On the Diagnostics of Neuroblastoma

Clinical and Experimental Studies

KLEOPATRA GEORGANTZI
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Abstract

Neuroblastoma (NB) is one of the most common childhood cancers. Patients with low stage tumor have high survival rate, while those with advanced stage and/or unfavorable molecular biology have poor prognosis. A correct histopathological diagnosis, clinical stage, and identified genetic aberrations are crucial for treatment stratification according to current protocol. The tumor sample is obtained either by fine needle aspiration, cutting needle biopsy or open biopsy. NB exhibits neuroendocrine differentiation by showing immunoreactivity for chromogranin A (CgA), synaptophysin (syn), and neuron specific enolase (NSE) and 90% of the patients have increased levels of urine catecholamine metabolites.

Experimental and clinical NB tumor samples were immunostained for somatostatin receptors (SSTRs) 1-5, somatostatin and CgA. Clinical tumor samples were also immunostained for syn, synaptic vesicle protein 2 (SV2), and vesicle monoamine transporter 1 (VMAT1) and 2 (VMAT 2). Blood samples from 92 patients were analyzed for level of CgA, NSE, and chromogranin B and compared with control groups. The urinary excretion of catecholamine metabolites was analyzed in samples collected at diagnosis. Clinical and laboratory data were extracted from patient records, including information on the diagnostic accuracy of ultrasound guided cutting needle biopsies (UCNB) and potential complications.

We found that NB expressed the different SSTRs and that receptor 2 was the most frequently expressed before chemotherapy. Furthermore, NB tumors showed immunoreactivity for SV2, VMAT 1 and VMAT2 alongside CgA and syn. The immunoreactivity of SV2 was comparable to CgA and superior to syn. Patients with NB had higher blood concentrations of CgA and NSE compared with controls. Patients with advanced stage disease, MYCN amplification and 1p deletion had higher concentrations of both CgA and NSE while only NSE was correlated to outcome with higher concentrations in the deceased patients.

A high urinary excretion of homovanillic acid and dopamine were correlated to inferior outcome. UCNB were found to be safe and may provide all necessary diagnostic requirements for adequate therapy stratification according to current treatment protocols.

Keywords: Neuroendocrine, Immunohistochemistry, Urinary Catecholamine Metabolites, Markers, Outcome

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To Georgina and Elena
List of papers

This thesis is based on the following papers:


III Georgantzi K, Tsolakis AV, Jakobson A, Christofferson R, Janson ET, Grimelius L. Synaptic vesicle protein 2 and vesicular monoamine transporter 1 and 2 are expressed in neuroblastoma. *Manuscript*

IV Georgantzi K, Sköldenberg E, Janson ET, Jakobson A, Christofferson R. Diagnostic Cutting Needle Biopsies in Neuroblastoma: a safe and efficient procedure. *Submitted*
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CgA</td>
<td>Chromogranin A</td>
</tr>
<tr>
<td>CgB</td>
<td>Chromogranin B</td>
</tr>
<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>FISH</td>
<td>Fluorescence In Situ Hybridization</td>
</tr>
<tr>
<td>FNAC</td>
<td>Fine Needle Aspiration Cytology</td>
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<tr>
<td>INRG</td>
<td>International Neuroblastoma Risk Group</td>
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<tr>
<td>INSS</td>
<td>International Neuroblastoma Staging System</td>
</tr>
<tr>
<td>HVA</td>
<td>Homovanillic acid</td>
</tr>
<tr>
<td>IR</td>
<td>Immunoreactivity</td>
</tr>
<tr>
<td>LD</td>
<td>Lactate Dehydrogenase</td>
</tr>
<tr>
<td>LDCV</td>
<td>Large Dense Core Vesicle</td>
</tr>
<tr>
<td>MIBG</td>
<td>Meta-Iodobenzylguanidine</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>MYCN</td>
<td>Avian MYeloCytomatosis and human Neuroblastoma derived homolog</td>
</tr>
<tr>
<td>NB</td>
<td>Neuroblastoma</td>
</tr>
<tr>
<td>NE</td>
<td>Neuroendocrine</td>
</tr>
<tr>
<td>NET</td>
<td>Neuroendocrine Tumors</td>
</tr>
<tr>
<td>NSE</td>
<td>Neuron-Specific Enolase</td>
</tr>
<tr>
<td>SS</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SSTR</td>
<td>Somatostatin Receptor</td>
</tr>
<tr>
<td>SSV</td>
<td>Small Synaptic Vesicle</td>
</tr>
<tr>
<td>SV2</td>
<td>Synaptic Vesicle protein 2</td>
</tr>
<tr>
<td>syn</td>
<td>Synaptophysin</td>
</tr>
<tr>
<td>UCNB</td>
<td>Ultrasound-guided Cutting Needle Biopsy</td>
</tr>
<tr>
<td>U</td>
<td>Urinary</td>
</tr>
<tr>
<td>VMA</td>
<td>Vanillylmandelic Acid</td>
</tr>
<tr>
<td>VMAT</td>
<td>Vesicular Monoamine Transporter</td>
</tr>
</tbody>
</table>
Introduction

Cancer is a rare condition in childhood but in Western countries it is still the second most common cause of death in children up to the age of 15 next to accidents. In Sweden approximately 300 children are diagnosed with a cancer annually (Gustafsson G et al, Report from the Swedish Childhood Cancer Registry; Cancer Incidence and Survival in Sweden, 2013). The incidence has been stable during the last 30 years but the prognosis for children with cancer has improved significantly the last decades. Today three out of four children with cancer will survive their disease (Fig 1) (Gustafsson G et al, Report from the Swedish Childhood Cancer Registry; Cancer Incidence and Survival in Sweden, 2013). However, many cancer survivors, especially those treated according to high-risk protocols, suffer from late side effects caused by the therapy. Improved survival with less treatment is therefore a primary concern in pediatric oncology.

The most common malignancies in children are acute lymphatic leukemia and brain tumors followed by neuroblastoma (NB), Wilms tumor, soft tissue and bone sarcoma.

NB is the most common extracranial solid tumor of childhood and accounts for 6% of all childhood cancers with 15-20 new cases in Sweden annually.
Fig. 1. Improvement of the estimated 5-year survival in childhood cancers in Sweden 1951-2010 (Gustafsson 2013).

Neuroblastoma
Background and Epidemiology

The histological characteristics of NB were first described by the German pathologist Rudolf Virchow in 1864 but the term “neuroblastoma” was first used in 1910 by the American pathologist James Homer Wright (1). NB is an embryonal cancer originating from the sympathetic neurons in the adrenal medulla, the sympathetic cord or paraganglia.

The annual incidence of NB is 10.5 cases per million children. NB affects children in the first years of life, 25% of these children are under 1 year of age at diagnosis and 75% are under the age of 5 (2) (3).

The incidence of NB in boys is higher than in girls, with a ratio of between 1.2-1.4:1 (2), a difference observed also in other childhood malignancies.

Staging

There have been different staging systems for NB. The first staging system was proposed by Evans et al. (4) in 1971 and allocates patients either to one
of four stages (I-IV) based on size and spread, or to a special infant stage with disseminated disease, (IVS; Table 1). Stages I-II and IVS have generally a favorable outcome while stages III and IV have a poorer prognosis.

The INSS (International Neuroblastoma Staging System) was established in 1988 and has been used in Sweden until 2010 (5) (6). The INSS system is a postsurgical staging system allocating the patients to one of six stages (1, 2A, 2B, 3, 4, or 4S; Table 2).

