence of 2-4 per 100,000 population [3], which is increasing at a rate of 3-10% per year, depending on the subtype [4]. In early stages, surgical resection is curative, but at the time of diagnosis, 50% of patients already have distant metastases [8, 9]. The prognosis in such cases is three years for G1 graded tumours and 24 months for G2 graded tumours; for NECs, the median survival is 3-10 months. Several drugs are available which improve the prolonged survival time for 10-15 months. Until now, little is known about the biological mechanisms of this kind of tumour. Further investigations are necessary to find new targets for therapy in advanced stages. DAXX and ATRX mutations were recently found in 43% of PANETs [70]. These mutations are combined with an ALT phenotype [75]. In our study, we examine the expression of DAXX/ATRX in insulinomas and NETs of the small intestine by using immunohistochemistry. In addition, we investigate tumours for the presence of ALT phenotype by FISH. Our aim is to examine the significance of DAXX/ATRX expression and ALT phenotype in insulinomas, which has been investigated in PANETs, but only for a small number of insulinomas. We also address this question in NETs of the small intestine, which has not been examined at all. In addition, we explore the relationship between clinical data and our findings.
3. Material and methods

3.1 Subject/case selection

**Insulinomas**

126 cases with well-differentiated insulinomas were included in our study. 62 cases were recruited from the archives of the Department of Pathology of the University Hospital in Zurich (Switzerland), Düsseldorf (Germany), Kiel (Germany) and Verona (Italy). All patients underwent surgery between 1975 and 2006. The other 64 cases were from the consultation archives of the Department of Pathology of the University Hospital in Kiel (Germany), G. Klöppel. The diagnosis was based on the WHO classification of 2010. The cohort consists of 120 primary tumours, 20 of which exhibited malignant behaviour. Lymph node metastases and liver metastases were available for four primary tumours. Furthermore, two lymph node metastases and four liver metastases were available despite the absence of tissue of the primary. 78 patients were female, 46 were male (no information about gender was provided in two cases). The mean age was 50 years, with a standard deviation of 17. The oldest patient was 84, and the youngest 16. The mean size of the tumour was 2.1 cm.

<table>
<thead>
<tr>
<th>Demographic features of patients with insulinoma</th>
<th>Mean (percentage)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Female</strong></td>
<td>78 (62%)</td>
<td></td>
</tr>
<tr>
<td><strong>Male</strong></td>
<td>46 (37%)</td>
<td></td>
</tr>
<tr>
<td><strong>Not known</strong></td>
<td>2 (1%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>50 years</td>
<td>16-84 years</td>
</tr>
<tr>
<td><strong>Tumour size</strong></td>
<td>2.1 cm</td>
<td>0.8-10 cm</td>
</tr>
</tbody>
</table>

Table 8: Demographic features of patients with insulinomas.

**siNETs**

135 cases with well-differentiated neuroendocrine tumours of the ileum were recruited from the archives of the Department of Pathology of the University Hospital in
Tübingen, Düsseldorf, Marburg and TU München (Germany). All tumours were diagnosed based on the WHO classification of 2010. Tissue of primary tumours of 127 patients was available. A total of 74 lymph node metastases and 15 liver metastases were available. 50 were female, 56 male; no information about gender was provided for the others. The mean age was 58 years; the youngest patient was 20, the oldest 87.

<table>
<thead>
<tr>
<th>Demographic features of patients with neuroendocrine tumours of the small intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (percentage)</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Not known</td>
</tr>
<tr>
<td>Age</td>
</tr>
<tr>
<td>Tumour size</td>
</tr>
</tbody>
</table>

Table 9: Demographic features of patients with neuroendocrine tumours of the small intestine.

<table>
<thead>
<tr>
<th>UICC stage</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage I</td>
<td>7</td>
</tr>
<tr>
<td>Stage II A</td>
<td>6</td>
</tr>
<tr>
<td>Stage II B</td>
<td>5</td>
</tr>
<tr>
<td>Stage III A</td>
<td>1</td>
</tr>
<tr>
<td>Stage III B</td>
<td>35</td>
</tr>
<tr>
<td>Stage IV</td>
<td>52</td>
</tr>
<tr>
<td>Not known</td>
<td>29</td>
</tr>
</tbody>
</table>

Table 10: UICC stage of patients with neuroendocrine tumours of the small intestine.
UICC = Union of International Cancer Control.

The study design was approved by the Local Ethics Committee in Tübingen (022/2014BO1).

3.2 Immunohistochemistry (IHC)

Immunohistochemistry (IHC) was established by Albert Coons in 1941, and is now an important part of pathology. IHC is an immunological method that uses binding between antibody and antigen. First, a primary antibody is used to bind on an epitope
consisting of a sequence of five to ten amino acids. Next, the antibody-antigen binding sites are detected either directly or indirectly by using a second antibody. When a further signal enhancement is required, the secondary antibody carries biotin which binds to a complex of avidin and a biotin carrying enzyme. In this case, more marker molecules are involved and the signal is enhanced.

For our study, we used antibodies to detect ATRX (Sigma/ # HPA001906) and DAXX (Sigma/# HPA008736).

