NEUROBLASTOMA INCIDENCE, BIOLOGY AND OUTCOME
With special emphasis on quantitative analysis of tyrosine hydroxylase mRNA in blood and bone marrow

Catarina Träger

Figure 1

Figure 2
NEUROBLASTOMA INCIDENCE, BIOLOGY AND OUTCOME;
With special emphasis on quantitative analysis of tyrosine hydroxylase mRNA in blood and bone marrow

Catarina Träger

Stockholm 2009
To Andrea, Catinka and Natasha.
ABSTRACT

Neuroblastoma, the most common malignant disease of early childhood, accounts for 6% of childhood malignancies but is responsible for 9% of paediatric cancer related deaths. The tumour originates from cells in the neural crest and has a very heterogeneous clinical course. A minority of tumours may differentiate or regress spontaneously whereas the metastatic form is difficult to cure which is related to difficulties in treating minimal residual disease (MRD).

This study aimed to characterise neuroblastoma in Sweden during 27 years as well as developing methods for detecting MRD and evaluate the clinical significance of detecting MRD in blood and bone marrow in children with neuroblastoma.

By now 359 children with neuroblastoma below the age of 15 years have been included in the Swedish Childhood Cancer Registry. The yearly age standardised incidence in Sweden was 1/100 000 children <15 years with no trend towards an increase or decrease. The median age at diagnosis was 18 months. The gender ratio was significant different with 1.16:1 male to female. The outcome for boys was slightly worse than for girls mainly due to more frequent metastatic disease, 1.41:1. Survival for all children with neuroblastoma improved from 57.8% (overall survival at 5 years for children diagnosed 1982-1990) to 74.1% (2000-2008). Survival for high-risk neuroblastoma increased significantly (p<0.001) from 8.6% (1982-1990, n=35) to 17.0% (1991-1999, n=53) and 54.5% (2000-2008, n=50).

In order to detect MRD in blood (PB) and bone marrow (BM) from children with neuroblastoma, we developed a quantitative real time RT-PCR (qRT-PCR) method with tyrosine hydroxylase (TH) mRNA as a target. PB and BM samples from 24 children with neuroblastoma of all different biological subsets and stages were analysed. Tyrosine hydroxylase mRNA analysis could significantly distinguish localised disease from metastatic disease and detect relapse before conventional methods.

We used this sensitive method for detection of TH in pigment cells to understand whether this enzyme can take an active part in pigment formation or not. Our results indicated that there is no TH that can contribute to pigment formation in melanocytes or melanoma cells.

In our fourth study we used qRT-PCR to compare the specificity, sensitivity and clinical usefulness of TH, dopa decarboxylase (DDC) and GD2 synthase (GD2S) mRNA for detection of minimal disease in PB and BM in neuroblastoma patients. In total 554 samples from 58 patients were analysed. TH and DDC mRNA could discriminate localised disease from metastatic disease both in PB and BM whereas GD2S could discriminate between localised and metastatic disease in PB but not in BM. Tyrosine hydroxylase and DDC higher than median at diagnosis predicted a worse outcome for 24 well-characterised uniformly treated high-risk neuroblastoma patients followed for a median of 63 months from diagnosis (survival probability 91% at 5 years for TH below median versus 33% for TH above median, p<0.009). These results suggest that TH and DDC transcript concentrations at diagnosis is a possible new way to stratify patients for different novel treatment strategies. In conclusion, high expression of TH and DDC both in PB and BM corresponds to metastatic neuroblastoma at diagnosis, residual disease, and poor outcome. The concentration of TH and DDC can distinguish at diagnosis children who can be cured with current intensive therapy from those in need for novel therapeutic strategies as one way forward to further improved outcome for children with neuroblastoma.
LIST OF PUBLICATIONS


II. Catarina Träger, Per Kogner, Magnus Lindskog, Frida Ponthan, Anita Kullman and Bertil Kägedal. Quantitative Analysis of Tyrosine Hydroxylase mRNA for sensitive Detection of Neuroblastoma Cells in Blood and Bone Marrow. Clinical Chemistry 2003; 49:104.112

III. Bertil Kägedal, Anita Kullman, LiseLotte Lenner, Catarina Träger, Per Kogner and Malin Farnebäck. Pterin-Dependent Tyrosine Hydroxylase mRNA is not expressed in Human Melanocytes or Melanoma Cells. Pigment Cell Res 2004;17: 346-351

IV. Catarina Träger, Åsa Vernby, Anita Kullman, Ingrid Ora, Per Kogner and Bertil Kägedal. mRNAs of tyrosine hydroxylase and dopa decarboxylase but not of GD2 synthase are specific for neuroblastoma minimal disease and predicts outcome for children with high-risk disease when measured at diagnosis. Int J Cancer 2008; 123: 2849-2855
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<td>AUC</td>
<td>Area Under Curve</td>
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<td>Bone Marrow</td>
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<td>MRD</td>
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<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
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<td>MS</td>
<td>Metastatic Special</td>
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<td>NF1</td>
<td>Neurofibromatosis Recklinghausen</td>
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<td>NOPHO</td>
<td>Nordic Society for Pediatric Haematology and Oncology</td>
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<td>PB</td>
<td>Peripheral Blood</td>
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<td>PBSC</td>
<td>Peripheral Blood Stem Cell</td>
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<td>Phox 2B</td>
<td>Paired like homebox 2b</td>
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<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription- Polymerase Chain Reaction</td>
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<td>ROC</td>
<td>Receiver Operator Curve</td>
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<td>SIOPEN</td>
<td>European Society of Pediatric Oncology Neuroblastoma</td>
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<td>SNP</td>
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1 INTRODUCTION

1.1 CANCER

Unrestrained growth is the hallmark of the cancer cell. This property is caused by abnormal regulation of genes which are involved in the control of cellular proliferation and cell death. Other disturbed cellular functions important for the evolution of cancer involve the control of DNA integrity, angiogenesis and cellular senescence (Hanahan et Weinberg 2000). It appears that the cause of these functional disturbances is mainly genetic rearrangements, although recently there is greater awareness of the possibility that stable epigenetic defects may contribute significantly to tumourigenesis – especially in the case of childhood tumours of embryonal origin. However, in most cases we do not know what initiates this process, except in some inherited forms of cancer and some types of adult cancer in which environmental factors play a crucial role (Hanahan et Weinberg 2000).

Childhood cancer is relatively rare and accounts for 0.5% of all human cancers (Soci-Alstyrrelsen; Cancer i siffror 2009). Cancer is the second most common cause of death in childhood in western countries after accidents, whereas infectious diseases are more important in developing countries (Reamonn et al 2006). Acute leukaemia and brain tumours are the most common malignancies in childhood, followed by Wilms’ tumour and neuroblastoma, the latter accounting for 6% each of childhood malignant diseases (Gustafsson et al 2007). The prognosis for children with cancer has improved significantly and currently more than 75% are cured, although this rapid development appears to have levelled out. Furthermore a significant portion of childhood cancer survivors suffer from illness or disability caused by the disease or the therapy indicating need for better management and treatment of these patients (Pritchard-Jones et al 2006, Gustafsson et al 2007, Oeffinger et al 2007, Hjern et al 2007).
2.0 NEUROBLASTOMA

2.0.1 HISTORICAL BACKGROUND
As early as 1864, Virchow described the pathology of a childhood tumour which, in all likelihood, was neuroblastoma (Virchow R. 1864-65), but the name neuroblastoma was first suggested by James Homer-Wright in 1910 (Rothenberg et al.2009). James Homer-Wright was a pathologist in Boston, who published a review of a series of previously misclassified cases. The children had adrenal tumours with bundles of cells which he believed resembled primitive neuronal cells of the fetal adrenal medulla. James Homer-Wright was thus the first to describe the tumour as being of primitive neural cell origin. Two other physicians at that time described the different patterns of spread in neuroblastoma: In 1901 William Pepper described a classical example of stage 4S disease in an infant suffering from an adrenal tumour with hepatic metastases (Pepper WA 1901), and in 1907 Robert Hutchinson reported older children with tumours of the adrenals with metastases to the orbit (Hutchinson R 1907, Rothenberg et al 2009). The early therapy for neuroblastoma was surgery and radiotherapy which was introduced by Gross and Wittenborg, both working at the Childrens Hospital Boston in the 1940s (Rothenberg et al 2009, Carachi R 2002). They also described the prognostic implication of age at diagnosis and of bone metastases (Gross et al. 1959). Chemotherapy was introduced in the 1950s by Farber (Farber, Toch. 1951) and in the 1960s by Thurman (Thurman et al 1964) with some success.

2.0.2 GENERAL BACKGROUND
The cells from which neuroblastoma originate are embryologically primitive cells with neuronal characteristics. It has been suggested that they may have features in common with neural crest cells, which are transient cells during embryonal development that give rise to the entire sympathetic nervous system. Neuroblastoma can arise in all anatomical sites harbouring sympathetic tissue including the adrenal medulla, ganglia of the sympathetic trunk, and other sympathetic ganglia in the abdomen. Neuroblastoma is the most common malignant disease in infancy and more than 50% of the tumours have been diagnosed before the age of two years. Neuroblastoma accounts for 6% of all childhood cancers in Sweden but it is responsible for 9% of all paediatric oncology deaths (Gustafsson et al 2007).

Neuroblastoma has a very diverse clinical course ranging from spontaneous regression to an incurable disease despite multimodal therapy (Park et al 2008). Biological markers, in particular of genetic nature, associated with the tumour in combination with age at diagnosis and stage of disease provide reliable prognostic information.