Due to the need of staging of the tumors preoperatively by identifying high-risk tumors, a new staging system, INRGSS (International Neuroblastoma Risk Group Staging System) was introduced in 2005 (7) (8). The stage of the disease is here based on the absence or presence of imaging-defined risk factors and/or metastatic disease at diagnosis. The patients are allocated to one of four stages L1, L2, M, or MS; Table 3). Stage L1 tumors can usually be excised while in stage L2 surgery as the primary option may be discouraged due to the risk factors present.

**Table 1.** Staging according to Evans and Children’s Study Group (Evans et al. 1971)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Tumor confined to the organ or structure of origin</td>
</tr>
<tr>
<td>II</td>
<td>Tumor extending in continuity beyond the organ or structure of origin but not crossing the midline. Regional lymph nodes on the ipsilateral side may be involved</td>
</tr>
<tr>
<td>III</td>
<td>Tumor extending in continuity beyond the midline, Regional lymph nodes may be involved bilaterally</td>
</tr>
<tr>
<td>IV</td>
<td>Remote disease involving the skeleton. organs. soft tissue and distant lymph node groups</td>
</tr>
<tr>
<td>IV-S</td>
<td>Special category. Patients who would be otherwise Stage I or II but who have remote metastases to liver, skin, or bone marrow, but who have no radiographic evidence of bone metastases on complete skeletal survey</td>
</tr>
</tbody>
</table>
Table 2. INSS (Brodeur et al. 1993)

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Localized tumor with complete gross excision, with or without microscopic residual disease, representative ipsilateral lymph nodes negative for tumor microscopically</td>
</tr>
<tr>
<td>2A</td>
<td>Localized tumor with incomplete gross resection, representative ipsilateral nonadherent lymph nodes negative for tumor microscopically</td>
</tr>
<tr>
<td>2B</td>
<td>Localized tumor with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumor. Enlarged contralateral lymph nodes must be negative microscopically</td>
</tr>
<tr>
<td>3</td>
<td>Unresectable unilateral tumor infiltration across midline, with or without regional lymph node involvement</td>
</tr>
<tr>
<td>4</td>
<td>Any primary tumor with dissemination to distant lymph nodes, bone, bone marrow, liver and/or other organs</td>
</tr>
<tr>
<td>4S</td>
<td>Localized primary tumor with dissemination limited to skin, liver, and/or bone marrow (less than 10% infiltration) limited to infants &lt; 12 months of age</td>
</tr>
</tbody>
</table>

Table 3. INRGSS (Monclair et al. 2009)

<table>
<thead>
<tr>
<th></th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>Localized tumor not involving vital structures and confined to one body compartment</td>
</tr>
<tr>
<td>L2</td>
<td>Locoregional tumor with presence of one or more image-defined risk factors</td>
</tr>
<tr>
<td>M</td>
<td>Distant metastatic disease (except stage MS)</td>
</tr>
<tr>
<td>MS</td>
<td>Metastatic disease in children younger than 18 months with metastases confined to skin, liver and/or bone marrow</td>
</tr>
</tbody>
</table>

Clinical presentation

The clinical presentation of NB varies depending on the age of the child, the localization of the primary tumor and the presence of metastases. In some cases the child may not have any symptoms at all, especially in infants where the tumor often is detected by the parents, by the physician at a rou-
tine abdominal palpation, or incidentally at ultrasound examination due to e.g. urinary tract infection, or as calcifications at plain abdominal radiograph due to abdominal pain.

In other cases, the child may have local symptoms depending on the site of the tumor, i.e. abdominal distension, respiratory tract infection or dyspnea, neurological problems and symptoms such as signs from compression of the spinal cord, or in rare cases opsoclonus myoclonus syndrome (rapid, involuntary twitching of eyes and muscles). Abdominal pain, fatigue, fever, weight loss, bone pain and diarrhea are general symptoms of the disease. In some rare cases hypertension may be present due to catecholamine secretion from the NB cells or following compression of the renal artery caused by a growing tumor. Most patients have a retroperitoneal primary tumor giving symptoms depending on the structures involved, i.e. kidney and ureter, diaphragm, or spine.

Tumors arising from the adrenal medulla or paraspinal ganglia can grow into the spinal canal through the intervertebral foraminal and compress the spinal cord causing weakness or paralysis of the legs, and dysfunction of the urinary bladder or the bowel.

Common metastatic sites are the bone marrow, bones, lymph nodes, and the liver.

**Neuroendocrine properties of Neuroblastoma**

NB tumors have some neuroendocrine (NE) properties in that they receive neuronal input and release hormones to the circulation. Examples of this is the secretion of catecholamines (i.e epinephrine, norepinephrine, and dopamine), chromogranin A (CgA) (9), and neuron-specific enolase (NSE) (10) by the NB tumor cells. NSE and CgA can be detected in the blood while catecholamines can be measured either in blood, as metanephrines, or as the metabolites homovanillic acid and (HVA) and vanillyl mandelic acid (VMA) in the urine. Catecholamine metabolites are used as NB markers. Ninety percent of all NB patients have elevated concentrations of catecholamine metabolites in the urine at diagnosis. In order to separate NB from other small-blue-round cell tumors of childhood, diagnostics of NB biopsies include immunohistochemical staining for CgA and synaptophysin (syn). (11) two general NE marker.

**Diagnostic procedures**

The diagnosis of NB is based on morphology and immunohistochemical staining of a tumor sample, taken either by a fine needle biopsy, or by a medium core (1.2 x 20mm) cutting needle biopsy, or by an open surgical biopsy. At Uppsala University Hospital almost all children with a solid tumor undergo diagnostic cutting needle biopsy, since this procedure is empirically
safe, is minimally invasive and usually provide sufficient material for histological diagnosis and for ancillary molecular profiling (12).

Meta-iodobenzylguanidine (MIBG; a synthetic catecholamine precursor, labeled with either $^{131}$I or $^{123}$I) scintigraphy is always performed for staging of the disease (13). Specific uptake of MIBG is observed in the primary tumor and metastases in 90% of patients.

Bilateral bone marrow aspirations and biopsies are also mandatory to identify or exclude bone marrow metastases.

Magnetic resonance imaging (MRI) or computed tomography (CT) investigations are also performed to identify NB image-defined risk factors.

Prognostic factors

The stage of the disease, the presence of metastases and the age of the child are known prognostic factors. In the past, the cut-off for age in having a better prognosis was at 12 months, but in the INGR staging system the cut-off was raised to 18 months since clinical data revealed that patients <18 months without cytogenetic risk factors have lower tumor stage, a more favorable histology, and a better prognosis (14).

Other important prognostic factors are cytogenetic aberrations and include the presence of 1p deletion, 17q gain, amplification of the proto-oncogene MYCN (avian MYeloCytomatosis and human Neuroblastoma derived homolog) amplification, diploidy, and an 11q aberration, all of which are associated with unfavorable outcome (15).

MYCN amplification is associated with more advanced stage and poor outcome also in low stage disease (16). About 25% of NB patients have MYCN amplification. The current staging system requires molecular profiling of the tumors for risk stratification (Table 4).

Deletion of the short arm of chromosome 1 is also correlated to advanced stage and poor outcome. Deletion of chromosome 1p is present in 30-35% of NB and is associated with MYCN amplification (17) (18).

Gain of genetic material at chromosome 17 is the most frequent cytogenetic abnormality in NB (present in 72%) and is associated with 1p deletion, MYCN amplification and advanced disease (19).

Deletion of 11q is associated to advanced disease, unfavorable histopathology, and poor outcome and is inversely related to MYCN amplification (20).

