LNESG2 STUDY:
GUIDELINES FOR THE TREATMENT OF PATIENTS WITH
LOCALIZED RESECTABLE NEUROBLASTOMA
AND ANALYSIS OF PROGNOSTIC FACTORS

A PROTOCOL OF THE SIOP-E NEUROBLASTOMA GROUP

FINAL DRAFT JUNE 2004

ACTIVATION: OCTOBER 1st 2004
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1. INTRODUCTION, BACKGROUND AND STUDY JUSTIFICATION

1.1. INTRODUCTION AND BACKGROUND

Treatment strategies for localized neuroblastoma have changed over recent years. Whereas, undoubtedly, intensive treatment is still necessary for advanced disease, the trend for localized NB has been towards a reduction of treatment intensity, and in particular, avoidance of chemotherapy (1, 2, 3). Several studies have shown that patients with localized INSS stage 1 and 2 neuroblastoma can be treated safely with surgery alone with a 5-year survival rate of at least 90% (4, 5, 6).

Neuroblastoma is characterized by heterogeneity of clinical and biological behavior. Besides the well established risk factors such as age and disease extension at diagnosis, several relevant biological and pathological prognostic factors have been identified in the last few years. The most important of these include amplification of the oncogene Nmyc (MYCN) (7, 8, 9), unfavorable histopathology (10), elevated serum neuron specific enolase (11), deletions of the short arm of chromosome 1 (13, 14, 15, 16) and 17q gain (16a, 16b). These factors have been investigated primarily in advanced stage disease. However, their significance in localized neuroblastoma is less clear.

Some studies have attempted to address risk factors in localized neuroblastoma specifically. MYCN has certainly been shown to be associated with an adverse prognosis (4, 6, 17, 18, 19, 20). The prognostic role of the deletion of the short arm of chromosome 1 (1pdel) is more controversial (14, 21). In a recent study, 1pdel correlated independently with decreased EFS (but not OS) in low-risk patients, thus defining a subgroup of patients with localized neuroblastoma and higher risk of relapse (22). Elevated lactate dehydrogenase (LDH) is a further, easily measurable, marker that reflects advanced disease status, recurrence (23) and correlates with adverse prognostic factors such as high histological grade, MYCN and diploidy (24). A large univariate analysis of 37 different variables in stage 1-3 NB, identified elevated LDH as an independent risk factor for localized disease (25). The histopathological classification by Shimada into favorable (FH) or unfavorable histology (UH) is another factor predicting the clinical behavior of disseminated and localized NB (18), even in infants (26), and correlates well with low risk and high risk patients defined by others (27).

1.2. RESULTS OF LNESG 1

The recently completed multicentre European Study for localized NB (LNESG 1) primarily evaluated the safety and efficacy of surgery as the only treatment in the management of INSS stage 2 neuroblastoma without MYCN amplification (Trial patients). Stage 1 and 3 patients as well as those with ganglioneuroma were followed as Study patients.

From January 1995 until September 1999, 905 patients were registered from 9 European countries. There were 740 patients with localized neuroblastoma or ganglioneuroblastoma, of whom 616 eligible for the study and 124 for the trial; their distribution is shown on Table 1. Seventy-one patients presented with ganglioneuroma and 94 were ineligible (32 NB, 62 for other diagnosis).
Table 1. Patient distribution

<table>
<thead>
<tr>
<th>INSS</th>
<th>trial</th>
<th>Study MYCN-</th>
<th>Study MYCN+</th>
<th>Study MYCN not evaluable</th>
<th>total pt</th>
</tr>
</thead>
<tbody>
<tr>
<td>stage 1</td>
<td>0</td>
<td>289</td>
<td>7</td>
<td>34</td>
<td>330</td>
</tr>
<tr>
<td>stage 2</td>
<td>124</td>
<td>22</td>
<td>10</td>
<td>13</td>
<td>169</td>
</tr>
<tr>
<td>stage 3</td>
<td>0</td>
<td>166</td>
<td>25</td>
<td>50</td>
<td>241</td>
</tr>
<tr>
<td>total</td>
<td>124</td>
<td>477</td>
<td>42</td>
<td>97</td>
<td>740</td>
</tr>
</tbody>
</table>

1.2.1 Relapse pattern and outcome

1.2.1.1. Stage 1 patients

Disease relapse occurred in 14/289 patients for whom the assay of MYCN gene was successfully performed and found to be normal. Five relapses occurred locally and 9 in distant or combined sites (of which 7 were in the bone and/or bone marrow, 1 in subcutaneous tissues, 1 in the liver). Relapses were treated with a variety of regimens.