2.0.3 AETIOLOGY
Despite the vast knowledge of somatically acquired genomic alterations in neuroblastoma and their correlations with clinical tumour phenotype, little is known about predisposing factors for these genetic events. Several hits early in embryogenesis or in the germ line are likely requisites for the development of neuroblastoma. Interestingly, germline mutations in the anaplastic lymphoma kinase tyrosine receptor gene were recently described which may constitute the genetic background for most familial cases of neuroblastoma (Moss et al.2008) and a subset of sporadic cases (Carén et al 2008). Another candidate gene for neuroblastoma is Phox2B in which mutations are associated with congenital central hypoventilation syndrome and Hirschsprungs.

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disease and a subset of these patients may develop neuroblastoma (Trochet et al 2004, Krona 2008). Another genetic syndrome with increased risk of developing neuroblastoma is neurofibromatosis (Satge et al 1998) in which the NF1 tumour suppressor gene may be homozygously inactivated (Martinsson et al 1997), whilst children with Down's syndrome seem to be protected from developing neuroblastoma tumours (Satge et al 1998). More common genetic traits increasing the risk of neuroblastoma were recently reported to be due to a common genetic variation at chromosome band 1p22 (Maris et al 2008) or to certain SNP polymorphisms of the MDM2 gene (Cattelan et al 2008).

2.0.4 EPIDEMIOLOGY
Several studies have found an increase in the incidence of neuroblastoma during recent decades (Spix et al. 2006, Kaatsch 2006). The reported global age-standardised annual incidence has been estimated to 3-12 per million children below 15 years of age (Stiller and Parkin 1992 and 1996).

This corresponds to an average of 1 child per 7,800; which contrasts to the report on neuroblastoma detected in situ in the adrenal medulla of approximately 1 in 250 newborns dying from non-neoplastic causes, (Beckwith and Perrin, 1963). The annual age-standardised incidence of neuroblastoma in the Caucasian population of North America has been estimated to 11.5 per million children, as compared to the African-American population with a corresponding incidence of 7 per million (Ries et al 1999). The incidence of neuroblastoma for boys is slightly higher than for girls with ratios ranging between 1.2 and 1.4, depending on the investigation (Stiller and Parkin 1992; Hale et al. 1994, Nyari et al 1999) which is a pattern observed in other childhood malignancies (1.2:1 for leukemias, 1.10 for CNS-tumours and 1.21 for other solid tumours, Gustafsson et al 2007) but hitherto without any satisfactory explanation.

Neuroblastoma is the most common malignancy of infancy and 40% of neuroblastoma patients are <1 year of age at diagnosis. In a comparative European investigation, there were significant differences between countries in the median age at diagnosis and distribution of the different stages of the disease at presentation (Powell et al 1998). Patients from the UK had the highest median age (24.8 months) and the highest proportion of stage 4 disease (61.5%). The median ages at diagnosis in France and Germany were 21.6 and 17.3 months and the proportions of stage 4 neuroblastomas were 44.9% and 39.4% respectively (Powell et al. 1998).

Infant screening programmes for neuroblastoma have been carried out in England, France, Germany, North America, and Japan. As a result, an increased incidence of low stage neuroblastoma was found in the screened cohorts. Most importantly, follow-up studies of these cohorts revealed that the procedure was not followed by a drop in the incidence of aggressive neuroblastoma. Thus, screening for neuroblastoma has revealed a high frequency of subclinical disease during infancy and the results implies that high-risk tumours are not derived from prognostic favourable infant tumours (Schilling et al. 2002, Woods et al. 2002). From an international perspective the incidence of neuroblastoma varies widely. For example, the native Alaskan population has a significantly lower incidence compared to the Caucasian US population (Lanier et al 2003); in some African countries neuroblastoma is rare (Parkin et al. 1998). Low figures are also reported from southern and eastern Asia, including India with an age standardised incidence ranging from 3.0 to 3.7 per million children. Higher incidence rates are reported from Hong Kong (7.3) and particularly from Japan (14.0) (Stiller, Parkin 1992, Parkin et al 1998).

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There are indications that the number of neuroblastoma cases detected reflects the amount of healthcare provided in a country. In a country where young children do not regularly visit health care institutions, the incidence of neuroblastoma is lower than in countries where most children attend well baby clinics or similar programmes, which supports the assumption that there are a number of subclinical neuroblastoma tumours that undergo spontaneous regression.

In Europe, children diagnosed with neuroblastoma between 1978 and 1997 had a 5 year overall survival of 59% ranging from 47% to 67% (Spix et al. 2006). The overall survival improved during this period from 37% in children diagnosed between 1978-1982 to 66% in those diagnosed in the 1993-1997 period (Spix et al.2006). It is possible that infant screening during the latter period may have contributed to these improved survival figures, as a result of increased detection of prognostically favourable subclinical cases.

Neuroblastoma is the most common malignancy in early childhood which makes a prenatal initiation of the tumour plausible. Prenatal factors studied include a potentially protective effect of vitamin intake during pregnancy, in particular folic acid (Olahan et al. 2002, Goh et al. 2007) and of being a twin (Puurmalta et al. 2009). Maternal intake of alcohol (Schutz et al.2001, Kramer et al.1987, Yang et. al.2000), diuretic drugs (Kramer et al. 1987) and use of hair dyes (Mc Call 2005) during pregnancy as well as caesarean delivery are associated with an increased risk (Hamrick et al. 2001). However, these statistically relatively weak epidemiological data are not conclusive with regard to potential environmental causes of neuroblastoma.

Previous spontaneous abortion has also been reported to decrease the risk of developing neuroblastoma, but this effect was confined to MYCN non-amplified infant tumours whereas congenital malformations were linked to a higher risk of, MYCN-amplified, infant tumours, in particular (Munzer et al 2008).

Environmental pesticides have been reported to increase the risk of developing neuroblastoma (Daniels et al 2001) as well as other childhood cancers, although reliable measurements of exposure are difficult to obtain for these and other compounds (Wigle et al 2007 and Wigle et al 2008).

In Scandinavia a population-based case control study, using data from participating NOPHO centres, concerning the significance of peri- and prenatal factors for the development of childhood solid tumours is ongoing, and might give some new clues to the tumourigenesis of neuroblastoma.

2.0.5 CLINICAL PRESENTATION

The very young child with neuroblastoma might not have any symptoms at all. The tumour may be incidentally detected on a routine check-up either by palpation of the abdomen by a physician or during a routine ultrasound of the abdomen. Local symptoms vary depending on the site of the tumour, and include abdominal swelling, respiratory symptoms, neuralgia and signs of spinal cord compression. General symptoms include abdominal pain, feeding difficulties, weight loss, fatigue, bone pain, and chronic diarrhoea. Hypertension is not common but when present it may be caused by renal artery compression or catecholamine excess (Weinblatt et al 1983, Maris et al 2007).

At diagnosis more than 50% of patients have metastases, which usually involves the bone marrow. (Maris et al 2007, Pizzo et Poplack 2002) Skeletal metastases are also frequently present, which may lead to pain and pathological fractures as presenting features.
symptoms. However, patients may also present with unexplained fever, weight loss, irritability, and periorbital ecchymosis secondary to metastatic spread to the orbits (Park et al. 2008). Approximately two thirds of patients with neuroblastoma have abdominal primary tumours and the symptoms depend on their proximity to vital structures and progress over time (Park et al. 2008). Tumours that arise from the paraspinal sympathetic ganglia can grow through the spinal foramina into the spinal canal and compress the spinal cord giving neurological symptoms, including weakness, limping, paralysis as well as bladder and bowel dysfunction (De Bernardi et al. 2001). Thoracic tumours may give symptoms from the airways. When the tumour masses extend into the neck they can produce a Horner syndrome (Mahoney et al. 2006). Primary cervical neuroblastoma is rare but may also give rise to a Horner syndrome and feeding and respiratory difficulties. In some infants, neuroblastoma presents with a small primary tumour and metastatic disease confined to the liver, skin, and bone marrow. This condition usually has a good prognosis except when it is associated with a large tumour burden in the neonate causing severe respiratory distress (Hsu et al. 1996).

Approximately 2% of patients present with an opsoclonus-myoclonus syndrome (Mitchell et al. 2002) which is an autoimmune paraneoplastic syndrome affecting the brain, the most prominent symptom of which is random myoclonic jerking eye movements. These patients often have localised disease with a favourable long-term tumour prognosis (Cooper et al. 2001) However, these tumours can be difficult to diagnose because of
their small size and some may not produce catecholamines. Unfortunately, the neurological symptoms can persist and progress to devastating neurological sequelae (Hayward et al. 2001). It is therefore extremely important to initiate immunosuppressive treatment promptly. Profuse diarrhoea due to tumour secretion of vasointestinal peptide (Kaplan et al. 1980) is a rare paraneoplastic symptom associated with histologically differentiated tumours with a good prognosis. An even rarer paraneoplastic phenomenon is associated with production of anti-HU antibodies (Graus et al. 2001, van Vuuren et al. 2005) resulting in encephalomyelitis and paraneoplastic sensory neuropathy and paraneoplastic cerebellar degeneration as well as autonomic dysfunction (Darnell et al. 2003).

2.0.6 CATECHOLAMINE METABOLISM

About 95% of the neuroblastoma tumours produce catecholamines (Candito et al. 2002). The catecholamines noradrenaline, adrenaline and dopamine are all derivatives of DOPA which is a catecholic amino acid. Tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis, converts the amino acid tyrosine to DOPA, dopa decarboxylase converts DOPA to dopamine, and dopamine hydroxylase converts dopamine to noradrenaline. Phenylethanolamine-N-methyltransferase catalyses the conversion to adrenaline (Klægeld B review 1988). Further metabolism of dopamine leads to homovanillinic acid (HVA), which is one of the catecholamine metabolites measured in urine and used for diagnostic purposes in neuroblastoma. The end product of noradrenaline metabolism is vanillylmandelic acid (VMA). Both HVA and VMA can be measured in urine (LaBrosse et al. 1980) as a diagnostic tool in neuroblastoma and as a way of following treatment response.