Screening for NB in infants has been undertaken in Canada, USA, Europe and Japan by assaying catecholamine metabolites in urine samples. Screening identified more infants with NB, but most of these were low stage disease with a high rate of spontaneous regression (21). Currently there is no country screening for NB.
Treatment

After taking stage, histology, imaging, and prognostic factors into consideration the therapy is individually stratified (Table 4)(22).

In some low risk tumors (age <18 months, tumor without any genetic aberrations or metastases) observation alone and regular follow-up can be sufficient. The reason for this is that some NB may undergo spontaneous maturation and regression, a phenomenon unique among human cancers. If the tumor does not regress, the child can be cured by a surgical resection, even if it is not radical.

The treatment of intermediate risk tumors may vary from limited chemotherapy and surgical resection to more intense chemotherapy.

High-risk tumors need more advanced therapy with pre-operative intensified chemotherapy, resection, high dose chemotherapy post-operatively with autologous hematopoietic stem cell transplantation (SCT) followed by retinoic acid maintenance therapy.


<table>
<thead>
<tr>
<th>INRG Stage</th>
<th>Age (months)</th>
<th>Histologic Category</th>
<th>Grade of Tumor Differentiation</th>
<th>MYCN Aberration</th>
<th>11q Aberration</th>
<th>Ploidy</th>
<th>Pretreatment Risk Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>LO/1</td>
<td>Any, except GNB maturing or GNB intermixed</td>
<td>NA</td>
<td>Amp</td>
<td>A Very low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>&lt; 18</td>
<td>Any, except GNB maturing or GNB intermixed</td>
<td>NA</td>
<td>Amp</td>
<td>No</td>
<td>B Very low</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>≥ 18</td>
<td>GNB nodular/ neuroblastoma</td>
<td>Differentiating</td>
<td>NA</td>
<td>No</td>
<td>E Low</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Poorly differentiated or undifferentiated</td>
<td>NA</td>
<td>Yes</td>
<td>H Intermediate</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>&lt; 18</td>
<td>NA</td>
<td>Hyperdiploid F</td>
<td>Low</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt; 12</td>
<td>NA</td>
<td>Diploid</td>
<td>Intermediate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12 to &lt; 18</td>
<td>NA</td>
<td>Diploid</td>
<td>J Intermediate</td>
<td></td>
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<tr>
<td></td>
<td>&lt; 18</td>
<td>Amp</td>
<td>I</td>
<td>O High</td>
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<tr>
<td></td>
<td>≥ 18</td>
<td>Amp</td>
<td>J</td>
<td>P High</td>
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</tr>
<tr>
<td>MS</td>
<td>&lt; 18</td>
<td>NA</td>
<td>Yes</td>
<td>C Very low</td>
<td></td>
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<td></td>
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</tbody>
</table>

Somatostatin receptors

Somatostatin (SS), also known as somatotropin release-inhibiting hormone is a regulatory peptide with two active forms (SS-14, SS-28), which are pro-
duced in the brain (hypothalamus) and by NE cells in the gastrointestinal tract. SS inhibits the release of growth hormone from the pituitary as well as release of several gastrointestinal hormones e.g. insulin and glucagon. SS also reduces pancreatic juice secretion (23, 24).

SS exerts its action by binding to somatostatin receptors (SSTRs). Today, five human SSTR subtypes (SSTR1-5) have been cloned and characterized. The transcript of the SSTR2 gene can be present in two splice variants that differ only in the length of the cytoplasmic portion of the receptor (SSTR2A and SSTR2B). SSTRs are widely expressed both in normal human tissues and in many different cancers (23).

Native SS-14 binds to SSTR 1–4 with higher affinity, while SS-28 is more SSTR5 selective.

The broad antisecretory properties of SS have made it an important pharmacological agent. Analogs structurally similar to SS (e.g. octreotide and lanreotide) have been developed and used clinically initially for the treatment of acromegaly and later for NE gastroenteropancreatic tumors.

Octreotide and lanreotide were the first two analogs available for clinical use. They bind preferentially to SSTR2, with moderate affinity for SSTR3 and SSTR5 compared to native SS. A recently developed SS analog pasireotide (SOM230) has a 39-, 30- and 5-fold higher binding affinity for SSTR5, SSTR1 and SSTR3, respectively, and a slightly lower affinity for SSTR2 compared with octreotide (25, 26).

**Neuron-Specific Enolase**

Enolase is a glycolytic enzyme present in many human tissues. Neuron-specific enolase (NSE) represents the enolase isoenzyme found in neuronal and NE tissue and is a well-established marker for NB and other tumors derived from the neural crest (27). NSE concentrations in the circulation in patients with neuroendocrine tumors (NETs) are correlated to tumor mass and metabolic activity. In NB, NSE is elevated in high stage disease and is a prognostic marker of poor outcome (10, 15, 28).

**Neuroendocrine markers**

There are two pathways of secretion in NE cells; the large dense core vesicle (LDCV) and the small synaptic vesicle (SSV) pathway. Neurons predominantly contain SSV, while NE cells frequently contain both LDCV and SSV. The chromogranins (Cgs) and the vesicular monoamine transporters (VMATs) are present in the LDCV, while synaptophysin (syn) and synaptic vesicle protein 2 (SV2) belong to the SSV pathway. VMATs, syn, and SV2
are used as vesicular markers, while proteins associated with subcellular structure (e.g. NSE) are used as cytosolic markers.

General neuroendocrine markers

Chromogranin A and chromogranin B

Granins constitute a family (currently eight members are identified) of single-chain glycoproteins consisting of Cgs, secretogranins (Sgs) and additional related acidic proteins. Cgs are co-stored and co-released with catecholamines from neurosecretory granules in NE cells. The Cgs consist of CgA, CgB, and peptides derived from them by proteolysis. CgA is the best-known member of the granin family and was the first granin to be sequenced.

Human CgA consists of 439 amino acids. Its biological functions have not been completely elucidated, but it acts as a precursor of many biologically active peptides generated by cleavage at specific sites (29). Because of its widespread distribution in NE cells, it can be used both as an immunohistochemical marker in tumor sections and a serum marker of NETs (30). CgA is produced and stored in the secretory granules of NE cells together with the specific peptide hormones produced by the cell and is secreted simultaneously with the hormones (29).

The human CgB molecule consists of 657 amino acids and as CgA it may go through proteolytic cleavage, which results in several smaller peptides. The CgB distribution in NETs is limited and less well investigated compared to CgA (31).

Synaptophysin

Syn is a glycoprotein and was the first integral membrane protein of synaptic vesicles to be isolated and cloned. Its distribution in NE cells is wide and it is considered a general NE marker. It is found in neurons, pancreatic islet cells, in adrenal medulla and in NETs (32).

Synaptic vesicle protein 2

SV2s are a family of three membrane proteoglycans (SV2A, B, and C), specifically found in the secretory vesicles of all neural and NE cells. They are transcribed by different genes. Concerning the neuron expression, SV2A is present in all types of neurons; SV2B and C have a more differentiated distribution. SV2A is localized in the pancreas, anterior pituitary lobe, and adrenal medulla where the relative incidence of immunoreactive (IR) cells is higher compared to syn-positive cells, and it is also used in the diagnostics of NETs (33).
Specific neuroendocrine markers

Vesicular monoamine transporters 1 and 2

VMAT 1 and 2 are a part of a larger family of transporters; toxin-extruding antiporter system (TEXANs). VMATs are acidic glycoproteins and are required for active transport of monoamines into synaptic and secretory vesicles in mammalian cells.