**Protocol for immunostaining ARTRX and DAXX:**

**Preparation**

**Deparaffination:**
- 3x 10 min xylol
- 2x 3 min 100% EtOH
- 2x 3 min 98% EtOH
- 1x 3 min 75% EtOH
- 1x 5 min 75% EtOH with 3% of H2O2 (20 ml 30% H2O2 in 180 ml 75% EtOH)
- 5 min washing in dH2O

**Pre-treatment**
- Ci pH6 or TEC pH9 5 min
- Anneal slides for 20 min
- Dip slides briefly in dH2O
- 2x 3 min in wash buffer

**TECAN**
- Put all slides into machine
- Fill buffer cuvette
- 1. Block, 2. secondary AK, 3. conjugate (HRP Polymer)
- 200 µl per slide

**Programme:**
- Wash buffer 5 min
Block 5 min
AK is pipetted (30 min)
Wash buffer 5 min
Secondary AK 30 min
Wash buffer 5 min
Conjugate 30 min
Wash buffer 5 min
DAB 2x 5 min
Wash buffer 5 min

End of process
Put slides from cover plates into dH2O
Counterstain with Papanicolaou for 15 min
3x wash in dH2O
Pan slides in EtOH/ammonium (200 ml 70% EtOH+6 ml NH₃)
1x 70% EtOH 2 min
1x 80% EtOH 2 min
2x 96% EtOH 2 min
4x 100% EtOH 2 min
2x xylol 2 min
Put into embedding machine

Evaluation of immunohistochemical findings
Three core biopsies of each tissue block were scored in order to avoid sampling errors. Two pathologists independently scored the immunohistochemistry staining. The tissue was only classified if there was a positive internal control, such as lymphocytes or stromal cells. If there was no positive internal control, staining was repeated, but this time the whole tissue was used. If there was still no positive internal control, the case was determined as not assessable (n.a.). It was noted whether there was nuclear or/and
cytoplasmic staining. Only nuclear staining was counted. No positive cells or less than 10% stained tumour cells were rated as loss of expression. If between 10% and 80% of the tumour cells were stained positive, staining was called heterogeneous. An excess of 80% of stained tumour cells was rated as positive.

3.3 Fluorescence in situ hybridisation (FISH)

Fluorescence in situ hybridisation, first used in 1980 [188], is a method for detecting sequences of DNA and RNA in metaphase and interphase cells by using probes with complementary sequences. The probes are either directly marked with a fluorescence stain or digoxigenin/biotin labelled probes are used, detected by fluorochrome labelled anti-digoxigenin or anti-biotin antibodies. These are plasmids, cosmids, phages, YACs (yeast artificial chromosomes) or complete chromosomes. For detection, several fluorochrome stains are provided for single colour FISH, Multiplex-FISH or SKY-FISH (Spectral Karyotyping), 24-colour karyotyping to detect structural or numerical aberrations [189]. Either fixed permeabilised cells or formalin-fixed paraffin-embedded slides can be used for this technique. FISH is applied for the purpose of research and diagnosis. With this technique, it is possible to:

- Locate genes and DNA sequences to detect translocations, deletions, inversions and insertions. One example of a translocation is the Philadelphia chromosome in chronic myeloid leukaemia, where there is a breaking point in chromosome 22 and this part fuses with chromosome 9.
- Provide evidence of RNA to detect gene expression.
- Provide evidence of pathogenic germ in tissues.
- Identify chromosomes. In prenatal diagnosis, for example, the Down’s syndrome can be detected by proving a third chromosome 21.
- Identify amplifications, for example, the Her2 coding ERBB2 gene in carcinoma of the breast. This is used in routine diagnostics when immunohistochemistry staining of Her2 is uncertain.
Figure 24: FISH technique: the target DNA is denaturated and then hybridised with the complementary PNA (peptide nucleic acid). In our study, the PNA was complementary to the mammalian telomere repeat sequence.

For our study, formalin-fixed, paraffin-embedded tissue microarrays were used. This FISH was performed at the Institute for Pathology, University of Bern (Switzerland). TMAs were hybridised with the complementary peptide nucleic acid to mammalian telomere repeat sequence CCCTAACCCCTAACCCCTAA (N- to C-terminus). This was done per protocol as listed below. The slides were counterstained with DAPI (4’, 6-diamidino-2-phenylindole dihydrochloride), which stains the nucleus. In order to detect telomere signals, fluorescein isothiocyanate (FITC) with anti-digoxigenin binds on the digoxigenin-labelled probe. A Zeiss Axio Imager M2 microscope with appropriate emission filters was used to analyse the samples.

**Protocol for telomere FISH**

**Pre-treatment:**

1 h 60°C ageing

3x xylene 10 min
100% EtOH 3x 2 min

Air dry

Immerse slides in 100 mM Tris/ 50 mM EDTA PH 7.0 (92.8°C for 15 min)

PBS rinse

250-300 µl

Digest all 37°C, pepsin 1:2, 14 min

PBS rinse

Dehydrate in EtOH 70%, 85%, 95%, 100% RT, 2 min each

Air dry

**Probe preparation:**

ERG: 10 µl HB+2 µl Cot-1+2 µl Probe-Bio+2 µl Probe-Dig,

    denat. 73°C 5min

**Hybridisation:**

Place 15 µl probe mix on slide for 18x18 mm coverslip

Rubber cement edges to maintain probe concentration

Place in crocodile 94°C for 3 min, 37°C overnight

**Post-hybridisation wash:**

Immerse slides in 2x SSC: RT for a few minutes, let coverslip swim off

    75°C for 6 min

**Detection:**

Rinse the slides in 0.5x SSC, RT, 3x 2 min

CAS-Block (10% normal goat serum), RT

FITC anti-Dig/594 SAV, 1:500, 1 h
Rinse slides in 0.5x SSC, RT, 3x 2 min

**Evaluation of telomere FISH**

Two pathologists independently gave scores to the TMAs. In order to classify as ALT-positive tumour cells, the following criteria had to be met: large heterogeneous ultrabright signals located in the nucleus of the tumour cells and coexistent normal telomere signals in surrounding lymphocytes and stromal cells. Tumour cells with normal telomere signals were scored as ALT-negative. Cores of TMAs without any signal were rated as not assessable (n.a.).
3.4 Statistical analysis