![Fig. 2 Catecholamine Metabolism](image-url)
2.0.7 DIAGNOSIS
Abdominal and thoracic tumours are usually found by radiology. Measurement of urinary excretion of catecholamine metabolites is an important diagnostic aid, but immunocytochemical verification from biopsy material is normally required for a definite diagnosis. The morphological requirements are described in International Neuroblastoma Pathology Committee (INPC) (Shimada 1999). Alternatively, the diagnosis can be made from tumour cells detected in the bone marrow combined with increased urine or serum catecholamines or catecholamine metabolites (Brodeur et al. 93). Bilateral bone marrow aspirates and biopsies should be analysed according to the recommendations of INSS (Brodeur et al. 1993). Despite that needle biopsies have been deemed suboptimal since they do not allow for a full histological classification according to INPC, they can be used in conjunction with specific staining and urine markers and also provide material for limited biological characterisation (Fröstad et al 1998 and 1999).
CT alternatively MRI investigations to assess image defined risk factors and an MIBG scintigraphy are mandatory for staging of the disease.

2.0.8 STAGING
Over the years, there have been several staging systems for neuroblastoma, which have sometimes been used in parallel to each other. The first system, initially used by the Children’s Cancer Study Group, was the Evans staging system (Evans et al. 1971, Evans 1980) (Table 1). This system was based on imaging, using roman numerals to distinguish localised and metastatic tumours (I-II vs. IV) and the infant special stage (IVS). The staging system currently in use in Sweden is the INSS system, which is a post-surgical staging system established after an international consensus in 1988 (Brodeur et al. 1988) and revised in 1993 (Brodeur et al. 1993) (Table 2). The patients are divided into six groups according to surgical excision and the spread of the tumour: Stage I, 2A, 2B, 3, 4 and 4S. S refers to “special” and 4S refers to a spreading disease with metastases confined to the liver, bone marrow and skin in a child younger than 12 months at diagnosis. A primary tumour should be at stage 1 or 2 and there should not be more than 10% tumour cells in the bone marrow (Evans 1971).

Table 1.
According to Evans and the Children’s Cancer Study Group (Evans et al., 1971).

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<td>Stage I</td>
<td>Tumour confined to the organ or structure of origin</td>
</tr>
<tr>
<td>Stage II</td>
<td>Tumour 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>Stage III</td>
<td>Tumour extending in continuity beyond the midline. Regional lymph nodes may be involved bilaterally</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Remote disease involving the skeleton, organs, soft tissue and distant lymph node groups</td>
</tr>
<tr>
<td>Stage IVS</td>
<td>(Special category). Patients who would be otherwise Stage I or II but who have remote disease confined to liver, skin, or bone marrow, and who have no radiographic evidence of bone metastases on complete skeletal survey</td>
</tr>
</tbody>
</table>

Table 1.
According to Evans and the Children’s Cancer Study Group (Evans et al., 1971).

<table>
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<th>Stage</th>
<th>Description</th>
</tr>
</thead>
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</tr>
</tbody>
</table>
Table 2. INSS Stage revised (Brodeur et al. 1993)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Localised tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically</td>
</tr>
<tr>
<td>2A</td>
<td>Localised tumour with incomplete gross resection, representative ipsilateral nonadherent lymph nodes negative for tumour microscopically</td>
</tr>
<tr>
<td>2B</td>
<td>Localised tumour with or without complete gross excision; with ipsilateral nonadherent lymph nodes positive for tumour. Enlarged contralateral lymph nodes must be negative microscopically</td>
</tr>
<tr>
<td>3</td>
<td>Unresectable unilateral tumour infiltration across midline, with or without regional lymph node involvement</td>
</tr>
<tr>
<td>4</td>
<td>Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver and/or other organs</td>
</tr>
<tr>
<td>4S</td>
<td>Localised primary tumour, 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>

INSS assessment is made after the initial surgical procedure and is therefore dependent on the work of the individual surgeon. To address these limitations and take the risk factors related to localised tumours into account (Ceccheto et al. 2005), the INRGSS was developed (Table 3). This new system makes it easier to compare the clinical material from different studies because of strict image defined risk factors. Localised tumours are distinguished as L1 or L2 pending presence of image defined risk factors (IDRF, Table 4, Monclair et al. 2008). Investigation of a large patient material has proved that an age cut off of 18 months at diagnosis is the prognostically most relevant age limit, as compared with the previously used limit of 12 months (London et al. 2005, Cohn et al. 2009).

Table 3. INRGSS Stage (Monclair et al. 2008)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>Localised tumour not involving vital structures and confined to one body compartment</td>
</tr>
<tr>
<td>L2</td>
<td>Locoregional tumour 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>

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Table 4. Image-Defined Risk Factors in Neuroblastic Tumors (Monclair 2008)

<table>
<thead>
<tr>
<th>Ipsilateral tumor extension within two body compartments</th>
<th>Neck-chest, chest-abdomen, abdomen-pelvis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neck</strong></td>
<td></td>
</tr>
<tr>
<td>Tumour encasing carotid and/or vertebral artery and/or internal jugular vein</td>
<td></td>
</tr>
<tr>
<td>Tumour extending to base of skull</td>
<td></td>
</tr>
<tr>
<td>Tumour compressing the trachea</td>
<td></td>
</tr>
<tr>
<td><strong>Cervico-thoracic junction</strong></td>
<td></td>
</tr>
<tr>
<td>Tumour encasing brachial plexus roots</td>
<td></td>
</tr>
<tr>
<td>Tumour encasing subclavian vessels and/or vertebral and/or carotid artery</td>
<td></td>
</tr>
<tr>
<td>Tumour compressing the trachea</td>
<td></td>
</tr>
<tr>
<td><strong>Thorax</strong></td>
<td></td>
</tr>
<tr>
<td>Tumour encasing the aorta and/or major branches</td>
<td></td>
</tr>
<tr>
<td>Tumour compressing the trachea and/or principal bronchi</td>
<td></td>
</tr>
<tr>
<td>Lower mediastinal tumour, infiltrating the costo-vertebral junction between T9 and T12</td>
<td></td>
</tr>
<tr>
<td><strong>Thoraco-abdominal</strong></td>
<td></td>
</tr>
<tr>
<td>Tumour encasing the aorta and/or vena cava</td>
<td></td>
</tr>
<tr>
<td><strong>Abdomen/pelvis</strong></td>
<td></td>
</tr>
<tr>
<td>Tumour infiltrating the porta hepatis and/or the hepatoduodenal ligament</td>
<td></td>
</tr>
<tr>
<td>Tumour encasing branches of the superior mesenteric artery at the mesenteric root</td>
<td></td>
</tr>
<tr>
<td>Tumour encasing the origin of the coeliac axis, and/or of the superior mesenteric artery</td>
<td></td>
</tr>
<tr>
<td>Tumour invading one or both renal pedicles</td>
<td></td>
</tr>
<tr>
<td>Tumour encasing the aorta and/or vena cava</td>
<td></td>
</tr>
<tr>
<td>Tumour encasing the iliac vessels</td>
<td></td>
</tr>
<tr>
<td>Pelvic tumor crossing the sciatic notch</td>
<td></td>
</tr>
<tr>
<td><strong>Intraspinal tumor extension</strong></td>
<td></td>
</tr>
<tr>
<td>whatever the location provided that:</td>
<td></td>
</tr>
<tr>
<td>More than one third of the spinal canal in the axial plane is invaded and/or the perimedullary leptomeningeal spaces are not visible and/or the spinal cord signal is abnormal</td>
<td></td>
</tr>
<tr>
<td><strong>Infiltration of adjacent organs/structures</strong></td>
<td></td>
</tr>
<tr>
<td>Pericardium, diaphragm, kidney, liver, duodeno-pancreatic block, and mesentery</td>
<td></td>
</tr>
<tr>
<td><strong>Conditions to be recorded, but not considered image defined risk factors</strong></td>
<td></td>
</tr>
<tr>
<td>Multifocal primary tumors</td>
<td></td>
</tr>
<tr>
<td>Pleural effusion, with or without malignant cells</td>
<td></td>
</tr>
<tr>
<td>Ascites, with or without malignant cells</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
# Table 5. The International Neuroblastoma Risk Group Consensus pre-treatment Classification Schema

<table>
<thead>
<tr>
<th>BRG Stage</th>
<th>Age (months)</th>
<th>Histological Category/Grade of Tumor</th>
<th>MYCN</th>
<th>Thy characterization</th>
<th>Peduli</th>
<th>Pre-treatment Risk Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1/L2</td>
<td>&lt;16</td>
<td>N, history of previous relapse</td>
<td>N</td>
<td>No</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>L1</td>
<td>&gt;16, &lt;12m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>No</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>L2</td>
<td>&gt;12m, &lt;18m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>Yes</td>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>L3</td>
<td>&gt;18m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>Yes</td>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>&lt;16</td>
<td>N, history of previous relapse</td>
<td>N</td>
<td>No</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>L5</td>
<td>&gt;16, &lt;12m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>No</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>L6</td>
<td>&gt;12m, &lt;18m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>Yes</td>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>L7</td>
<td>&gt;18m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>Yes</td>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>&lt;16</td>
<td>N, history of previous relapse</td>
<td>N</td>
<td>No</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>MS</td>
<td>&gt;16, &lt;12m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>No</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>MS</td>
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<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>Yes</td>
<td>Intermediate</td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>&gt;18m</td>
<td>E, nodal +, differentiated or undifferentiated</td>
<td>N</td>
<td>Yes</td>
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<td></td>
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</tbody>
</table>
2.0.9 PROGNOSTIC FACTORS

It has long been recognized that clinical stage and age at diagnosis has prognostic significance in children with neuroblastoma (Breslow and McCann 1971). A wide array of clinical, biochemical and biological factors have been shown to have a prognostic value (Cotterill et al. 2000, Hartmann et al. 1999). In a recent INRG study comprising 8,800 children several of these factors were evaluated and age was still the most powerful prognostic variable in stage 4 patients (Cohn et al. 2009). As discussed in the INRG Task Force report it would be optimal to evaluate age as a continuous variable for risk stratification because outcome gradually worsens with increasing age (London et al. 2005). There are also studies showing that children aged 12-18 months at diagnosis with INSS stage 4 tumours without MYCN amplification had a better outcome than older patients with the same characteristics (Schmidt et al. 2005, George et al. 2005). In the new INRG staging system (Table 3 and Fig. 1) the age cut off for higher risk is 18 months (Monclaire et al. 2009).