Both VMATs are responsible for the uptake and storage of the monoamines dopamine, norepinephrine, epinephrine, and 5-hydroxy-tryptophane (serotonin) (34, 35). VMAT1 is expressed in the enterochromaffin cells of the gastrointestinal tract and in the chromaffin cells of the adrenal medulla. VMAT2 is expressed in neurons of the central and peripheral nervous system, as well as in endocrine (e.g. chromaffin cells of the adrenal medulla, enterochromaffin-like cells and pancreatic β cells) and in non-endocrine cells (eg mast cells) (36, 37).

Urine catecholamine metabolites

The catecholamines dopamine, norepinephrine, and epinephrine are neurotransmitters formed in the nervous system and the adrenal medulla. In the adrenal medulla the catecholamines are produced from L-tyrosine and released by sympathetic stimulation. Catecholamines are stored in the synaptic vesicles and use the VMATs for their transportation (34, 35). Catecholamines are metabolized to Vanillylmandelic Acid (VMA) and Homovanillic acid (HVA) which are secreted by the kidneys (Fig. 2). VMA and HVA concentrations are measured in the urine and are used for diagnostic purposes in NB. Approximately 90% of all patients with NB have elevated catecholamine metabolites concentrations in the urine at diagnosis (6). Measurements of urine catecholamine metabolites during chemotherapy or at follow-up are used for control of response to treatment. Urine catecholamine metabolites have been measured in newborns and infants in the past for early detection of NB by screening (21). High-performance liquid chromatography (HPLC) is used for the measurement of metabolites in the urine.
Ultrasound cutting needle biopsies

The use of ultrasound-guided cutting needle biopsies (UCNB) is reported to be well-established and safe procedure that can ensure sufficient tissue material for pre-treatment histological diagnosis of solid tumors in children (12). Usually 3-5 biopsy cores are taken per tumor. Larger blood vessels and necrotic areas can be avoided with the help of ultrasound with Doppler so that biopsies are taken from representative viable areas of the tumor (Fig. 3). This is an advantage compared with open, surgical biopsies, which require laparotomy or thoracotomy and still carry a risk of not being representative. Cutting needle biopsies are small (1.2×20 mm, 18 gauge), which means that there are limitations for the number of immunohistochemical and molecular profiling analyses of tumor tissue. These analyses are important discriminating between high-risk and low-risk tumors. In patients with low-risk tumors, surgery alone can be appropriate, while high-risk patients need intensive chemotherapy, irradiation and high dose chemotherapy with stem cells rescue for best results. In Uppsala ultrasound-guided cutting needle biopsies are used pre-treatment in the diagnosis of all pediatric solid tumors since 1988.

Fig. 2. The metabolism of catecholamines.
Fig. 3. The Biopty® cutting needle. A spring ejects the needle into the tumor and the sharpened case cuts out the biopsy. The needle and the biopsy core are immediately internalized into the case.
Aims of the study

The overall aim of the study was to explore the NE phenotype of NB.

The specific aims of each paper were:

Paper I
To investigate the expression of SSTRs by receptor-specific immunohistochemistry on sections from experimental and clinical NB in order to explore the feasibility of future SS-analog treatment in therapy-resistant NB.

Paper II
To compare serum and plasma explore concentrations of CgA, CgB, and NSE in serum and plasma from patients with NB and to compare them with those of healthy children and patients with non-NB tumors or other malignancies, and to correlate the concentrations to NB stage, tumor size, other prognostic factors, and outcome.

Paper III
To explore if other NE markers such as SV2, VMAT1 and 2 are expressed in human NB and, if so, to compare the usefulness of these markers in the diagnostics of NB. Furthermore, to investigate if there is any correlation between elevated u-catecholamine metabolites and outcome.

Paper IV
To investigate if pre-treatment ultrasound-guided cutting needle biopsies are safe and give adequate material for both immunohistochemical studies and molecular profiling in the diagnosis and staging of NB.
Materials and methods

Patients

Paper I

Formalin-fixed paraffin-embedded, pre- and post-treatment NB tumor samples from 11 children with INSS stages 2-4 were retrieved from the Dept. of Pathology at Uppsala University Hospital. The tissue taken before therapy was by an 18-gauge (1.2 mm) ultrasound-guided cutting needle biopsy cores (except in one patient where the whole tumor was removed before chemotherapy). The tissue taken after chemotherapy was obtained from the resected tumor specimen.

Paper II

Blood samples from 104 patients with NB (n=92) or benign ganglioneuroma (GN) (n=12) were analyzed and clinical data extracted from their hospital records (Table 5). Blood samples were also collected from 69 healthy control children. The control group consisted of healthy neonates after birth (n=10), healthy neonates that underwent metabolic screening the first week of life (n=32), and infants and children undergoing anesthesia due to non-systemic disease (e.g. inguinal hernia repair, extraction of transfixation pins) (n=27). Furthermore, 31 patients without NB or GN, but suffering from other solid tumors or leukemia were included in a separate control group.

Table 5. Clinical data on neuroblastoma and ganglioneuroma patients in paper II

<table>
<thead>
<tr>
<th></th>
<th>Neuroblastoma (NB)</th>
<th>Ganglioneuroma (GN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (n)</td>
<td>92</td>
<td>12</td>
</tr>
<tr>
<td>Age (y) range</td>
<td>0-18</td>
<td>2.6-11</td>
</tr>
<tr>
<td>Age median</td>
<td>1.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Male/Female</td>
<td>48/44</td>
<td>10/2</td>
</tr>
<tr>
<td>Stage (NB) 1/2/3/4/4S</td>
<td>12/18/15/41/6</td>
<td>-</td>
</tr>
<tr>
<td>MYCN present/absent/N.D.</td>
<td>24/59/9</td>
<td>0/9/3</td>
</tr>
<tr>
<td>1p LOH present/absent/N.D.</td>
<td>19/43/30</td>
<td>0/5/7</td>
</tr>
<tr>
<td>Aneuploidy: yes/no/N.D.</td>
<td>30/26/36</td>
<td>-</td>
</tr>
<tr>
<td>Follow-up (y): mean (range)</td>
<td>20 (3-27)</td>
<td>13.4 (10-24)</td>
</tr>
<tr>
<td>Deaths</td>
<td>40 (43%)</td>
<td>0</td>
</tr>
</tbody>
</table>

y: years, N.D.: not determined
Paper III
Thirty-four formalin-fixed paraffin-embedded tumor samples before and/or after treatment from 21 patients with NB were included. The tumor was MYCN amplified in six patients. The specimens were either from a UCNB at diagnosis or obtained at surgery before or after chemotherapy and were immunostained for NE markers. In 18 of those patients the urinary concentration of HVA, VMA, and dopamine were analyzed by HPLC at diagnosis, and related to the concentration of creatinine in the same sample.

Paper IV
The medical records of 29 patients with NB that underwent pre-treatment, diagnostic, ultrasound guided needle biopsy at the Uppsala University Hospital were reviewed. The information extracted from the patient records included: age at diagnosis, gender, tumor site, INSS stage, cytogenetic analyses (MYCN status, aberrations at 1p, 11q, or 17q) and any biopsy complication (bleeding, pain, and any anesthesia-related complications).

Experimental tumors (paper I)
Tumors derived from five human NB cell lines xenotransplanted to nude mice (NMRI nu-nu) were kindly donated by Ulrika Bäckman, Dept. of Medical Cell Biology at Uppsala University. The cell lines used were; SH-SY5Y, SK-N-DZ, SK-N-AS, IMR-32, and KELLY (ATCC, Rockville, MD) (38)

Methods
Tissue samples
All the tissue samples in paper I and paper III were fixed in 10% buffered neutral formalin and then processed to paraffin wax. Consecutive 4-micrometer thick sections were cut and attached to positively charged glass slides. The sections were then dewaxed in decreasing concentrations of alcohol and rehydrated in distilled water.