Two of these 289 patients died; 1 after bone and bone marrow relapse and 1 after primary surgery.

The overall survival (OS) at 36 months is 99.2%. The relapse rate (RR) was 4.4% at 12 months (CI 1.9-6.8) and 4.7% at 24 and 36 months (CI 2.2-7.2), and was significantly lower than in stage 2 (p=0.0004).

The pattern of relapses and patient outcome are presented in table 2. Median follow-up for these patients is 50 months (range 0 - 84 months).

Table 2. Stage 1 patients with normal MYCN. Relapse pattern and outcome

<table>
<thead>
<tr>
<th>Total number of patients</th>
<th>289 (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>relapses</td>
<td>14 (1)</td>
</tr>
<tr>
<td>local</td>
<td>5</td>
</tr>
<tr>
<td>distant/comboined</td>
<td>9 (1)</td>
</tr>
<tr>
<td>deaths</td>
<td>2</td>
</tr>
<tr>
<td>of disease</td>
<td>1</td>
</tr>
<tr>
<td>surgery-related</td>
<td>1</td>
</tr>
</tbody>
</table>

( ) number of deaths

1.2.1.2. Stage 2 patients

Of 169 INSS stage 2 patients, 124 were MYCN negative and were registered as trial patients. Twenty-one patients relapsed. Relapse was local in 12 patients, combined in 8 and only distant in 1 patient. Metastases involved the bone and/or bone marrow in 7 cases. Two patients had no metastatic work-up performed. As for stage 1 patients, relapses were treated with a variety of regimens. Seven out of 21 patients who relapsed died of disease, 6/7 after a subsequent relapse. An additional patient (who never relapsed) died of sepsis more than 1 year after surgery that had included splenectomy (table 3). The overall survival for the trial patients is 94.8% at 3 years (90.7-98.9) indicating that the majority of INSS stage 2 patients (MYCN negative) can be cured with surgery alone. The RR was 13.2% (CI 7.2-19.3) at 12 months, 15.8% (CI 9.3-22.3) at 24 and 17.8% (CI 90.7-98.9) at 36 months.
Table 3. Stage 2 patients with normal MYCN. Relapse pattern and outcome

<table>
<thead>
<tr>
<th>Total number of patients</th>
<th>124 (8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relapses</td>
<td>21 (7)</td>
</tr>
<tr>
<td>Local</td>
<td>12</td>
</tr>
<tr>
<td>distant/combined</td>
<td>9 (7)</td>
</tr>
<tr>
<td>Deaths</td>
<td>8</td>
</tr>
<tr>
<td>of disease</td>
<td>7</td>
</tr>
<tr>
<td>of infection</td>
<td>1</td>
</tr>
</tbody>
</table>

( ) number of deaths

1.2.2. Biochemical, biological and histological risk factors in Trial patients

The presence at diagnosis of each of the following three factors (among others) - elevated serum LDH level, deletion of the telomeric portion of 1p chromosome and unfavorable histology according to Shimada criteria - were analyzed for their ability to increase the risk of relapse. Despite the impression that these three factors are all associated with a greater propensity to relapse (table 4), statistically they are not considered conclusive enough to justify treating patients with these features with adjuvant therapy. This decision is based upon a considerable amount of missing data (16% for LDH, 22% for 1p deletion and 15% for histology), resulting in small numbers and the small number of total events. However, out of the three factors, unfavorable histology seems to be the strongest in predicting relapse and mortality. Among the trial patients, 8/21 patients with UH relapsed 5 of whom died, whereas 12/84 patients with FH relapsed 3 of whom died. This observation justifies consideration of heavy treatment for patients with UH at relapse.