Genomic amplification of MYCN is the genetic aberration most consistently associated with poor outcome and advanced disease stage (Brodeur et al. 1984, Seeger 1985). But MYCN amplification is also associated with poor outcome in patients with low stage disease (Cohn et al. 1995, Perez et al. 2000, Katzenstein et al. 1998). MYCN is an oncogene localised distally on the short arm of chromosome 2 and is amplified in approximately 25% of primary cases leading to overexpression of the gene. Deletion of the short arm of chromosome 1 is identified in about 30% of neuroblastomas and correlates with MYCN amplification and advanced disease stage (White et al. 1995, Gehring et al. 1995, Martinsson et al. 1995). The short arm of chromosome 1 likely harbours a tumour suppressor gene or genes that control neuroblast differentiation. Deletion of 1p is more common in near-diploid tumours and is associated with a more advanced stage of disease. Most of the deletions of 1p involve the 1p36 region. Allelic loss of the long arm of chromosome 11 is rarely seen when the tumour is MYCN amplified but is nevertheless associated with poor outcome and is present in approximately 40% of primary tumours (Guo et al. 1999, Plantaz et al. 2001, Spitz et al. 2003).

Gain of genetic material on the long arm of chromosome 17 is very common in aggressive tumours irrespective of presence or absence of MYCN amplification (Bown et al. 1999). In a recent INRG investigation of prognostic factors a cohort of 8,800 children were included and histological category turned out to be the most powerful prognostic factor for INSS stage 1, 2, 3 and 4S. In patients with MYCN non-amplified stage 4S tumours, 1q gain was the most important prognostic factor for children younger than 18 months with stage 4 disease MYCN amplification was the most powerful prognostic factor.

DNA content is also of prognostic significance (Bagattelli et al. 2009). Near triploid tumours are assumed to have a defect in the regulation of mitosis and usually have whole chromosome gains and losses. These tumours are prognostically favourable in contrast to tumours with a near diploid DNA content which seems to have a defect in genomic stability resulting in unbalanced translocations and chromosomal rearrangements which contribute to their more malignant behaviour (Brodeur 2003).

2.1.0 STRATIFICATION AND TREATMENT

Before deciding what type of treatment a child is going to receive, thorough risk stratification must be performed, taking age, stage, and genetic aberrations into consideration (Cohn et al. 2009).

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2.1.0 STRATIFICATION AND TREATMENT

Before deciding what type of treatment a child is going to receive, thorough risk stratification must be performed, taking age, stage, and genetic aberrations into consideration (Cohn et al. 2009).
For diagnostic purposes and for analysis of genetic aberrations, a tumour biopsy is required. For low risk tumours surgical resection may be curative (Perez et al. 2000, Matthay 1989, Evans et al. 1996) also when surgery is incomplete (De Bernardi et al. 2008) and for some tumours even observation alone could be the best approach (Yamamoto et al. 1998).

Children with high-risk disease have a poor prognosis but it has been shown that they need multimodal therapy with intensive induction chemotherapy (Pearson et al. 2008), followed by an attempt to radical surgery and irradiation of the primary tumour to achieve local control (Stanton Adkins et al. 2004, Haas Kogan et al. 2003). High dose chemotherapy with stem cell rescue has a significant advantageous effect (Pritchard et al. 2005, Matthay et al. 1999, 2009, Ladenstein et al. 2008) usually preceding the local radiotherapy and maintenance therapy with retinoic acid (Matthay et al. 1999). Although survival for the high-risk group has improved during recent decades, still the majority of these patients do not survive their disease (Matthay et al. 2009). There is thus still a need for new therapeutic approaches also for infants with MYCN – amplified neuroblastoma (Canete et al. 2009) whereas infants with widespread disease (4/M or 4S/MS) without MYCN-amplification do well with limited therapy (Schmidt et al 1997, De Bernardi et al. 2009).

MIBG is also a useful treatment modality (Dubois et al. 2008, Matthay et al. 2009) and at the moment there are ongoing trials in which different types of MIBG treatment are evaluated (Matthay et al. 2009).

For children with tumours in the intermediate risk group the treatment decision may be more complicated, in the view of the possibility that the tumour may undergo spontaneous maturation or regression (Kushner et al. 1996, Hero et al. 2006).

They may favour from limited chemotherapy, in particular before surgery if there are image defined risk factors (Cecchetto et al. 2005) possibly combined with radiotherapy and maintenance therapy with retinoic acid.

### 2.1.1 MINIMAL DISEASE

In neuroblastoma minimal disease (MD) or minimal residual disease (MRD) has not yet been used for stratification of therapy, although metastatic disease at diagnosis is a poor prognostic factor and indicates that the patient should receive multimodal therapy.

To detect MRD a very sensitive and specific method is required. Immunocytological tests of the bone marrow can detect one tumour cell in 105 normal mononuclear cells (Moss 1999 and 91). Using the standardised immunocytological detection method developed by the SIOPEN bone marrow subcommittee, as little as one neuroblastoma cell in 106 mononuclear cells can be detected, provided 3x106 cells are analysed (Sweerts et al. 2005, Beiske et al. 2005). With RT-PCR one tumour cell in 106 haematopoietic cells may be detected (Cheung et al. 2001, Lambooy et al. 2003).

Earlier studies have shown that detection of neuroblastoma cells in bone marrow, blood, or peripheral blood stem cells (PBSC) during or after therapy correlated significantly with worse outcome (Moss et al. 1991, Bremner et al. 1993, Moss and Sanders 1999, Seeger et al. 2000, Fukuda et al. 2001, Burchill et al.1995, 2001, Horibe et al. 2001). Seeger et al. and Fukuda et al examined the bone marrow during therapy and showed that...
the detection of tumour cells in bone marrow after completion of induction therapy was associated with decreased EFS. Differences in the therapeutic approach after induction therapy can influence the impact of MRD that persists in the bone marrow. The prognostic value of analysing bone marrow, post therapy, with RT-PCR has been reported for patients who had not been treated with retinoic acid (Cheung et al. 2000). There are studies claiming that tumour contamination of stem cells does not have any prognostic implication (Corrias et al 2006). Different targets for PCR detection of neuroblastoma cells have been used, for example GAGE (Cheung et al 2000), GD2 synthase (Hoon et al 2001, Lo Piccolo et al 2001, Cheung et al. 2003), dopa decarboxylase (Bozzi et al. 2004) and tyrosine hydroxylase mRNA (Burchill et al. 1994, 2001, Paper II).

The lack of investigations with sufficiently large patient cohorts and the lack of uniformly designed investigations have resulted in inconclusive results regarding the usefulness of MRD detection in neuroblastoma. At present there are ongoing high-risk studies in both Europe and North America in which evaluation of the impact of MRD in bone marrow and blood before, during, and after therapy is an integral part. The detection of MRD in these studies is performed at various times during therapy, and both immunocytoLOGY and RT-PCR for applied to treat patients when MRD is detected, either with GD2 antibody and GM-CSF (or multiple gene products are used for the analyses. In these studies different approaches are applied to treat potential MRD, either with GD2 antibody and GM-CSF (Gilman et al. 2009) or with IL-2 in alternating courses) followed by 13-cis-RA, or with 13-cis-RA alone (Matthay et al).

Very recently a report with recommended criteria for sensitive detection of MRD in neuroblastoma has been published by the International Neuroblastoma Risk Group Task Force, to address the weakness with earlier studies such as lack of conformity regarding laborative methodology (Beiske et al 2009).

In 90% of neuroblastoma patients, radiolabelled MIBG is concentrated in tumour tissue making mIBG scintigraphy a sensitive and specific way to detect tumour cells (Matthay et al 2003, Kushner et al. 2003, Lebthai et al. 1997). MIBG uptake also occurs in well differentiated tumours, which means that MIBG uptake does not provide conclusive evidence of active malignant neuroblastoma (Braun et al 1998). The prognostic value of detection of mIBG-positive lesions soon after the completion of myeloablative therapy remains to be defined (Reynolds 2004). Both MIBG scintigraphy and qRT-PCR are useful tools for the detection of MD in neuroblastoma and the methods complement each other.

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3 AIMS OF THE STUDY

The overall aim of this thesis was to improve survival for children with neuroblastoma, and the specific aims were:

• To characterise neuroblastoma in Sweden throughout almost 27 years
• To develop a qRT-PCR method for sensitive detection of MRD in neuroblastoma
• To show the applicability of our developed qRT-PCR method for detection of TH mRNA in other cells than neuroblastoma cells
• To compare the clinical usefulness of three different targets for MRD detection in neuroblastoma with qRT-PCR
4 MATERIALS AND METHODS

The patients studied in papers I, II and IV were all included in the Swedish Childhood Cancer Registry, whereas the patients included in paper II and IV required parental informed consent, which was given prior to inclusion of the children into the study.

4.1.1 A 27 YEAR SURVEY OF NEUROBLASTOMA IN SWEDEN

Ethical approval for paper I was obtained from the ethical committee at the Karolinska University Hospital (Stockholm, Sweden).

Paper I is a register-based study of data from the Swedish Childhood Cancer Registry. All children from 0 to 14 years of age who were reported in the registry with a diagnosis of neuroblastoma from 1982 to November 2008 were included in the study and parental consent was obtained prior to registration. Their diagnoses were classified according to the international classification of childhood cancer (Steliarova-Foucher et al. 2005).