Statistical analysis was performed using SPSS (version 22.01). The Pearson chi-squared test was used to compare DAXX/ATRX and ALT phenotype with biological behaviour. The Mann Whitney U test was used to test for correlation of DAXX/ATRX loss and ALT phenotype with tumour size. The relationship between loss of DAXX/ATRX or ultrabright signals in FISH and overall survival was examined using the log-rank test depicted as Kaplan-Meier curve. P values below 0.05 were denoted as statistically significant.
4. Results

HE staining

All tissue microarrays (TMAs) were stained with haematoxylin & eosin (HE) to identify the morphology of the tumour for recognising tumour cells in DAXX and ATRX staining and to distinguish tumour cells from other cells such as lymphocytes in the FISH.

Telomere FISH in normal cells

Lymphocytes and pancreas acini exhibit normal telomere signals. A few small round signals are detectable in each cell. The signals are slight and have a homogeneous distribution.

Figure 25: Lymphocytes (A) and pancreas acini (B) with homogeneous green detecting telomeres. A, B: 1000x telomere FISH.
4.1 Insulinomas

4.1.1 ALT phenotype

There were four cases (3%) of insulinomas that exhibited ultrabright signals. All cases were insulinomas with malignant behaviour. In three of these cases tissue of the primary and the metastasis were available. One ALT-positive case was a liver metastasis; the tissue of the primary was not available. All cases were associated with loss of expression of DAXX/ATRX. One of them (25%) was correlated with a loss of DAXX expression; the other three (75%) were correlated with a loss of ATRX expression. The mean age of patients with ALT-positive tumours was 52 years, similar to the mean age of all patients with insulinoma. Three were female and one was male. The mean tumour size in ALT-negative cases was 2.1 cm and 5.5 cm in ALT-positive cases. The biggest tumour measured 8 cm. The cases with ALT-positive phenotype are listed below (Table 12).

<table>
<thead>
<tr>
<th>FISH</th>
<th>normal</th>
<th>ub</th>
<th>n.a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour</td>
<td>74 (61%)</td>
<td>3 (3%)</td>
<td>43 (36%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>2 (40%)</td>
<td>2 (40%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>1 (13%)</td>
<td>4 (40%)</td>
<td>3 (37%)</td>
</tr>
</tbody>
</table>

Table 11: FISH results of insulinomas. FISH = fluorescence in situ hybridisation, ub = ultrabright signals, n.a. = not assessable.

<table>
<thead>
<tr>
<th>Case Nr</th>
<th>FISH</th>
<th>FISH</th>
<th>FISH</th>
<th>DAXX</th>
<th>DAXX</th>
<th>DAXX</th>
<th>ATRX</th>
<th>ATRX</th>
<th>ATRX</th>
<th>Gender</th>
<th>Age</th>
<th>Tumour size</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>ub</td>
<td>ub</td>
<td>ub</td>
<td>norm</td>
<td>norm</td>
<td>norm</td>
<td>loss</td>
<td>loss</td>
<td>loss</td>
<td>f</td>
<td>56</td>
<td>8</td>
</tr>
<tr>
<td>57</td>
<td>ub</td>
<td>x</td>
<td>ub</td>
<td>norm</td>
<td>x</td>
<td>norm</td>
<td>loss</td>
<td>x</td>
<td>loss</td>
<td>f</td>
<td>63</td>
<td>1.7</td>
</tr>
<tr>
<td>61</td>
<td>ub</td>
<td>ub</td>
<td>x</td>
<td>norm</td>
<td>loss</td>
<td>norm</td>
<td>norm</td>
<td>norm</td>
<td>norm</td>
<td>f</td>
<td>55</td>
<td>8</td>
</tr>
<tr>
<td>56</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>norm</td>
<td>x</td>
<td>x</td>
<td>loss</td>
<td>m</td>
<td>36</td>
<td>4.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 12: All insulinoma cases with the ALT phenotype. P =Primary, LNM = lymph node metastasis, LM = liver metastasis, ub = ultrabright signal in FISH, norm = normal expression of DAXX/ATRX, loss = loss of DAXX/ATRX expression, x = no tissue, f = female, m = male.
Figure 26: Distribution of cases with ALT phenotype. n.a. = not assessable.

The following images show cases classified as ALT-positive.