In paper II peripheral venous blood samples were collected either using sterile Vacutainer® 2 or 5 mL serum tubes without additives (BD, Franklin Lakes, NJ). Alternatively 2 or 5 mL sodium-heparin tubes (BD) were used. Samples from other hospitals were transported on dry ice. The samples were transferred to the Dept. of Clinical Chemistry at Uppsala University Hospital and spun at 1,300 g for 10 min at 4°C. The supernatant was divided into aliquots. The samples were stored at -70°C until further processing.
Primary antibodies for immunohistochemistry

The primary antibodies used in **paper I** were in–house produced polyclonal rabbit antibodies against SSTR 1-5, and CgA (33, 39), while SS antibodies were commercially available (A0566; DakoCytomation, Glostrup, Denmark).

The primary antibodies used in **paper III** were: mouse monoclonal vs. CgA (LK2H10, 1199021, Boehringer, Mannheim, Germany, dilution 1:16000) and vs. SV2 (NCL-SV2, 15E11, NovoCastra, Newcastle upon Tyne, UK, 1:50); rabbit polyclonal vs. synaptophysin (A0010, DakoCytomation, Glostrup, Denmark, 1:400) and VMAT2 (AB1767, Chemicon International, Temecula, CA, 1:2400); and finally, goat polyclonal vs. VMAT1 (C-19, sc-7718, Santa Cruz Biotechnology®, Santa Cruz, CA, 1:4000).

**Routine immunostaining in paper I and III**

The sections were heated for 2 x 5 min at 750 W in a microwave oven in their retrieval solution; Tris-buffered saline at pH 8.0 for SSTR, and citrate buffer at pH 6.0 for CgA. Endogenous peroxidase activity was quenched by incubating the sections for 20 min with 2% hydrogen peroxide in phosphate-buffered saline (PBS), pH 7.4. After washing with PBS all sections were incubated at room temperature for 30 minutes with serum from the same species the secondary antibody was raised in before applying the primary antibody. The serum, diluted 1:5 with PBS, was either normal horse serum (S-2000, Vector) or normal goat serum (S-1000, Vector). The sections stained for SSTR were then incubated overnight at +4°C, while the other antibodies were incubated at room temperature, with the different primary antibodies diluted in PBS with 1% BSA.

After washing, a second incubation with the secondary antibodies in PBS, was carried out for 30 min followed by further washing with PBS. Finally, the sections were incubated for 30 min with an avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector) according to the manufacturer’s instructions. Diaminobenzidine was used as final chromogen (5 min incubation; Fig. 4). All the incubations were carried out in a humidified chamber at room temperature unless otherwise stated.
Fig. 4. Immunohistochemistry using Avidin Biotin complex (ABC kit). Peroxidase oxidizes DAB substrate to a brown pigment that precipitates at the site of the antigen.

Analysis of immunoreactive cells
All the slides were examined in a Zeiss Axioscope 40 light microscope (Carl Zeiss Microimaging GmbH, Jena, Germany) at x25 to x400. Both the experimental and the clinical tumors were evaluated by two independent observers.

In paper I at least 50% of 1,000 NB cells had to show immunoreactivity for the investigated antigen to be considered as positive. In biopsy material all NB cells were counted.

In paper III we used a semiquantitative method for all the NE markers.

Immunohistochemical controls
The negative controls of the immunostaining included omission of the primary antibody or replacement of the primary antibody with non-immune serum. Sections of normal human pancreas were used as positive controls for the primary antibodies in paper I. Sections from macro- and microscopically normal human gastric corpus and antral mucosa from a stomach resected due to adenocarcinoma were used as positive controls in paper III.
Sample analysis (paper II)

The blood samples were analyzed for CgA and CgB as described previously and the results were expressed in nmol/L (39, 40). NSE was analyzed using a commercial kit (Delfia NSE, Wallac Oy, Turku, Finland) according to the manufacturer’s instructions and the results were expressed in µg/L. Since the sample volume in some cases was small, a priority was given to primarily CgA, then CgB, and finally NSE. Thus, in some cases, only one or two of the three variables were analyzed. Only six NSE concentrations were hence available from the group of 32 neonates seen at a routine post-partum health revisit.

Statistics

McNemar’s test for paired nominal data was used to compare the expression of SSTRs pre- and after-treatment in paper I. The software used was SAS (SAS institute, Cary, NC) version 9.1.

In paper II comparisons between groups were made using the Mann-Whitney U test and Spearman’s rank correlation was used to assess the correlations between variables. Cox proportional hazards regression was used to associate the prognostic variables to the outcome. To avoid losing data due to missing analyses in the multivariable model, we performed multiple imputation of the baseline variables. We created 20 imputed data sets and performed the regression analysis in each imputed data set. The results were then combined using Rubin’s rules.

The number of cases per variable in the multivariable model was low. Confounder adjustment was done using ridge regression to reduce the effective degrees of freedom (41). The amount of shrinkage was determined by maximizing a corrected Akaike Information Criterion (AIC).

In paper III we tested the correlation of the different catecholamine metabolites in urine to outcome using a permutation test. This very general test correlates the ranked u-catecholamine values to survival. It provides optimal adjustment for multiple testing by considering the correlations between the test statistics. The null distribution for this multivariate test statistic was obtained through by randomly permuting the response variable, i.e. the survival time and status, 100,000 times while keeping the u-catecholamine values fixed thereby breaking any associations that may exist.

For paper II and III data was analyzed in IBM SPSS Statistics 20 (Armonk, NY), and R version 3.2.4 (Vienna, Austria).
Ethics

All four studies were approved by Regional Ethical Review Board in Uppsala, Sweden.
Results

Immunohistochemical analyses (papers I and III)
Somatostatin receptors, somatostatin and chromogranin A

Clinical tumors
All tumors showed specific immunoreactivity (IR) for CgA while none of the tumors expressed somatostatin (SS). The most frequently expressed SSTR was SSTR2 while SSTR4 was the least frequently expressed SSTR pre-treatment. The pattern of SSTR expression did not change significantly post-treatment. The SSTR IR was localized both to the cell membrane and in the cytoplasm.

Experimental tumors
All tumors derived from KELLY, SK-N-DZ, and SK-N-AS were immunoreactive for CgA. Some SH-SY5Y and IMR-32 tumors were negative for CgA in our hands. None of the experimental tumors expressed SS. All the experimental NBs expressed at least one of the SSTRs but the expression was patchy in individual experimental tumors. All tumors were immunoreactive for SSTR4 while SSTR1 was expressed less consistently. SK-N-DZ tumors expressed all SSTR subtypes except SSTR1 and exhibited the least heterogeneous IR for these receptors compared with the other experimental NBs. When comparing different experimental tumors derived from SH-SY5Y cells a more variable pattern of SSTR subtype expression was detected.

General neuroendocrine markers
Thirteen samples before chemotherapy and five samples after chemotherapy were stained for all the general NE markers (CgA, SV2 and syn). One patient had samples both before and after chemotherapy.

In samples before chemotherapy the frequency of tumor cells IR for CgA and SV2 was higher than 50% in all tumor samples while only eight tumors expressed syn in a majority of tumor cells. Nine tumor samples showed CgA and SV2 IR in more than 90% of the cells.