4.1.2 Development of a quantitative RT-PCR method for sensitive detection of tyrosine hydroxylase mRNA

This study was approved by the ethical committees at the Karolinska University Hospital and at the Linköping University hospital as well as at the University Hospital in Umeå (Sweden).

The first step in the development of this method was to design primers and probe specific for tyrosine hydroxylase (TH) mRNA, which was performed using the program primer express (PE Applied Biosystems).

RNA from the cell line IMR 32 was used to prepare a calibrator for the qRT-PCR method. From the absorbance of the calibrator we calculated the number of molecules, which made it possible to prepare a solution containing 1 transcript/2.5 μL. A final stock calibrator solution containing 106 transcripts/μL was then made from an earlier stock of the preparation. (for a more precise description of calibrator preparation see paper II), which was frozen in small aliquots for later use when analysing patient samples. We used the stock solution to generate a calibration curve (fig 2).
Both the calibrator and the patient samples were analysed in triplicate in all PCR experiments. The samples were amplified in an ABI prism 7700 Sequence Detector System (Perkin-Elmer). The IMR 32 cell line was also used to prepare RNA and cDNA control samples. Thus, IMR 32 cells were added to blood samples from healthy individuals, RNA was extracted and pooled and a part of the pooled RNA was used for cDNA synthesis.

Patients included in the study were children treated at Linköping University Hospital, Umeå University Hospital, and at Astrid Lindgren Children’s Hospital in Stockholm from January 1999 to April 2001. A total of 25 children were enrolled in the study (24 with neuroblastoma and 1 with ganglioneuroma). Some of the children were already diagnosed when the study started and others were diagnosed during the study period. The children were between 2 months and 6 years of age at the time of diagnosis. All patients were treated according to international protocols in a risk-based fashion depending on age, stage, and biological risk factors.

As normal controls, blood from 98 healthy children of different ages, blood from 34 adult blood donors as well as blood (n=12) and bone marrow (n=19) from children with other diseases than neuroblastoma was used.

4.1.3 Using the RT-PCR method to evaluate whether tyrosine hydroxylase is expressed in melanoma cells and melanocytes

Three pigmented melanocyte cell lines (one from Cascade Biologies Inc. and two from in-house primary cultures from foreskin circumcisions) and 12 human melanoma cell lines were used (Johansson et al. 2002). Four of the melanoma cell lines were pigmented and the other 8 were amelanotic. Eight neuroblastoma cell lines were used (ATCC, USA).

In addition, TH mRNA; we also analysed mRNA corresponding to 4 melanocyte specific proteins (tyrosinase, Tyrp1, Tyrp2, and Melan-A). Calibrators for the different transcripts were prepared as described in 4.1.2. qRT-PCR to detect the transcript was then performed using the method previously described in 4.1.2. As biological controls we used blood from 27 healthy children 6 months to 16 years of age. These samples were run through the whole procedure including the cDNA synthesis. The housekeeping gene HPRT was used to control the quality of the RNA.

4.1.4 Comparative study concerning the usefulness of sensitive detection of tyrosine hydroxylase mRNA, dopa decarboxylase mRNA and GD2 synt-hase mRNA in blood and bone marrow from children with neuroblastoma

This is a prospective Swedish multicentre study to which the children or the parents gave informed consent prior to inclusion. The study was approved by the local ethical committees at Lund University Hospital, Gothenburg University Hospital, Uppsala University Hospital, Umeå University Hospital and Linköping University Hospital as well as Karolinska University Hospital (Sweden).

We analysed 554 samples (365 blood samples and 198 bone marrow samples) from 58 unselected children with neuroblastoma of all different stages and biological subsets. Forty-two of the patients were investigated at diagnosis, during treatment and after cessation of therapy. The samples from the remaining 16 patients were taken during and after treatment. All patients were uniformly treated according to risk adapted treatment protocols. Twenty-four children with high-risk neuroblastoma were included in the study (SNS Stage 4 >2 years of age and/or MYCN amplification). Survival for high-risk patients was 62.2 % from diagnosis to last follow-up (n=15; median 63 months; range 24-156 months).
25-88) or to death (n=9), median 14 months; range 6-37). All but one of the surviving patients was followed for >3 years.

Ten neuroblastoma cell lines were used for validation of the method and as positive controls. A total of 78 healthy children (newborn to 16 years of age) were used as controls for blood samples. In total, 34 children with other diseases than neuroblastoma served as additional controls for biological background activity. From these children we obtained blood from 28 and bone marrow from 30.

A multiplex qRT-PCR was used to analyse GD2 S mRNA, DDC mRNA and TH mRNA from samples from blood and bone marrow. HPRT (a housekeeping gene) was used only for evaluation of the integrity of the samples, not for normalisation of data. Calibrators were synthesised for each transcript as described in section 4.1.2.

4.2 CALCULATIONS AND STATISTICS

4.2.1 A survey of neuroblastoma in Sweden

All incidence calculations were based on patients who were diagnosed between January 1982 and December 2007. Data on the Swedish population 0-14 years of age was obtained from Statistics Sweden (www.scb.se). These data and the world standard population were used to calculate the annual incidence per 100 000 children 0-14 years of age (Doll et al 1966). Time trends in the incidence were investigated using the US NCI Join point regression software, by which the annual percentage change and the average annual percentage change in incidence were estimated. The method also examines the presence of join points; points that divide time into segments (Kim et al 2001). The overall significance level was set to 0.05.

Survival curves and estimates of 5 and 10 year survival probability were calculated using the Kaplan-Meier method (Kaplan, Meier. 1958). All 359 children diagnosed between January 1982 and November 18, 2008 were included in the survival analysis. The Log rank test was used for significance testing (Ries et al) and the Fisher exact test was used for analysis of 2x2 tables.

4.2.2 Development of a quantitative real time reverse transcriptase PCR method

The concentration of tyrosine hydroxylase mRNA was calculated by multiplication of the reading on the calibration curve (transcripts/μL) with the factor 53. This constant was derived from the experimental design as follows: during RNA purification the sample was concentrated by a factor 37.5 (500 μL/40 μL), and during cDNA synthesis the sample was diluted to the double volume. The reading of the concentration on the calibration curve should thus be multiplied by the factor 0.053 (2/37.5) to give the sample concentration in transcripts/μL and by 53 to give the concentration in transcripts/mL blood. The results from series of duplicates and triplicates, were stratified into different groups (ranks) according to the transcript concentration in the sample, and the variation was calculated by ANOVA using SPSS statistical software. The mean standard deviation of each group was defined as the square root of the within-sample variance. Differences between groups were tested by the Wilcoxon rank-sum test and/or two-sided Fisher exact test.

4.2.3 Sensitive detection of tyrosine hydroxylase in melanoma cells and melanocytes

The concentrations of the transcripts were calculated as described in 4.2.2. Since the expected concentration of tyrosine hydroxylase mRNA if any, would be very low, the
method had to be very sensitive. Therefore we focused on the detection limit in the validation of the method and exclusion of possible false positives by analysis of controls analysed without template in the reaction tube. The cDNA in these control samples was replaced with water. There were positive signals in seven of 120 wells in the 40 last consecutive routine runs, with the highest value corresponding to 1.1 transcripts/µL (mean of triplicate) of cDNA from the sample. A total of nine transcripts were detected in these 40x3 rounds, which gave a mean of 0.075 transcripts/µL. This corresponds to six transcripts from a sample run through the whole procedure. Blank samples from 106 cells should thus give a mean of 0.0000066 transcripts/cell. The detection limit was set to 0.000088 transcripts/cell, which corresponded to the highest blank value (1.1 transcripts/µL of cDNA). The biological non-template control (originally blood samples from healthy children) run through the whole procedure including the cDNA synthesis yielded a mean of 10.3 transcripts per sample resulting in a mean blank value of 0.000001-0.00001 per leukocyte.

4.2.4 Comparative study evaluating the usefulness of sensitive detection of tyrosine hydroxylase mRNA, dopa decarboxylase mRNA and GD2 synthase mRNA in blood and bone marrow from children with neuroblastoma

The concentrations of the transcripts were calculated as described in 4.1.2. Only one measurement from each patient was used for statistical analyses to ensure independence between observations. Measurements during therapy were used descriptively. Fisher’s exact test was performed for descriptive comparisons between patients with localised disease and metastatic disease regarding levels of TH, GD2S and DDC, because of small expected frequencies. Because of the extremely skewed distribution of the transcript levels and the correlation between the transcripts, we used non-parametric tests to compare differences between transcript levels between groups (Spearman’s rank correlation coefficients and the Mann-Whitney U-test). ROC curves (Akobeng 2007) and AUC (Area under the curve) estimates were prepared to determine which of the transcripts was best in defining presence of minimal residual disease. ROC curves were also used to calculate a cut off level which could discriminate disseminated disease from local disease. Overall survival curves were calculated using the Kaplan-Meier method (Kaplan, Meier 1958). The last day of follow-up was January 1, 2007. The log-rank test was used to test the significance of the difference between the median and those with measurements equal to or above the median.

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5 RESULTS AND DISCUSSION

5.1 SURVEY OF NEUROBLASTOMA IN SWEDEN

This retrospective study includes 359 children diagnosed with neuroblastoma from January 1982 to November 2008 and identified in the nationwide Swedish Childhood Cancer Registry. According to the INRG staging system (Monclair et al. 2009), 201 children had localised stage (L), and 142 children had metastatic stage (M) and 16 infants were diagnosed with metastatic special stage (MS) of the disease.

The cases were analysed according to sex, age, stage and chromosomal alterations in relation to incidence and survival. Chromosomal aberrations were not completely analysed for the whole population, mainly due to the fact that the analytical methods were not available at the early part of the period investigated, and storage of biological material was not complete. The incidence was also analysed according to month of birth to investigate whether seasonal perinatal factors could be of significance or not.