Figure 27: Case number 59: primary tumour of a 56-year-old woman with a tumour size of 8 cm. Loss of ATRX expression with positive internal control and normal DAXX expression. The telomere FISH shows heterogeneous ultrabright signals. A: HE 400x, B: ATRX 400x, C: DAXX 400x, D: FISH 1000x.
Figure 28: Case number 59: lymph node metastasis of the primary tumour shown above. The metastasis also shows a loss of ATRX expression and DAXX showed a strongly nuclear expression in the tumour cells, neighbouring strongly positive lymphocytes. The telomere FISH is positive for the ALT phenotype. A: HE 400x, B: ATRX 400x, C: DAXX 400x, D: FISH 1000x.
Figure 29: Case number 59, liver metastasis. Loss of ATRX expression with neighbouring positive lymphocytes. DAXX expression is normal. FISH reveals spotted ultrabright signals. A: HE 200x, B: ATRX 400x, C: DAXX 400x, D: FISH 1000x.
Figure 30: Case number 57: primary tumour of a 63-year-old woman with a tumour size of 1.7 cm. Tumour cells with loss of ATRX expression and normal DAXX staining. The telomere FISH shows heterogeneous ultrabright signals, classified as positive for the ALT phenotype. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x.
Figure 31: Case number 57, liver metastasis. As the primary tumour, the liver metastasis also shows a loss of ATRX expression. DAXX was positive in about 85% of the tumour cells, classified as normal expression. In the telomere FISH, ultrabright heterogeneous signals typical for ALT phenotype were present. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x.
Figure 32: Case number 61, primary tumour of a 55-year-old female with a tumour size of 8 cm. In the primary tumour, ATRX and DAXX staining were classified as normal. The telomere FISH was ALT-positive. A: HE 400x, B: ATRX 400x, C: DAXX 200x, D: FISH 1000x.
Figure 33: Case number 61, lymph node metastasis. ATRX staining was normal and DAXX expression, different to the primary, was lost in the tumour cells. FISH signals were positive for ALT. A: HE 400x, B: ATRX 400x, C: DAXX 400x, D: FISH 1000x.
Figure 34: Case number 61, liver metastasis. Similar to the lymph node metastasis, the tumour cells stained positive for ATRX and showed a loss of DAXX expression. FISH signals were strongly heterogeneous with spotted ultrabright signals. A: HE 400x, B: ATRX 400x, C: DAXX 400x, D: FISH 1000x.
Figure 35: Case number 56, liver metastasis of a 36-year-old man with a primary tumour of 4.5 cm in diameter. ATRX expression was lost in tumour cells neighbouring positive liver cells with background staining. DAXX expression was normal. Telomere FISH revealed spotted ultrabright signals. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x.
One case, a primary tumour of a 79-year-old woman with a tumour size of 3 cm, was normal for DAXX and ATRX expression, but yielded a different distribution of telomere signals in FISH to ALT-positive or ALT-negative cases. As the figure below shows, there are bright homogeneous signals of the same size and some spotted bigger signals, which are somehow smaller than ALT-positive signals, but brighter than normal signals. For this reason, we classified this case as unclear.

Figure 36: Case number 102, insulinoma of a 79-year-old woman with a size of 3 cm. The tumour had not metastasised. The telomere signal is predominantly bright and homogeneous, but also shows sporadic spotted ultrabright signals. This case was classified as not assessable.

Figure 37: Case number 102, the ATRX and DAXX expression is normal with a strongly nuclear positivity. A: HE 200x, B: ATRX 200x, C: DAXX 200x.
4.1.2 Loss of DAXX expression

Only one (0.8%) of the 126 insulinomas yielded a loss of DAXX expression. DAXX staining was normal for the primary tumour, but a loss of expression was yielded in the lymph node and liver metastasis. The primary, the lymph node and the liver metastasis showed ultrabright abnormal signals in FISH. The ATRX expression was normal in the primary and liver metastasis; the lymph node metastasis was classified as heterogeneous. This patient was female, 55 years old, and had an 8 cm sized tumour.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Heterogeneous</th>
<th>Loss</th>
<th>n.a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour</td>
<td>93 (77%)</td>
<td>0</td>
<td>0</td>
<td>27 (23%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>3 (60%)</td>
<td>0</td>
<td>1 (20%)</td>
<td>1 (20%)</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>4 (50%)</td>
<td>1 (13%)</td>
<td>1 (13%)</td>
<td>2 (25%)</td>
</tr>
</tbody>
</table>

Table 13: DAXX results in insulinomas. n.a. = not assessable.

<table>
<thead>
<tr>
<th>Case Nr.</th>
<th>DAXX P</th>
<th>DAXX LNM</th>
<th>DAXX LM</th>
<th>ATRX P</th>
<th>ATRX LNM</th>
<th>ATRX LM</th>
<th>FISH P</th>
<th>FISH LNM</th>
<th>FISH LM</th>
<th>Gender</th>
<th>Age</th>
<th>Tumour size</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>norm</td>
<td>loss</td>
<td>loss</td>
<td>norm</td>
<td>het</td>
<td>norm</td>
<td>ub</td>
<td>ub</td>
<td>ub</td>
<td>f</td>
<td>55</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 14: A single case with loss of DAXX expression. P = primary, LNM = lymph node metastasis, LM = liver metastasis, norm = normal expression of DAXX/ATRX, het = heterogeneous expression of DAXX/ATRX, loss = loss of expression of DAXX/ATRX, ub = ultrabright signals in FISH, f = female.

Loss of DAXX expression

Figure 38: Distribution of cases with loss of DAXX expression. n.a. = not assessable.
4.1.3 Loss of ATRX expression

Four cases (3%) showed a loss of expression of ATRX. Two of these had lymph node and/or liver metastasis. In two of the cases, tissue of the liver metastasis was available, but not of the primary. There was no simultaneous loss of DAXX expression. One case did not correlate with an ALT phenotype. This case was a liver metastasis for which no tissue of the primary tumour was available. The mean age was 59 years. The average size of the primary tumour was 4.1 cm compared to the mean tumour size of all insulinomas, which was 2.1 cm.

<table>
<thead>
<tr>
<th>ATRX</th>
<th>Normal</th>
<th>Heterogeneous</th>
<th>Loss</th>
<th>n.a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour</td>
<td>84 (70%)</td>
<td>0</td>
<td>2 (2%)</td>
<td>34 (28%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>4 (80%)</td>
<td>0</td>
<td>1 (20%)</td>
<td>0</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>2 (25%)</td>
<td>0</td>
<td>4 (50%)</td>
<td>2 (25%)</td>
</tr>
</tbody>
</table>

Table 15: ATRX results in insulinomas. n.a. = not assessable.