In samples obtained after chemotherapy, CgA was expressed in more than 50% of tumor cells in all the samples while SV2 was expressed in four and
syn in three out of five. Both CgA and SV2 showed more than 90% IR in four out of five samples after chemotherapy while syn in only two.

**Vesicular monoamine transporters VMAT1 and VMAT2**

Fourteen samples before and seven samples after chemotherapy were immunostained for VMAT1 and 2. In the samples before chemotherapy VMAT1 was expressed in the most tumor cells in five out of 14 cases while VMAT2 was expressed in 12 samples.

VMAT1 was expressed in most of tumor cells in two and VMAT2 in three out of five samples after chemotherapy. In summary, VMAT2 was more frequently expressed compared to VMAT1 in samples both before and after chemotherapy.

**Comparison between all five neuroendocrine markers**

In a total of 15 cases, 12 before and four after chemotherapy the samples were immunostained for all the NE markers, both general and VMATs. In one case immunostained sections were available both before and after chemotherapy. The two markers most frequently expressed were CgA and SV2. Syn and VMAT2 showed a similar staining frequency but inferior to that of CgA and SV2, while VMAT1 was least frequently expressed of all the markers.

**Circulating biochemical markers (paper II)**

CgA and NSE concentrations in blood were higher in patients with NB compared with controls. Concentrations were higher in patients with high stage disease (stages 3 and 4) compared to them with low stage disease (stages 1 and 2), and higher in patients with loss of heterozygosity of the chromosome 1 and with MYCN amplification. NSE concentrations correlated to death of disease and had a linear correlation to the maximal diameter of the primary tumor. Neither CgA nor CgB had such a correlation. Concentrations of CgB were not different in low vs. high stage disease.

Deceased patients had larger tumors at diagnosis compared with the survivors. Higher stage and metastases were correlated to increased risk of death of disease.
Urine catecholamine metabolites (paper III)

The u-catecholamine metabolite concentrations were elevated in 13 of the 18 patients as a sign of NE activity. Ten patients had increased concentrations of u-HVA/creatinine, eight u-VMA/creatinine, and eight of u-dopamine/creatinine at the time of diagnosis. Three patients had increased concentrations of both u-HVA/creatinine and u-dopamine/creatinine, two of both u-HVA/creatinine and u-VMA/creatinine, and four patients had elevated concentrations of all three u-metabolites. Five out of six patients with MYCN amplification had elevated u-catecholamine metabolites. All patients with MYCN amplification are deceased. The u-catecholamine metabolite concentrations correlated to survival were u-HVA (p=0.009) and u-dopamine (p <0.001). U-VMA did not correlate to outcome.

Ultrasound-guided cutting needle biopsies (paper IV)

The included 29 patients with NB underwent in total 34 procedures. Between two and six cores were collected at each procedure. The diagnostic sensitivity of UCNB in NB in our material was 90% (26/29). In three patients more than one UCNB was performed for varying reasons. One patient was initially misdiagnosed as a Wilms’ tumor and in two other patients the first UCNB was inconclusive. In 25 out of the 29 patients (86%) the biopsies were sent for molecular profiling. In all patients after 2008, a full molecular status could be performed on the biopsy material with the Single Nucleotide Polymorphism (SNP) array. Three of the four patients lacking molecular profiling had stage IV disease with bone and/or bone marrow metastases and any result of a molecular profiling would not have influenced the treatment strategy.

Two infants had a clinically significant bleeding in the tumor following UCNB. Both required transfusion of erythrocyte concentrate and for one of them also emergent surgical intervention (thoracotomy) due to bleeding and compromised airway. Two patients required analgesics due to pain after UCNB. Both patients were off analgesics 24 h after UCNB. No other complications were recorded during the first 24 h. There was no macroscopic tumor growth in the biopsy tract at surgery, relapse, or during follow-up (median follow-up: 11.2 years). No infections related to the UCNB were recorded.
Discussion

NB survival has increased, but the increase in survival has tapered off. There is still a clinical need for new treatment options to reduce late-effects and to improve survival in patients with a poor prognosis. This thesis aims at adding knowledge of diagnostics and NE properties of NB, and intends to facilitate development of new therapies.

In paper I we examined the expression of the five SSTR subtypes in clinical and experimental NB.

We showed that clinical and experimental NBs do not produce SS but do express SSTRs. SS immunoreactivity has previously been demonstrated in benign GN, and in a subset of NB with advanced disease but with a favorable prognosis (42). However, none of the tumors examined in this study expressed SS. The absence of detectable SS in high-risk tumors in the present study indicates that there is no autocrine loop for SS in NB.

We used polyclonal antibodies specific for each receptor to immunostain both experimental and clinical NBs for the first time. We found that certain cell lines express all SSTR subtypes uniformly while others show a more complex pattern. In the clinical tumors, we could not find a difference in SSTR expression before or after chemotherapy. SSTR expression has previously been detected in both cell lines and human NBs (43-45). SSTR1 and SSTR2 were studied by immunohistochemistry (46) in NB tumors at diagnosis and at relapse and all tumors expressed SSTR1 and 84% expressed SSTR2. These results were similar to ours.

We also studied the other three SSTRs and could show that both SSTR3 and 5 are frequently expressed. This might be of importance for future treatment studies. In two previous studies, the expression of mRNA for SSTR2 was identified as an independent favorable prognostic factor in NBs, and was negatively correlated to amplification of MYCN and to metastatic dissemination (47, 48).

It has previously been reported (49) that the absence of receptor expression at SSTR scintigraphy is correlated to more advanced stages and unfavorable prognosis. Expression of receptor mRNA and receptor protein does, however, not always correlate. The small number of patients in the present study does not allow conclusions about the prognostic value of the different
SSTRs, but the presence of a differentiated SSTR expression in NB merits further investigation. We believe that the frequent expression of the SSTRs indicates that treatment with SS analogs is feasible in high-risk NB (50).

The SSTR expression was patchy in individual experimental tumors despite their monoclonal origin. This finding suggests interactions between tumor cells, the tumor stroma and its blood vessels. The least heterogeneous immunoreactivity for SSTR was in SK-N-DZ, which lacks MYCN amplification and 1p deletion. We speculate that the more unfavorable characteristics the tumor has, the more heterogeneous is the expression of SSTRs. The IR of SSTRs in individual cells was mainly cytoplasmic, suggesting a high turnover of these surface receptors (51).

SSTR scintigraphy is routinely used for diagnosis and staging of small intestinal and pancreatic NETs in adults, while MIBG scintigraphy is traditionally used in NB (13, 52). Similarly, the presence of SSTRs in NB suggests that SSTR scintigraphy may be useful to evaluate SSTR expression also in NB, especially when MIBG scintigraphy is negative. It has also been shown that there is a strong correlation between SSTR IR and uptake of the radioligand at SSTR scintigraphy (53). A specific uptake at SSTR scintigraphy would thus indicate that treatment with an SS analog is feasible (52).

SS analogs are used in the treatment of adult NETs with good biochemical and clinical response (54). Recently, it has also been shown that the SS analog octreotide LAR has an antiproliferative effect in small intestinal neuroendocrine tumors, significantly prolonging the time to progression significantly (55). Another therapeutic alternative might be the use of radiolabeled SS analogs for tumor targeting. Treatment with $^{177}$Lu-octreotate induces objective responses in about 30% of adult patients with NET (25). Such treatment could be considered in patients with refractory or relapsed NB. The currently available SS analogs, octreotide and lanreotide, have a high binding affinity to SSTR2 and an intermediate affinity to SSTR5 and SSTR3. All these receptors are expressed in NBs at a variable frequency. A recently approved SS analog in clinical trials is SOM-230, with a broader receptor binding profile, binding with high affinity to SSTR1, 2, 3, and 5, indicating a better antiproliferative effect than the currently used SS analogs (26). Although significant progress has been made in improving the outcome of patients with NB, there is still a need for more effective and less toxic therapies. Our study shows that NB expresses SSTR1–5.