In contrast to other European Studies (Spix et al. 2006), in which a slight increase in incidence has been reported, the incidence of neuroblastoma in Sweden was constant throughout the study period.

In Sweden the incidence was slightly higher in males (1.16:1) than in females, in accordance with the general difference for childhood cancers (1.18:1) in the Swedish population (Gustafsson et al. 2007) and similar to the results from a large European neuroblastoma study (Spix et al. 2006) as well as a recent Danish report (Schroeder et al. 2008). More males than females presented with metastatic disease (ratio 1.41:1) at diagnosis, but the frequency of local disease was equal in both sexes. The higher frequency of metastatic disease in males is a plausible explanation as to why male patients had a worse clinical outcome than female (5 years survival probability boys vs. girls 58.3% vs. 70.0%). There were no other known biological differences between the tumours in males and females.
Boys with localised stage were younger than girls ($p<0.01$), the median age at diagnosis was 8 months for boys and 12.5 months for girls. Boys with metastatic stage were slightly older than girls with metastatic stage (median age 30 vs. 29 months).

The frequency of MYCN amplification among stage M patients was in concordance with other investigations (Park et al. 2008). Analysis of 11q deletion and 17q gain were performed only in some cases which provides little information about the frequency in the whole cohort.

Survival analysis showed that high age (>12 or >18 months of age at diagnosis), stage M, MYCN amplification, 1p or 11q deletion and gain of chromosome 17q were all associated with a worse outcome.

The 5 year survival probability for all children with neuroblastoma, during the investigated period was 63.7% ($\pm$ 2.7%). When dividing the children into three different time periods for diagnosis it becomes clear that the survival has improved significantly over the years studied. The 5 years survival probability for children diagnosed 1982-1990 was 57.8% and for children diagnosed 1991-1999, 61.5% and for children diagnosed 2000-2008 the 5 years survival probability was 74.1% ($p<0.001$).

Improved survival probability was most pronounced for children with high-risk disease (stage M >18 months or MYCN amplification) and their survival at 5 years increased from 8.6% (1982-1990, n=35) to 17.0% (1991-1999, n=53) and 54.5% (2000-2008, n=50, $p<0.001$).

The median age at diagnosis was 18 months, and the fact that this is a disease of the very young child makes it plausible that prenatal and perinatal factors are important in the tumorigenesis of neuroblastoma. Incidence according to year of birth and month of birth showed considerable variation over time with no definite trend. Thus, certain periods of birth were associated with low incidence of neuroblastoma, and these were similar in both boys and girls indicating that girls and boys may have a perinatal common tumour-initiating factor. Although a few epidemiological studies have been performed concerning prenatal and perinatal factors in the pathogenesis of neuroblastoma, no conclusive results have been reported with regard to environmental causes of neuroblastoma (Kramer et al. 1987, Yang et al. 2000, McCall et al. 2005, Olshan et al. 2002, Puumula et al.2009, Björge et al. 2008).

We conclude that the incidence of neuroblastoma in Sweden from 1982 to 2008 has been stable. There was a significant difference between boys and girls both in incidence and in outcome, during the time period studied. The probability of survival improved for all disease stages, but was most pronounced for high-risk neuroblastoma. The improved survival of children with high-risk neuroblastoma was strongly related to the risk-based therapeutic approach used for treating children with neuroblastoma. Intensified multimodal therapy has made it possible for the majority of children with high-risk disease to be long-term survivors.

5.2 DEVELOPMENT OF A SENSITIVE QUANTITATIVE REAL TIME REVERSE TRANSCRIPTASE METHOD FOR THE DETECTION OF TYROSINE HYDROXYLASE mRNA

Our quantitative real time RT-PCR method for analysis of tyrosine hydroxylase mRNA can produce a detectable signal even when only one cDNA molecule is present in the final PCR step. This corresponds to 50 transcripts/mL of blood. Due to the stochastic nature of the data (Bustin 2004) the imprecision of the PCR step is not possible to calculate at such a low concentration; the mRNA signal is either there or not. We therefore calculated the detection limit from samples with 50-100 transcripts/mL of blood and found a mean
standard deviation of 36 transcripts/mL. If 2 standard deviations were used as cut-off for
the detection limit the lowest detectable concentration would be 72 transcripts/mL.
The results in our study varied from <72 to 4.6x10^4 transcripts/mL in blood and from
<72 to 8x10^6 transcripts/mL in bone marrow. If the true differences are small, low im-
precision is needed. Blood samples from healthy children were analysed as controls, and
none of these samples contained detectable TH mRNA. This indicates a high specificity
of the method for the analysis of blood. Bone marrow from children with leukaemia was
analysed for detection of TH mRNA and detectable concentrations were found in 5 of
19 cases. It has been shown that catecholamines are synthesised by lymphocytes (Ber-
qvist et al 1994). This indicates that bone marrow from leukaemia patients may not be
a suitable system to evaluate the analytical specificity of the method. The quantitative
analysis of tyrosine hydroxylase is not a measure of the number of neuroblastoma cells
in samples from different patients, since the number of transcripts per tumour cell may
vary from child to child. However, we assume that the number of transcripts is reason-
ably constant in the tumour cell population of one patient. We therefore assess that the
present method is appropriate for monitoring the progression and regression of micro
metastases in blood and bone marrow in children with neuroblastoma.

Tyrosine hydroxylase may be up-regulated in tumours treated with the angiogenesis
inhibitor TNP-470 (Wassberg et al 1999). This may indicate that the number of tran-
scripts per cell may change due to hypoxia in the tumour. This hypothesis should be
studied more systematically with quantitative methods.

In the present study, we show that children with metastatic disease (blood, range; 203-
46000 transcripts/mL, bone marrow, range; 6000-793000) have significantly higher
levels of tyrosine hydroxylase mRNA in blood and bone marrow at diagnosis than those
with locoregional disease (blood < 83 transcripts/mL, bone marrow not detectable). This
makes it possible to discriminate between metastatic and local disease at diagnosis with
our method. Furthermore, the analytical data followed the clinical course closely and it
was possible to detect relapse from blood samples before the appearance of symptoms
or any other sign of relapse. See fig 6a and 6b below describing two different children
with high-risk neuroblastoma with serial blood samples analysed for TH mRNA.

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or any other sign of relapse. See fig 6a and 6b below describing two different children
with high-risk neuroblastoma with serial blood samples analysed for TH mRNA.
Blood samples were collected from ten children before, during and after surgical manipulation of the tumour. In these children, the highest concentrations were observed one hour after surgery, indicating the release of neuroblastoma cells into the circulation during surgical manipulation of the tumour. The transcript concentration in blood after the preoperative induction chemotherapy was normal in six out of seven high-risk neuroblastoma patients. This indicates that chemotherapy may eradicate systemic disease and that the described method is useful for monitoring this effect. Interestingly, the only child among these seven who did not achieve a normalised preoperative transcript concentration in blood relapsed and died from progressive disease (after conclusion of the present study). This may indicate an adverse prognostic effect of remaining MRD after therapy, which is similar to results reported by Burchill et al (2001).

In conclusion this is a reliable and robust method to detect remaining minimal detectable disease in neuroblastoma. The method can distinguish locoregional disease from metastatic disease and predict relapse before any other sign of relapse occurs.

5.3 DETECTION OF TYROSINE HYDROXYLASE IN MELANOMA CELLS AND MELANOCYTES

Tyrosine hydroxylase mRNA was undetectable or detected at very low concentrations in melanocytes and melanoma cells. Thus, in melanocytes the tyrosine hydroxylase mRNA ranged from not detectable to 0.000492 transcripts/cell. In the melanoma cells, the median yield was 0.000352 transcripts/cell (range: not detectable - 0.005340 transcripts/cell). There was no significant difference in the number of tyrosine hydroxylase transcripts/cell between pigmented and non-pigmented cell lines (Student’s t-test).

The presence of the housekeeping gene HPRT indicated adequate analytical performance. We used neuroblastoma cell lines as positive controls and detected a median number of 0.4 tyrosine hydroxylase mRNA transcripts/cell (range 0.02 - 25) indicating up-regulation of this gene in neuroblastoma cells.

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We conclude from our analysis that tyrosine hydroxylase mRNA is undetectable in melanocytes and melanoma cells irrespective of whether the melanocytes are pigmented or not. Thus, tyrosine hydroxylase does not contribute to pigment formation in human melanocytes or melanoma cells.

5.4 COMPARATIVE STUDY OF THREE DIFFERENT TRANSCRIPTS FOR SENSITIVE DETECTION OF MINIMAL DETECTABLE DISEASE IN NEUROBLASTOMA

Eight neuroblastoma cell lines were investigated with quantitative real time RT-PCR for tyrosine hydroxylase, dopa decarboxylase and GD2 synthase mRNA. All transcripts were detectable in all cell lines. The range of tyrosine hydroxylase expression correlated with MYCN amplification and cell phenotype with the highest and lowest transcript concentration in SK-N-BE(2) (25.0/2500) and SH-SY-5Y (0.02/2 cell) respectively. None of the transcripts were detectable in any blood sample from the healthy controls. In children with other diseases than neuroblastoma, elevated transcript concentrations in blood or bone marrow was more common for GD2 synthase than either tyrosine hydroxylase or dopa decarboxylase. In blood samples from children with localised neuroblastoma at diagnosis, there was no significant difference between the three transcripts. However, positive results for GD2 synthase (10/16) were more common compared with tyrosine hydroxylase (5/16) or dopa decarboxylase (2/16) in bone marrow samples from children with localised disease (p=0.0012). The increased GD2S transcript level in bone marrow did not correspond to neuroblastoma cells in the bone marrow analysed by conventional methods (these children did not have infiltration of neuroblastoma cells in the bone marrow), or to MYCN amplification, subsequent relapse, or worse clinical outcome. The majority of children with metastatic disease at diagnosis had levels above the cut-off level for all three transcripts in both blood and bone marrow. Two children with stage 4S had elevated levels of all transcripts in blood and bone marrow.