<table>
<thead>
<tr>
<th>case Nr.</th>
<th>ATRX P</th>
<th>ATRX LNM</th>
<th>ATRX LM</th>
<th>DAXX P</th>
<th>DAXX LNM</th>
<th>DAXX LM</th>
<th>FISH P</th>
<th>FISH LNM</th>
<th>FISH LM</th>
<th>Gender</th>
<th>Age</th>
<th>Tumour size</th>
</tr>
</thead>
<tbody>
<tr>
<td>59</td>
<td>loss</td>
<td>loss</td>
<td>loss</td>
<td>norm</td>
<td>norm</td>
<td>norm</td>
<td>ub</td>
<td>ub</td>
<td>ub</td>
<td>f</td>
<td>56</td>
<td>8</td>
</tr>
<tr>
<td>57</td>
<td>loss</td>
<td>x</td>
<td>loss</td>
<td>norm</td>
<td>x</td>
<td>norm</td>
<td>ub</td>
<td>x</td>
<td>ub</td>
<td>f</td>
<td>63</td>
<td>1.7</td>
</tr>
<tr>
<td>58</td>
<td>x</td>
<td>x</td>
<td>loss</td>
<td>x</td>
<td>x</td>
<td>norm</td>
<td>x</td>
<td>x</td>
<td>norm</td>
<td>m</td>
<td>82</td>
<td>2.5</td>
</tr>
<tr>
<td>56</td>
<td>x</td>
<td>x</td>
<td>loss</td>
<td>x</td>
<td>x</td>
<td>norm</td>
<td>x</td>
<td>x</td>
<td>ub</td>
<td>m</td>
<td>36</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Table 16: All cases with loss of ATRX expression. P = primary, LNM = lymph node metastasis, LM = liver metastasis, norm = normal expression of DAXX/ATRX, normal FISH signal, loss = loss of expression of DAXX/ATRX, ub = ultrabright FISH signals = no tissue, n.a. = not assessable.
Figure 39: Case number 58, liver metastasis of an 82-year-old man with a tumour size of the primary tumour of 2.5 cm. ATRX expression is lost in tumour cells, DAXX expression is normal. FISH was classified as normal telomere signal. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x
4.1.4 Correlation between the results and clinical data

4.1.4.1 Correlation between our results and biological behaviour

A total of 59 cases were investigated for DAXX expression and biological behaviour. 47 cases showed benign behaviour and 12 cases were malignant. There was one case with loss of DAXX expression, which was malignant.

![Figure 40: Malignant and benign insulinomas with a loss of DAXX expression.](image)

A total of 59 cases were investigated for loss of ATRX expression and biological behaviour. 13 cases were malignant, 46 were benign. All four cases with loss of ATRX expression showed malignant behaviour.

![Figure 41: Malignant and benign insulinomas with a loss of ATRX expression.](image)

Altogether, 56 cases with assessable expression of DAXX and ATRX were investigated for clinical behaviour. 11 cases were malignant; 45 were benign. 5 cases showed loss of either DAXX or ATRX expression, all of them were malignant insulinomas. None was benign.
Figure 42: Malignant and benign insulinomas with ultrabright signals in telomere FISH.

In total 60 cases were assessable for telomere FISH signals. 49 cases of them were benign insulinomas; 11 cases showed malignant behaviour. 4 cases exhibit ultrabright signals in FISH, these cases were all malignant.

Figure 43: Number of cases of ALT phenotype, DAXX/ATRX expression loss in malignant insulinomas.
4.1.4.2 Correlation between our results and prognosis

A total of 56 cases (45 with benign behaviour, 11 with malignant behaviour) were investigated for correlation between our results and clinical data. Cases with no clinical information or without fully assessable data were excluded. 51 patients had insulinomas with normal expression for DAXX/ATRX. Five patients had insulinomas that showed a loss of DAXX/ATRX expression. By using the log-rank test, we revealed a correlation between loss of DAXX/ATRX expression and a worse outcome with a significance of \( p = 0.012 \). There was no significance (\( p = 0.129 \)) between ultrabright signals and a worse outcome. One explanation could therefore be the small number of cases with ultrabright signals in FISH.
Figure 44: Kaplan Meier curve showing overall survival time of patients with insulinomas with DAXX/ATRX loss and normal expression of DAXX/ATRX. Green: loss of DAXX/ATRX. Blue: normal DAXX/ATRX expression.
### 4.2 Neuroendocrine tumours of small intestine

#### 4.2.1 ALT phenotype

Only one case of 135 showed heterogeneous and ultrabright signals in telomere FISH. The patient was 67 years old; the primary tumour measured 6 cm. The primary tumour was normal in FISH, but the lymph node metastasis showed an ALT-positive FISH signal. DAXX and ATRX expression was normal.

<table>
<thead>
<tr>
<th>FISH</th>
<th>Normal</th>
<th>ub</th>
<th>n.a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour</td>
<td>117 (92%)</td>
<td>0</td>
<td>10 (8%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>64 (86%)</td>
<td>1 (1%)</td>
<td>9 (12%)</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>14 (93%)</td>
<td>0</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

Table 17: FISH results in NETs of the small intestine. ub = ultrabright signals, n.a. = not assessable.