We conclude that NB express SSTRs, and hence that treatment with somatostatin analogs or radiolabeled somatostatin is feasible. We suggest that they should be tested clinically in patients with NB when current treatment modalities have failed.
In paper II we explored if CgA, CgB and NSE are reliable circulating tumor markers in NB. CgA is a well-established tumor marker in NETs where it can successfully identify NETs and their metastases regardless of origin (56, 57). CgA is expressed in both functioning and non-functioning NETs and has a diagnostic sensitivity and a specificity of 70-95% and 70-80%, respectively (29). Hsaio et al found that circulating CgA had 100% specificity in discriminating 34 NB from 15 non-NB patients. In our study two of the non-NB patients with elevated CgA had renal impairment (one kidney clear cell sarcoma and one acute lymphatic leukemia with bilateral renal involvement. Both patients had S-creatinine above 115 µmol/L), a condition known to give a false-positive increase in CgA (58).

NSE is a reliable circulating marker in both NETs and NB. High concentrations are correlated to large tumor burden and to poor outcome in both NETs and NB (59, 60).

We demonstrated that patients with NB had significantly higher concentrations of CgA in their blood compared to their controls as well as to patients with other malignancies, and to patients with GN. We also showed that elevated concentrations were correlated to higher stages and the presence of negative prognostic markers, such as MYCN amplification and 1p deletion. High NSE concentrations were correlated to tumor size and to survival. We also noticed that patients with large tumors at diagnosis had a worse outcome, which indicates that the maximal tumor diameter at diagnosis could be used as a clinical tool.

It has been shown that the serum concentration of CgB is increased in patients with pheochromocytoma (31). We could not find increased concentrations of CgB in our patients, which indicate that NB cells, although secreting catecholamines, are biologically different from chromaffin cells of the adrenal medulla.

We conclude that NSE is a clinically valuable tumor marker in NB, and our data suggest that CgA may be a valuable marker too and hence merits prospective clinical studies.

In paper III we investigated the expression of different NE markers in NB tumors, and compared their expression with the already established and used markers in clinical diagnostics i.e. CgA and syn. Furthermore, we investigated if there was a correlation between elevated u-catecholamine metabolites (as a sign of NE activity) and outcome.

Tumor samples from the patients included in this study were immunostained for CgA, syn, SV2, VMAT1 and VMAT2. Also the u-catecholamine metabolites HVA, VMA and dopamine were quantified and correlated to outcome.

In our study SV2 showed an IR like CgA and both were superior to syn in identifying NB tumor cells. Previously, syn was suggested to be a better NE marker than CgA (61). The difference between our and their study could
depend on tissue processing and choice of antibodies. They used frozen sections and a monoclonal antibody while we used formalin-fixed, deparaffinized sections and a polyclonal antibody.

SV2 has been reported to be comparable to CgA in identifying most NE cell types and NE tumors, and is thus an important NE marker, (62, 63). There are even NETs (e.g. rectal NETs, L-cell type) where SV2 is more reliable diagnostically than both CgA and syn.

Chemotherapy did not influence the marker IR in an obvious way, possibly with the exception of VMAT1. This marker showed a larger proportion of immunoreactive cells before than after chemotherapy. The cases were, however, too few to make any certain conclusions.

A previous study of children with high risk, metastatic NB, explored the expression of VMAT1 and VMAT2 in tumor samples and showed that the number of tumor samples expressing VMAT2 exceeded that of VMAT1 (75% vs. 62%) (64). Our result was similar to theirs, with 84% vs. 57%, respectively. In our study we included patients of all stages with or without metastases but our cohort was more limited.

Thirteen patients had elevated levels of u-catecholamine metabolites at the time of diagnosis. It is well known that NB tumor cells do produce and secrete catecholamines to the circulatory system, and their metabolites are excreted in the urine. Elevated concentrations of u-catecholamine metabolites at diagnosis are seen in approximately 90% of NB patients (6). The relevance of u-elevated catecholamine metabolites for survival was investigated in a study including 114 patients. The u-catecholamine metabolite concentrations were correlated to survival and to certain prognostic markers such as MYCN amplification and 1p deletion (65). Patients with high levels of u-HVA had a significantly worse outcome in their study while elevated u-VMA and u-dopamine concentrations did not affect the outcome. In our material deceased patients had significantly elevated u-HVA and u-dopamine concentrations. Due to the small size of our cohort, no correlation to stage or other prognostic markers, such as MYCN amplification, could be tested.

In our study SV2 was a useful general NE marker for NB, similar to CgA, while syn was somewhat inferior. VMAT1 and VMAT2 were also expressed in several NB and VMAT2 expression exceeded that of VMAT1. VMAT2 immunoreactivity was similar to syn as a NE marker. Chemotherapy did not influence the IR in an obvious way. Elevated u-HVA and u-dopamine concentrations were correlated to poor outcome. Our material and cohort was limited. Therefore, large, multicenter and prospective studies are needed to confirm our results.
In paper IV we tested if UCNB is a safe way to obtain tumor tissue for the diagnosis of the NB and if the tissue obtained is appropriate for molecular profiling.

Pre-treatment biopsies can be obtained by UCNB, open - or laparoscopic - surgical biopsy, or FNAC. The traditional sampling in NB has been the surgical biopsy but there is an increasing interest in and support for core needle biopsies for the diagnosis of NB in children (12, 66). The use of ultrasound-guided cutting needle biopsies appears to be well established and safe procedure that can ensure enough tissue samples for histological diagnosis of solid tumors in children (12, 67). Larger blood vessels and necrotic areas can be avoided with the help of ultrasound and biopsies are taken from representative viable areas of the tumor. Usually 3-5 cores are taken at UCNB. Cutting needle biopsies are quite small (1,2x20mm) which may limit immunohistochemical and molecular biological analyses of the tumor tissue prior to chemotherapy. These analyses are very important for the characterization of the tumor and for discriminating high-risk from low-risk tumors, and are mandatory in the new NB protocol. In Uppsala ultrasound-guided cutting needle biopsies are used in the diagnosis of pediatric solid tumors since 1988 (12, 68).

Surgical biopsy was reported (67) to have a diagnostic sensitivity of 95% when the biopsy was representative. On the other hand FNAC had a diagnostic sensitivity of 92% in NB in a study of Gupta et al. (69). Studies that compared the sensitivity of different sampling techniques in NB were not able to find any significant differences (67, 69-72).

In both FNAC and cutting needle biopsies the radiologist/surgeon uses ultrasound guidance to identify the tissue, large vessels and necrotic areas. The surgical specimen is taken from the area that the surgeon considers appropriate. Both UCNB and surgical biopsy offer more material for the immunohistopathological and morphological diagnosis that FNAC which only yields cells. Due to that fact, UCNB or surgical biopsy can usually give enough material even for the molecular characterization of the tumor and for biobanking.

In our study the diagnostic sensitivity of UCNB was 90%, similar to other studies on UCNB in NB and our earlier reports. (66, 73).