We used all samples that were available for analysis to compare the three transcripts in blood and bone marrow. Most samples were drawn during and after treatment and the majority of the results were below the cut-off level. The levels of tyrosine hydroxylase and dopa decarboxylase mRNA in blood were closely correlated when all samples were taken into account (rTHODDC = 0.924; p < 0.001). When the concentration of tyrosine hydroxylase mRNA in bone marrow samples was low, there was no correlation with GD2 synthase mRNA. High expression of tyrosine hydroxylase mRNA correlated with active neuroblastoma disease and high expression of GD2S, but the concentration of GD2S mRNA was around a tenth of the concentration of tyrosine hydroxylase mRNA (rTHODGDS = 0.865; p < 0.001). No patients with a level of GD2S mRNA >10 times higher than tyrosine hydroxylase mRNA in bone marrow, showed any sign of active neuroblastoma disease. High expression of tyrosine hydroxylase and dopa decarboxylase in blood and/or bone marrow was detected in neuroblastoma patients with metastatic disease at diagnosis, or with residual relapsing disease. The concentrations in blood of tyrosine hydroxylase mRNA and dopa decarboxylase mRNA, but not of GD2S mRNA differed significantly between children with neuroblastoma and children with other diagnoses (TH p = 0.001; GDS p = 0.453; DDC p = 0.001). In bone marrow, quantitative concentrations of all three transcripts differed significantly between children with neuroblastoma and children with other diagnoses. The level of GD2 synthase could not discriminate between localised neuroblastoma and metastatic disease, which might be due to the presence of GD2 and GD2S in mesenchymal stromal cells (Martinez et al. 2007).

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The cut off level used is mathematically calculated and does not correspond to disease severity, nor does it imply a clinical decision limit (Paper II). When the bone marrow is infiltrated by neuroblastoma cells, the concentration of TH mRNA is 10 to 100 times as high as this theoretical cut off. We used ROC curves (Akobeng AK, 2007) to choose a decision level which discriminates between localised and disseminated disease. The level for tyrosine hydroxylase in blood was 288 transcripts/ml and for bone marrow, 2126 transcripts/ml calculated by this method. The corresponding levels for dopa decarboxylase were 37.5 in PB and 22.5 in bone marrow. Both of these levels are very low and in the range where the method is not reliable. Therefore dopa decarboxylase might not be as useful as tyrosine hydroxylase for detecting MD/MDR in neuroblastoma. If the cut off for tyrosine hydroxylase was set to 2100 transcripts/ml for bone marrow samples, none of the children with other diagnoses than neuroblastoma would have been considered positive for tyrosine hydroxylase.

When selecting the high-risk patients only, all of whom were uniformly treated according to European protocols, high tyrosine hydroxylase mRNA concentrations in the bone marrow at diagnosis clearly indicated a worse outcome with similar results for dopa decarboxylase mRNA. This indicates that both tyrosine hydroxylase mRNA and dopa decarboxylase mRNA could be useful to stratify patients into different treatment groups, in future clinical trials. A larger cohort of patients is needed to establish the concentration of the transcript indicating that the patients can be cured from high-risk neuroblastoma using current therapy.

Fig 7 Tyrosine hydroxylase, GD 2 synthase and dopa decarboxylase mRNA, in upper panel blood and lower panel bone marrow. The children in upper panel with tyrosine hydroxylase and dopa decarboxylase in bone marrow below median had a 5 year survival probability of 91% but the group with TH and DDC transcript concentration above median had a 5 year survival probability of 33% p=0.009.

The cut off level used is mathematically calculated and does not correspond to disease severity, nor does it imply a clinical decision limit (Paper II). When the bone marrow is infiltrated by neuroblastoma cells, the concentration of TH mRNA is 10 to 100 times as high as this theoretical cut off. We used ROC curves (Akobeng AK, 2007) to choose a decision level which discriminates between localised and disseminated disease. The level for tyrosine hydroxylase in blood was 288 transcripts/ml and for bone marrow, 2126 transcripts/ml calculated by this method. The corresponding levels for dopa decarboxylase were 37.5 in PB and 22.5 in bone marrow. Both of these levels are very low and in the range where the method is not reliable. Therefore dopa decarboxylase might not be as useful as tyrosine hydroxylase for detecting MD/MDR in neuroblastoma. If the cut off for tyrosine hydroxylase was set to 2100 transcripts/ml for bone marrow samples, none of the children with other diagnoses than neuroblastoma would have been considered positive for tyrosine hydroxylase.

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It is not clear whether the level of tyrosine hydroxylase mRNA expression reflects the tumour load alone or whether it also reflects the biological type of disease. High tyrosine hydroxylase expression/cell in the cell lines used in our study correlated with MYCN amplification, cellular phenotype of I-type stem cells, and resistance to chemotherapeutic drugs in vitro (Walton et al. 2004, Ponthan et al. 2007). A recent study reported that a tumour initiating cell isolated from bone marrow metastases in neuroblastoma showed high expression of tyrosine hydroxylase mRNA (Hansford et al. 2007).

In conclusion, tyrosine hydroxylase and dopa decarboxylase are the most sensitive and specific of the three markers included in the study. The concentration of tyrosine hydroxylase mRNA and dopa decarboxylase mRNA at diagnosis predicts outcome for high-risk patients, suggesting this as a novel way to stratify for different treatment strategies in these children.

Fig 8 Receiver operation characteristic curve from blood. In discriminating neuroblastoma from other diagnosis in blood THI and DDC gave results different from chance whereas GD2S was not better than chance to discriminate neuroblastoma from other diagnosis.

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Neuroblastoma is such a heterogeneous disease that it eventually has to be divided at least into two different diseases but there has to be clear genetic evidence for how to divide the disease into these groups. It is clear in the first epidemiological study that there are two populations of patients: first the very young child with the localised disease whom are diagnosed before 12 months, and then the older child with the median age around 30 months at diagnosis having a much more severe disease. Another interesting finding is that more boys than girls are diagnosed with the disease and that they more often suffer from metastatic disease, that makes one wonder if the male fetus are responding in a different way to oestrogen during pregnancy or if the extra x chromosome girls carry, are protective in some way. Our incidence curves regarding birth year and month are indicating that there is a common prenatal cause to boys and girls tumours.

The first study also shows us that the last 30 years work with improvement of treatment for the high-risk group has resulted in a significantly better chance to survival, with a 5 year survival probability raising from 8.6% to 54.5%. In study IV we found that it is possible to distinguish a group of homogeneously staged and treated high-risk children with a 5 year survival probability of 91% from another group with a 5 year survival probability of 33% with the median concentration of Tyrosine hydroxylase in bone marrow at diagnosis. The question arises: what is the difference between these two groups? Are we over-treating one of the groups? Would they still survive with a bit less intensive treatment? The other group with much worse outcome, are we giving them treatment which makes their neuroblastoma cells more aggressive? There is an invitro study showing that Cisplatin treatment upregulates expression of factors leading to survival and expansion of a highly tumorigenic fraction of neuroblastoma cells, and also induces enhanced clonogenic capacity and increased expression of stemness associated genes (Tsuchida et al 2008). Could it be that the group with high expression of tyrosine hydroxylase is more sensitive to Cisplatin induced upregulation of stemness associated genes? All children treated for high-risk neuroblastoma today receives Cisplatin. Perhaps, there are certain patients who should not get this drug.

Another question is what genetical differences there are between these cell populations? According to the standard analysis performed, as MYCN amplification and 1p deletion, there are no differences between cell populations, but there need to be further genetical analysis performed to address this question.

The usefulness of measuring MRD is still questioned, but in our limited material it turns out to have a great clinical significance for distinguishing children with local disease from metastatic disease, follow treatment efficiency and detecting relapse at an earlier time point than with other methods. Another possible application is for stratifying children for new treatment trials. One could speculate in using the concentration of tyrosine hydroxylase at diagnosis combined with the sex of the child for stratifying to different treatment arms when evaluating new treatment modalities.

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Fig 9 “Do I dare to jump?” Future challenges. Ill: © Marit Törnqvist
7 CONCLUSIONS

- The neuroblastoma incidence was stable in Sweden during the period studied with significant sex differences.
- Outcome improved during the investigated period related to a risk-based therapeutic approach with particular improvement for children with high-risk disease. The majority of children with high-risk neuroblastoma can now be long-term survivors thanks to intensified multimodal therapy.
- The quantitative RT-PCR method for tyrosine hydroxylase mRNA detection is reliable and easy to perform and the method could discriminate children with stage 4 disease from children with stage 1, 2 and 3 disease.
- With the quantitative RT-PCR method for tyrosine hydroxylase mRNA it was possible to detect relapse earlier than with conventional methods. It was also possible to follow small changes in TH mRNA concentrations in blood during tumour manipulation during surgery.
- It was easy to follow the treatment efficiency with analysis of TH in blood and bone marrow. The transcript concentrations decreased significantly after treatment with chemotherapy, 131I-meta-iodobenzylguanidine, and surgery, respectively.
- Our results indicated that there is no TH that can contribute to pigment formation in melanocytes or melanoma cells.
- TH and DDC mRNA as compared to GD2S, were the most sensitive and specific markers for MRD of neuroblastoma in PB and especially in BM.
- The concentrations of TH mRNA and DDC mRNA at diagnosis predict outcome for the high-risk NB patients in our study, suggesting a possible novel way to stratify patients to different treatment strategies.
Sammanfattning på svenska;

8 DEN GÅTFULLA SJUKDOMEN HOS DET GÅTFULLA FOLKET...

Barncancer är ovanligt, även om det inte känns så när man drabbas, 250-300 barn per år drabbas av cancer, vilket utgör 0,5% av all cancer i Sverige. Det vanligaste dödsorsaken under barnåldern är olyckor och den näst vanligaste är barncancer.