<table>
<thead>
<tr>
<th>Case Nr.</th>
<th>FISH P</th>
<th>FISH LNM</th>
<th>FISH LM</th>
<th>DAXX P</th>
<th>DAXX LNM</th>
<th>DAXX LM</th>
<th>ATRX P</th>
<th>ATRX LNM</th>
<th>ATRX LM</th>
<th>Gender</th>
<th>Age</th>
<th>Tumour size</th>
</tr>
</thead>
<tbody>
<tr>
<td>123a</td>
<td>norm</td>
<td>ub</td>
<td>x</td>
<td>norm</td>
<td>x</td>
<td>norm</td>
<td>x</td>
<td>n.a.</td>
<td></td>
<td>67</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

Table 18: All cases with ALT phenotype. P = primary, LNM = lymph node metastasis, LM = liver metastasis, norm = normal, ub = ALT phenotype, n.a. = not assessable, x = no tissue.

**Figure 45:** Distribution of cases with ultrabright signals in telomere FISH. n.a. = not assessable.
Figure 46: Case number 123a, primary tumour. ATRX staining was normal. More than 80% of tumour cells were positive for DAXX. FISH was classified as normal. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x.
Figure 47: Case number 123a, lymph node metastasis. ATRX and DAXX were strongly positive in tumour cells. FISH showed heterogeneous ultrabright signals, classified as ALT-positive. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x.
4.2.2 Loss of DAXX expression

There was one patient with a loss of DAXX expression. This patient was a 50-year-old woman with a tumour size of 0.8 cm and no metastasis. ATRX staining and telomere FISH were both detected as normal.

<table>
<thead>
<tr>
<th>DAXX</th>
<th>Normal</th>
<th>Heterogeneous</th>
<th>Loss</th>
<th>n.a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour</td>
<td>102 (80%)</td>
<td>18 (14%)</td>
<td>1 (0.7%)</td>
<td>6 (5%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>69 (93%)</td>
<td>1 (1%)</td>
<td>0</td>
<td>4 (6%)</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>14 (93%)</td>
<td>0</td>
<td>0</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

Table 19: DAXX results in NETs of the small intestine. n.a. = not assessable.

<table>
<thead>
<tr>
<th>Case Nr.</th>
<th>DAXX</th>
<th>DAXX</th>
<th>DAXX</th>
<th>ATRX</th>
<th>ATRX</th>
<th>FISH</th>
<th>FISH</th>
<th>FISH</th>
<th>Gender</th>
<th>Age</th>
<th>Tumour size</th>
</tr>
</thead>
<tbody>
<tr>
<td>I003</td>
<td>loss</td>
<td>x</td>
<td>norm</td>
<td>x</td>
<td>0</td>
<td>norm</td>
<td>x</td>
<td>x</td>
<td>f</td>
<td>50</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Table 20: One case with a loss of DAXX expression. P = primary, LNM = lymph node metastasis, LM = liver metastasis, norm = normal ATRX expression/normal FISH: loss = loss of expression, n.a. = not assessable, x = no tissue, f = female.

Figure 48: Distribution of cases with a loss of DAXX expression. n.a. = not assessable.
Figure 49: Case number 003, primary tumour. Less than 10% of tumour cells are positive for DAXX, and therefore classified as a loss of DAXX expression. ATRX was stained normal, and telomere FISH showed slightly homogeneous signals. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x.
Figure 50: Case number 33a, primary tumour with heterogeneous DAXX staining and normal ATRX staining. FISH was classified as normal. A: HE 200x, B: ATRX 200x, C: DAXX 200x, D: FISH 1000x.
4.2.3 Loss of ATRX expression

There was no case with a loss of ATRX expression.

<table>
<thead>
<tr>
<th>ATRX</th>
<th>Normal</th>
<th>Heterogeneous</th>
<th>Loss</th>
<th>n.a.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour</td>
<td>120 (94%)</td>
<td>0</td>
<td>0</td>
<td>7 (6%)</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>67 (91%)</td>
<td>0</td>
<td>0</td>
<td>7 (9%)</td>
</tr>
<tr>
<td>Liver metastasis</td>
<td>13 (87%)</td>
<td>0</td>
<td>0</td>
<td>2 (13%)</td>
</tr>
</tbody>
</table>

Table 21: ATRX results in NETs of the small intestine. n.a. = not assessable.

Figure 51: Distribution of cases with a loss of ATRX. n.a. = not assessable.
5. Discussion