The molecular profile of NB with UCNB in our study was possible in 86% (25/29 patients) which is in accordance with Hassan et al (66), while Gupta et al (69) found that 63% of UCNB was insufficient for complete histological and molecular profiling. Even FNAC has been used to estimate the molecular status of NB using fluorescent in situ hybridization (FISH) and image cytometry (74) but nowadays when SNP arrays and whole genome sequencing (WGS) are used, FNAC is less suitable (75). The lack of material for full molecular profiling limits biobanking of material for future studies and therefore makes FNAC a less attractive as a diagnostic option in NB.
In our study four complications (14%) occurred in three patients (10%). All three patients were infants. Two patients had significant bleeding and both needed erythrocyte transfusion. One of those was subjected to emergency thoracotomy due to breathing difficulties after the tumor bleeding. This patient and another one were in need of analgesics after UCNB. The pain in the first case could be due to bleeding and in the other case due to the bone marrow and skeletal metastases and not from postprocedural pain. Especially in infants it is difficult to distinguish between pain and other discomfort. The risk of major complications to UCNB is reported to be low in children (76). There is a potential risk of complications such as bleeding, wound and incisional infections, postsurgical bowel paralysis, and pain in surgical biopsies bleeding (70). FNAC is a safe method for sample taking in children with NB (75). All three methods for obtaining a tumor sample in children with NB have a low risk for complications (77).

In conclusion, UCNB is a minimally invasive and safe method that usually yields adequate tissue for morphological, immunohistochemical, molecular and genetic studies for the diagnosis and characterization of NB. Therefore, UCNB could be considered as the golden standard for diagnostic tumor sampling of NB.
Sa

manfattning på svenska

Bakgrund
Neuroblastom (NB) är en malign embryonal tumör som utgår från celler i det sympatiska nervsystemet. Tumören är den vanligaste extrakraniella solida maligna tumören hos barn och har en extremt varierande biologi. Tre fjärdedelar av alla NB diagnostiseras före 4 års ålder och hälften före 2 års ålder. Incidensen av NB i Sverige är cirka 15 nya fall per år. Prognosen är relaterad till kliniskt stadium, histologisk bild samt olika molekylärbiologiska markörer, såsom förekomst av amplifiering av onkogenen MYCN, förekomst av deletioner i tumörsuppressorgenerna på kromosomerna 1p och 17q etc. Trots intensifierad behandling med cytotatika och kirurgi har prognosen inte förbättrats för de med avancerad sjukdom. Den totala femårsöverlevnaden vid NB i Europa ligger på ca 45 %. NB har en hel del biokemiska likheter med neuroendokrina tumörer (NET) hos vuxna, som utgår från endokrina celler.

I min avhandling har jag fokuserat på de neuroendokrina egenskaperna av NB och dess betydelse i diagnostik och uppföljning. Ett delmål var att undersöka om ultraljudsvägledda mellannålbiopsier vid NB var en säker metod för diagnostik samt för att utföra molekylära studier.

Delarbete I
hos 74%, SSTR3 hos 68%, medan SSTR4 bara uttrycktes hos 21% av vävnadproverna från patienter. Även experimentella NB uttryckte SSTR1-5, men i varierande frekvens. Studien bekräftar att NB har neuroendokrina egenskaper, och talar för att somatostatinanaloger är en tänkbar ny terapeutisk strategi vid NB.

**Delarbete II**

Kromograninfamiljen består av chromogranin A (CgA), chromogranin B (CgB) och relaterade peptider. Kromograniner lagras i och frisätts från neurosekretoriska vesiklar. CgA används som markör för NET i vävnadssnitt, men också som en biokemisk markör för monitorering av patienter med NET. Även icke-NET med neuroendokrin differentiering (prostatacancer etc.) kan uttrycka CgA. CgB används främst som en biokemisk markör för patienter med feokromocytom. Intresset för CgB har ökat efter rapporten om att CgB till skillnad från CgA inte är förhöjd hos patienter med njursvikt, kronisk atrofisk gastrit eller vid syrahämmande medicinering. Neuron specific enolase (NSE) är ett enzym som finns i nerv- och neuroendokrinavvävnad och en välattablerad markör för NET. NB har visat sig uttrycka båda CgA och NSE, och patienter med NB har ofta förhöjda koncentrationer av kromograniner samt NSE i blodet.

Serumnivåer av CgA, CgB och NSE analyserades hos 92 patienter med NB och hos 12 patienter med GN samt hos en kontrollgrupp som bestod av 31 barn med andra maligniteter (tex leukemier) och 69 friska individer.

Nivåer av CgA, CgB och NSE i NB gruppen korrelerades till stadium, outcome, tumörstorlek samt kända prognostiska faktorer som MYCN amplifiering, 1p deletion etc.

Vi visade att CgA och NSE var förhöjda i patienter med NB samt att CgA och NSE var kopplade till kliniskt stadium och de kända prognostiska faktorerna men att bara NSE var kopplad till ökad risk för död och till tumörstorlek. Ingen korrelation till varken stadium, tumörstorlek, prognostiska markörer och överlevnad kunde ses för CgB. Därmed anses NSE var en bättre markör än CgA i vårt material men större studier behövs för att fastställa detta.

**Delarbete III**

CgA, synaptofysin (syn), synaptic vesicle protein 2 (SV2) är generella NE markörer och tillsammans med vesicular monoamine transporter 1 (VMAT1) och 2 (VMAT2) som är mer specifika NE markörer används vid diagnostik av NET. CgA och syn ingår även i panelen vid rutinfärtnings av NB.

Vi ville undersöka förekomst av de övriga neuroendokrina (NE) markörerna i NB tumörer och om någon annan markör förutom de som redan an-
vänds idag skulle kunna vara användbar i diagnostiken. Dessutom ville vi undersöka om nivåer av katekolaminmetaboliter i urin är kopplade till överlevnad.

Tumörmaterial på 21 patienter i olika stadier före och/eller cytostatikabehandling färgades för de generella NE markörerna CgA, syn, synaptic vesicle protein 2 (SV2), samt för de mer specifika markörerna VMAT 1 och 2. I vårt materiel visade sig SV2 vara en lika bra markör för NB som CgA och något bättre jämfört med syn i tumörer både före och efter behandlingen. VMAT1 och VMAT2 uttrycktes också i våra tumörer. VMAT2 uttrycktes i en högre frekvens jämfört med VMAT1. Vid en jämförelse före och efter behandlingen såg vi ingen skillnad i uttryck av markörerna.

De urin- katekolaminmetaboliter som är av intresse vid NB är homovanillimsyra (HVA), vanillylmandelsyra (VMA) och dopamin. Vi korrelerade dessa till överlevnad. Vi fann att de avlidna hade högre nivåer av HVA och dopamin i urin vid diagnos. I vårt material fann vi ingen sådan koppling för VMA.

Delarbete IV


Vi identifierade 29 patienter med NB som hade genomgått en mellannålsbiopsi, och retrospektivt extrahirade vi information om komplikationer, diagnostisk säkerhet och i hur många fall molekylärbiologiska analyser (ploïditet, amplifiering av onkogenen MYCN och påvisade av deletioner i tumorsuppressorgenerna på kromosomerna 1p och 17q) kunnat göras respektive misslyckats.

I vårt material var mellannålsbiopsierna diagnostiska i 90% av fallen och biopsimaterialet var tillräckligt för att tillåta komplet molekylär diagnostik i alla fall efter 2008. Komplikationer bestod av blödning i 2 fall (7%) och smärta efter ingreppet i 2 fall (7%). Inga andra större komplikationer och inga anestesikomplikationer fanns i vårt material. Ingen tumörspridning i biopsikanalen.
Mellannålsbiopsier är en säker och tillförlitlig metod för att erhålla material till diagnos men även till molekylärdiagnostik vid misstanke om NB.
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References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)