Neuroblastom drabbar 15-20 barn per år och utgör 6% av alla barncancerfall i Sverige. Sjukdomen förorsakar 9% av den cancerrelaterade mortaliteten hos barn. Det är framförallt spädbarn och småbarn som drabbar av neuroblastom och drar sig vid hälften av alla barn som diagnoseras med neuroblastom har fått sin diagnos vid två års ålder. Neuroblastom är inte en sjukdom utan flera! Cellerna ser visserligen ungefär likadana ut; små runda och blåskimrande, men det är en stor skillnad för det lilla barnet om det drabbar av ett ”snällt” eller ”elakt” neuroblastom.

BAKGRAUND TILL STUDIEN

Sjukdomen utgår från omoga nervceller i det autonoma nervsystemet. Tumören kan utvecklas utmed hela ryggraden eller i biken. Buktumöterna utgör oftast från nerverceller i binjuren och det finns en liten risk att tumören förväxlas med en njurttumör när den växer i direkt anslutning till njuren. Tumören kan växa in i ryggsäckskanalen och därigenom trycka på ryggsäck och nervor och ge symptomer i form av känslortillstånd i ben och fotter, svårigheter med avföring och vattenkastning samt förhållande av benen. Det finns en ovanlig form av neuroblastom som ger upphov till märkliga ögon rörelser och ”ostadiitet”. Själva tumörn brukar i dessa fall vara lättbotad men det kan vara svårt att komma till rätta med de neurologiska symptomen.

Hos spädbarnet kan det vid en rutinundersökning vara en ren tillfällighet att man känner en tumör i boken och barnet behöver inte ha haft några symptom allls. Amans är det inte ovanligt att barnen är gnälliga, inte vill äta, går inte upp i vikt som de ska och kanse slutar gå. Symtom kan vara vildlitliga olikän från fall till fall och speciell var i koppen tumörn sitter. Barn som är under 18 månader när diagnosen ställs har oftast en bättre prognos än de barn som är äldre när tumörn uppställs. Prognosen har främst att göra med tumörcellernas inre egenska- per, deras förmåga att sprida sig och överleva behandling alternativt deras förmåga att av sig självt mogna och bli ôlfrliga celler som i vissa fall även kan tillbakabillas och helt försvin- na. Prognosen har mycket lite att göra med när familjen kommer till sjukvården. Tumörn har sina biologiska egenskaper oavsett om man får vård tidigt eller sent i sjukdomsförföppet.

När barnet fått diagnosen måste man utreda vilken risknivå sjukdomen har och därefter utgår man ifrån barnets ålder (över eller under 18 månader), om barnet har en lokalisierad eller språdd sjukdom och om tumörcellerna har några genetiska förändringar som speciell en mer aggressiv alternativt mindre aggressiv sjukdom. Vidare utförs en rad rötanunder- sökningar för att ta reda på hur tumören växer i förhållande till andra organ vilket kan vara helt avgörande för om man kan operera bort tumören.

Barn med lokalisierad sjukdom indelas i två grupper; en grupp utan rötanunderlogiskt verifie- rade riskfaktorer (L1) och en grupp med riskfaktorer (L2). Alla barn delas in i låg-, mellan-
och högriskgrupp och beroende på vilken risknivå barnet tillhör bestäms vilken behandling barnet ska få. Barn med lokaliserad sjukdom där neuroblastom cellerna inte har någon genetisk markör för aggressiv sjukdom behandlas enbart med kirurgi. För en del barn räcker det med "aktiv expostetän" vilket betyder att barnet får komma på regelbunda kontroller men att man avvatkar med all typ av behandling och det gäller de barn där man förväntar sig att tumören ska mognas och försvinna spontant. För vissa barn med lokaliserad sjukdom värker tumören på ett sådant sätt att den skadar omkringliggande vävnad och en operation skulle kunna medföra ytterligare skador på omkringliggande organ. För att undvika skador i samband med operation behandlas barnet med cellgifter för att krympa tumören så att tumören därefter blir möjlig att avlägsna kirurgiskt utan ytterliggare skador.

Alla barn med spridd sjukdom (sjukdom med dottertumörer i skelett, hennning eventuellt ytterligare organ) som är över 18 månader gamla vid diagnos får en tuff behandling där intensiv cytostatikabehandling ges i 10 dagarsinterval under 70 dagar. Därefter följer operation och högdosbehandling med cytostatika samt stammcellstransplantering för att försöka sörja om att barnet återfår immunkompetenta celler (bland annat vita blodkroppar som försvinar kroppen i samband med infektioner. Därefter får barnet A-vitamin i 14 dagarsinterval under 6 månader där tanken är att eventuellt minimal kvarvarande sjukdom (=MRD) ska behandlas bort.

MÅL MED STUDIEN
- Att kartlägga förekomsten och arten av neuroblastom i Sverige under de år som det Svenska Barncancerregistret funnits (från och med 1982)
- Att ta fram en mycket specifik och känslig molekylärbiologisk metod (=PCR) för att hitta minimal kvarvarande sjukdom (=MRD) i blod och bennägg hos barn med neuroblastom
- Att testa användbarheten av ovanstående metod med analys av andra celler än neuroblastom (i detta fall för att ta reda på om melanomceller använder sig av samma enzym vid pigment metabolism som neuroblastom celler vid ämnesomsättningen av katekolaminer)
- Att jämföra känsligheten, specifiteten och den kliniska användbarheten av tre olika markörer för MRD i blod och bennägg

RESULTAT
Under den tidperiod som det svenska barncancerregistret funnits har 359 barn yngre än 15 år med neuroblastom registrerats. Något fler poajar än flickor har insjuknat (i överensstämmelse med andra liknande studier gjorda i andra länder) och det är något fler poajar än flickor som har drabbats av spridd sjukdom.

I den andra artikeln utvecklade vi en mycket känslig och specifik molekylär biologisk metod för att påvisa MRD i blod och bennägg. Metoden går ut på att man söker upp en genetisk sekvens av en markör, i vårt fall valde vi det hastighetregistrerande enzymet i ämnesomsättningen hos neuroblastomceller, tyrosinhydroxylas, som sjukdomsmärke. Prover från 25 barn med neuroblastom analyseras. För kontrolländamål analyseras blodprov från 34 friska blodsivar och 98 friska barn samt bennägg från 19 barn med andra sjukdomar än neuroblastom.

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I det tredje arbetet testade vi om det även i melanocyter (pigmenterade celler i huden) och melanomceller (hudcancer) fanns tyrosinhydroxylas. Vi gjorde då våra molekylärbiologiska analyser på prover där vi tillförde en viss mängd celler till ett prov. Neuroblastomceller, melanocyter och melanomceller analyserade. Vi kom fram till att melanocyter och melanomceller inte använde sig av tyrosinhydroxylas vid pigmentproduktion.

I det fjärde och sista arbetet utvärderades GD2 syntas (GD2S), som är ett enzym som är engagerat vid produktion av en cellväggsstruktur, GD2, hos neuroblastomceller men även melanomceller och även i vissa bindvävsceller. En amerikansk forskargrupp har ansett att denna markör är den bästa för att påvisa neuroblastomceller i blod och bennmag och utvecklat en PCR metod för detta. Vi valde även att ta fram en PCR metod för dopadekarboxylas som också är ett enzym i nedbrytningen av katekolaminer. Nivåer av dessa tre jämftöres hos 40 barn från diagnos och under behandling, en del barn analyserades även efter avslutad behandling. Tyrosinhydroxylas och dopadekarboxylas var de känsligaste och mest specifika för MRD vid neuroblastom av dessa tre markörer. Tyrosinhydroxylas och dopadekarboxylas kunde redan i samband med diagnosstillfället dela upp de 24 barnen med hög risk neuroblastom i två grupper med viss olika chans till överlevnad. En grupp med fem års överlevnad på 91%, det var barn med en koncentration av markörer i bennmag under medianen och en grupp barn med en fem års överlevnad på 33% som hade tyrosinhydroxylas och dopadekarboxylas nivåer över medianen vid diagnos.

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- Studien visar att vi har ungefär lika många som insjuknar i neuroblastom per år i Sverige som i övriga Europa och att antalet inte tenderar att öka eller minska.
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- De påvisade förändringar som finns i neuroblastom cellernas genetiska kod har samma betydelse hos svenska barn som hos barn med neuroblastom i andra internationella studier. Överlevnaden för barn med neuroblastom har blivit allt bättre över åren och främst är det överlevnaden för barn med högrisksjukdom som förbättrats. Förutom att behandlingen har intensifierats under åren har behandlingen av minimal kvarvarande sjukdom (MRD) förbättrats.
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Tack Per Kogner, som varit min huvudhandledare sedan jag kom till Stockholm, för din entusiasm och intressanta diskussioner kring neuroblastom på både en mer basal biologisk nivå och kring patient fall. Och tack för att du stöttade mig så att jag avslutade detta projekt.

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Tack Göran Gustafsson för att du introducerade mig till barnonkologer och delade med dig av din stora kunskap.

Tack Åsa Vernby för att du delade med dig av dina statistik kunskaper och för roliga och intressanta diskussioner och för ”systerskap”.

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Tack Fredrik Hedborg för att du läste min text när jag var som mest uppgiven och Susan Pfeifer för att all uppmuntran och stötning.


Alla som på något sätt bidragit till den här studien. Några kommer jag att nämna här men många fler har varit delaktiga.

Alla barn som drabbats av neuroblastom under dessa år och som på olika sätt ingått i min studie, utan er hade inte studien kunnat vara möjlig. Min förhoppning är att en del av detta arbete kan vara till nytta för andra barn med neuroblastom.

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Tack Tom Monclair för att jag fick använda INRG’s överlevnadsträd.

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10 REFERENCES


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