Neuroendocrine tumours (NETs) arise from neuroendocrine cells. Most NETs are found in the pancreas (34.2%); the second most common location is the small intestine (25.8%) [1]. The incidence of 2-4 per 100,000 population is increasing by 3-10% per year, depending on the subtype [3, 4]. One reason for this could be the improvement of diagnostic methods. Nevertheless, 50% of patients already had distant metastases at the time of diagnosis [8, 9]. For patients in the early stages, surgical resection is a curative therapy. In advanced disease, the following drugs are available: somatostatin analogues, targeted agents such as mTOR inhibitors, inhibitors of angiogenesis and interferon α. Somatostatin analogues such as ocreotide are administered when somatostatin receptors on the tumour cells are proved by SRS. In functional NETs, it is useful to prevent clinical symptoms, but recurrence of the symptoms are common [89]. A stabilisation of tumour growth is seen in 24-57% of cases [134]. The mTOR inhibitor everolimus is approved by the FDA for PANETs, but not for siNETs [137]. In PANETs, the prolonged survival time is 10 vs. 4.6 months compared to the placebo [79]. Inhibitors of angiogenesis such as sunitinib, a tyrosin kinase inhibitor, exhibited a longer survival time of 10.2 vs. 5.4 months compared to a placebo group [96]. In conclusion, a better understanding of the biological mechanisms of this tumour type is necessary in order to find drugs for targeted therapy. Recent studies reported that about 40% of sporadic insulinomas show mutations in the MEN1 gene [70]. MEN1 is a tumour suppressor gene; mutations in this gene are a hallmark of the autosomal dominant disease MEN1. 15% of PANETs harbour mutations in the mTOR pathway (PTEN, TSC2, PIK3CA) [70]. Inhibitors of this pathway have already been approved for advanced PANETs [137]. Jiao et al. recently determined that 45% of PANETs show mutations in DAXX/ATRX genes. Both DAXX and ATRX proteins are involved in remodelling chromosomes. Studies investigating these genes [70, 75, 184] and our results show that mutations/loss of expression in these genes are mutually exclusive. A Chinese study confirmed that alterations in DAXX/ATRX genes (54%) and genes of the mTOR pathway (54%) are common in PANETs. They also found mutations in KRAS (10.85), SMAD4/DPC (2.7%) and VHL (40.5%). In their study cohort, five insulinomas were
included with the following frequencies of gene mutations: 20% ATRX, 20% DAXX, 40% MEN1, 20% PTEN and 40% TSC2 [185]. In addition, Heaphy et al. found a perfect correlation between loss of DAXX/ATRX expression and ultrabright signals in telomere FISH, established as a hallmark for ALT phenotype [75]. ALT is an alternative mechanism of the cell for telomere maintenance. 85% of tumours activate the telomerase. Examples of tumour types that use the ALT pathway are osteosarcomas, soft tissue sarcomas, glioblastomas, medulloblastomas, oligodendrogliomas and astrocytomas [165-167]. Features of the ALT pathway are extrachromosomal telomeres, the exchange of telomeres between sister chromosomes, increased t-circles and the presence of PML-NBs [168-170]. It has been suggested that the DAXX and ATRX complex binds on G-rich regions, such as telomere repeats, and deposits the histone H3.3. The consequences of this are not yet understood [147-151].

So far, very few cases of insulinomas have been investigated for DAXX/ATRX mutations, and the reported studies did not include any NETs of the small intestine, which are the second most common NETs. Our purpose was to examine these subtypes for expression of DAXX/ATRX and to explore the relationship with ALT phenotype and clinical data.

In insulinomas, we found one case (0.8%) with a loss of expression of DAXX and four cases (3%) with a loss of ATRX expression. These losses were exclusive; we detected no cases with a loss of expression in both types of staining. This finding is in accordance with several other studies [70, 75, 185, 186]. In telomere FISH, we found four cases (3%) with ultrabright heterogeneous signals. In line with Heaphy et al., we detected a 100% correlation between ALT phenotype and DAXX or ATRX loss expression. One case (25%) yielded a loss of DAXX expression; three cases (75%) had a loss of ATRX. One difficult case, a primary tumour of a 79-year-old woman with a tumour size of 3 cm which was normal for DAXX and ATRX expression, showed a different distribution of the telomere signals in FISH to ALT-positive or ALT-negative cases. The signals were bright, evenly distributed, homogenous and of the same size. In addition, a number of spotted bigger signals were detected that were somehow smaller than ALT-positive signals, but brighter than normal signals. It is unclear whether this tumour was able to activate telomerase or whether it was positive for ALT and also had
activated telomerase. Another explanation could be that this tumour started to activate the ALT phenotype, so the signals get brighter and the distribution will become increasingly heterogeneous. For this reason, we classified this case as unclear.

In addition, we found one case with a loss of ATRX and a normal telomere signal in FISH. This case was an 82-year-old man with a liver metastasis, with the primary tumour measuring 2.5 cm. One explanation of this finding could be that the tumour already had a loss of ATRX and the ALT mechanism will be activated subsequently, but was not yet detectable [186]. Moreover, we detected no case with loss of DAXX expression and normal FISH signals. Another aim of this thesis was to investigate the correlation between our results and the clinical data. We found a strongly significant correlation (p< 0.001) between DAXX/ATRX loss and malignant behaviour. This result conforms with the study group around de Wilde [76]. Our finding also matches the study involving metastases of NETs, where they found 56% ALT-positive cases in metastases of PANETs [187]. In addition, we revealed a correlation between DAXX/ATRX loss and a greater tumour size with a strong significance (p= 0.005). This was also shown by the study group around Heaphy [75] and De Wilde [76]. Concerning the prognosis, we found an association between DAXX/ATRX loss and a shortened overall survival time (p= 0.012). This finding is in agreement with the finding of Yuan et al. [185]. Marinoni et al. came to the conclusion that DAXX/ATRX loss predicted a decreased relapse-free survival; no shortened tumour-specific survival was observed [186]. In contrast, Jiao et al. revealed that patients with PANETs that contained altered DAXX/ATRX mutations yielded a longer survival time. This discrepancy can be ascribed to a different cohort, which included metastasised PANETs of patients with palliative therapy [186]. The correlation between ultrabright signals in FISH and the shortened overall survival time was not significant in our study. This can be attributed to the fact that the case number with ALT-positive FISH is too small. In conclusion, previous studies and our own study suggest that DAXX/ATRX loss and ALT phenotype is a late event in tumour progression, and is associated with larger tumours with malignant behaviour. In addition, according to Marinoni et al., chromosome instability (CIN) is induced in that kind of tumour, as part of tumour progression.
In siNETs, we revealed one case with a DAXX loss and no cases with a loss of expression of ATRX. The case with a DAXX loss was the primary tumour of a 50-year-old woman with a tumour size of 0.8 cm. FISH revealed normal telomere signals. Further, we found one case with ALT phenotype; DAXX/ATRX expression was normal. The tissue of this case was a lymph node metastasis of a 6 cm primary tumour of a 67-year-old patient. The primary tumour also stained normal for DAXX/ATRX, and showed a normal signal in telomere FISH. This result is unique and contrasts with all our remaining cases and the studies undertaken to date. One explanation could therefore be that either DAXX or ATRX is mutated, but still positive in immunohistochemical staining. Another reason could be that another protein, which is altered, is involved in the regulation of telomeres. Histone H3.3 may be a candidate because decreased histone H3.3 leads to the destabilisation of telomeres [149]. Compared to our findings in insulinomas, the ALT phenotype seems to play a less important role in the pathogenesis. This is in line with the study by Dogeas et al., which revealed a 4% ALT pathway in liver metastasis of gastrointestinal NETs with carcinoid syndrome [187].

In conclusion, we found that a loss of DAXX/ATRX expression correlated with ALT phenotype in insulinomas. Compared to studies with PANETs, there were fewer ALT-positive cases in insulinomas since most insulinomas (90%) exhibit a benign behaviour. We revealed that a loss of DAXX/ATRX is correlated with malignant behaviour, a bigger tumour size and a shortened overall survival. In other words, finding a loss of expression of DAXX/ATRX in insulinomas is a sign of malignancy and a poor prognosis. In siNETs, loss of DAXX/ATRX expression and ALT phenotype play a minor role in the tumourgenesis. For insulinomas and other PANETs, mutations in the DAXX and ATRX gene represent a biological subtype, making it important to identify new targeted therapies. While inhibitors of the enzyme telomerase already exist, inhibitors of the ALT phenotype are lacking. For this reason, the mechanism of the ALT phenotype and the role played by DAXX and ATRX have to be investigated further.
6. Abstract

Neuroendocrine tumours (NETs) are rare neoplasms with an incidence of 2-4% per 100,000 population. Most NETs are found in the pancreas (34.2%) and the small intestine (25.8%). At time of diagnosis, 50% of all patients with NETs already have metastasis and systemic therapy is indicated. There are drugs available like somatostatin-analogues, inhibitors of the mTOR pathway and inhibitors of angiogenesis and interferon alpha. For Somatostatin-analogues a stabilisation of tumour growth is reported. The other drugs showed a prolonged survival time for months compared to placebo. However, a better understanding of the biological mechanisms of this tumour type is necessary in order to find drugs for targeted therapy. Recent studies reported that mutations in the MEN1 gene, in the DAXX/ATRX gene and in genes related to the mTOR pathway are found in pancreatic neuroendocrine tumours (PANETs). Our aim is to examine the significance of DAXX/ATRX expression and alternative lengthening of telomeres (ALT) in insulinomas, which has been investigated in PANETs, but only for a small number of insulinomas. We also address this question in NETs of the small intestine (siNETs), which has not been examined at all. We found a loss of DAXX expression in one insulinoma and a loss of ATRX expression in four insulinomas. These results correlate with ALT phenotype (p<0.001). We revealed that a loss of DAXX/ATRX is strongly correlated with malignant behaviour (p<0.001), a bigger tumour size (p=0.005) and a shortened overall survival (p=0.012). In other words, finding DAXX/ATRX expression loss in insulinomas is a sign of malignancy and a poor prognosis. In siNETs, loss of DAXX/ATRX expression and ALT phenotype play a minor role in the tumourgenesis. The ALT pathway plays an important role in insulinomas and PANETs in general, therefore further studies are required to find new agents for a targeted therapy.
7. Zusammenfassung

8. Erklärung zum Eigenanteil der Dissertationsschrift

Die Konzeption der Studie erfolgte durch Prof. Dr. med. Bence Sipos, stellvertretender ärztlicher Direktor (Institut für Pathologie und Neuropathologie, Universitätsklinik Tübingen).


Die Auswertung der immunhistochemischen Untersuchungen und der FISH sowie die statistische Auswertung erfolgte eigenständig durch mich unter Supervision durch Herrn Prof. Dr. Sipos.

Ich versichere, das Manuskript selbstständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.


Dr.med.univ. Bettina Neumayer
9. Danksagung

An erster Stelle möchte ich mich bei Prof. Dr. Sipos bedanken, für die Möglichkeit an einem spannenden Thema zu arbeiten und dieses auch zeitnah abschließen zu können, was in meinem Fall als Österreicherin für die Weiterbeschäftigung an einer deutschen Universitätsklinik wichtig ist. Und besonders dafür dass er sich immer Zeit genommen hat meine Fragen und Anliegen mit mir zu besprechen. Ich bedanke mich für die ausgezeichnete Betreuung und freue mich auf die weitere Zusammenarbeit!

Mein herzlicher Dank geht an Maike Nieser und Karen Greif für die tatkräftige Unterstützung, ohne den beiden wäre es nicht möglich gewesen. Ich danke Ihnen auch für die freundschaftliche Aufnahme in das Team und ich freue mich auf weitere Zusammenarbeit und Running after Work!

Mein Dank geht auch an die immunhistochemische Abteilung des Instituts für Pathologie der Universitätsklinik Tübingen.

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Bei Perikles Kosmidis möchte ich mich gerne für die Tipps und Tricks zum Fotografieren der FISH bedanken.

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10. References


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