Table 4. Relapse or progression in stage 2 patients

<table>
<thead>
<tr>
<th></th>
<th>UH</th>
<th>FH</th>
<th>unknown H</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1pL</td>
<td>1pN</td>
<td>1p?</td>
<td>1pL</td>
</tr>
<tr>
<td>high LDH</td>
<td>2/2</td>
<td>1/3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>normal LDH</td>
<td>1/2</td>
<td>1/8</td>
<td>3/4</td>
<td>0/4</td>
</tr>
<tr>
<td>? LDH</td>
<td>-</td>
<td>0/2</td>
<td>-</td>
<td>1/2</td>
</tr>
<tr>
<td>total</td>
<td>3/4</td>
<td>2/13</td>
<td>3/4</td>
<td>1/6</td>
</tr>
<tr>
<td>cumulative</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

UH: unfavorable histology    FH: favorable histology
1pL: 1p deletion    1pN: no deletion    1p?: missing data
high LDH: ≥ 2xN    normal LDH: < 2xN    ?LDH: missing data
1.2.3. Stage 1 and 2 disease and MYCN amplification (Study patients)

Patients with INSS stage 1 and 2 disease and MYCN amplification were followed as study patients. Amplification of MYCN was found in 7/296 INSS stage 1 and in 10/156 INSS stage 2 patients (Table 1) which represents 2.4% for stage 1 and 6.4% for stage 2 disease. Their primary treatment and outcome are presented in table 5.

Table 5. Primary treatment, relapse and outcome in MYCN + patients

<table>
<thead>
<tr>
<th></th>
<th>Stage 1</th>
<th>Stage 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total patient number</strong></td>
<td>296</td>
<td>156</td>
</tr>
<tr>
<td><strong>MYCN + cases</strong></td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>S + CT</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>S + RT</td>
<td>0</td>
<td>0</td>
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<td>S + CT + RT</td>
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<td>5</td>
</tr>
<tr>
<td>unknown</td>
<td>1</td>
<td>0</td>
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<tr>
<td><strong>Relapse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>L + D</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>unknown</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><strong>Outcome</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 DOD*</td>
<td>2 DOD**</td>
</tr>
<tr>
<td>S: surgery</td>
<td>CT: chemotherapy</td>
<td>RT: radiotherapy</td>
</tr>
<tr>
<td>D: distant relapse</td>
<td>DOD: dead of disease</td>
<td></td>
</tr>
</tbody>
</table>

The age distribution at diagnosis in this MYCN amplified group was as follows: in INSS stage 1 patients 4 were < 1 year of age and 3 > 1 year of age, 2 from each group died of disease; in INSS stage 2 patients 4 were < 1 year of age and 6 > 1 year of age, with both patients who died of disease being > 1 year. The numbers are too small and do not allow any definite conclusions. However, the high relapse rate (5/7 patients) and the high death rate (4/5 relapsed patients) in spite of mostly local relapse in INSS stage 1 patients raises the question of whether they may need chemotherapy after surgery from the very beginning - more data is required to give a definitive answer.

The combined local and distant relapses in INSS stage 2 MYCN amplified patients who received adjuvant treatment after primary surgery support the idea that this patient subgroup needs more aggressive treatment from the very beginning. At present, these patients will be treated according to the current European high risk protocol.
1.2.4 Surgical risk factors

The preoperative evaluation of surgical risk factors was introduced to avoid aggressive procedures with a predictably high risk of postoperative complications and any operation with a high risk of leaving a macroscopic residue. Operation should only have proceeded when complete excision appeared feasible without damage or risk to the patient. These precautions allowed some patients to undergo operation even for tumours crossing the midline (stage 3 patients).

Surgical data were examined to validate the risk factors for operation with regard to 1) postoperative complications, 2) results of excision and 3) the effect of the amount of tumour excised on outcome.

1.2.4.1. Postoperative complications of operations with or without surgical risk factors were evaluated according to stage (Table 6) as well as tumour site (table 7).

Table 6. Preoperative surgical risk and postoperative complication rate in operated neuroblastoma patients according to stage

<table>
<thead>
<tr>
<th>INSS stage 1</th>
<th>Risk</th>
<th>Patients operated</th>
<th>complication in pts operated</th>
<th>p value</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>not known</td>
<td>25(100%)</td>
<td>1 (4%)</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>no risk</td>
<td>240 (100%)</td>
<td>12 (5%)</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>risk</td>
<td>65(100%)</td>
<td>7 (11.1%)</td>
<td>0.084</td>
</tr>
<tr>
<td>total: 330</td>
<td></td>
<td>total: 330</td>
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<tr>
<td>INSS stage 2</td>
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<tr>
<td>pt number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>not known</td>
<td>19 (95%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>82</td>
<td>no risk</td>
<td>79 (96.3%)</td>
<td>5 (6.3%)</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>risk</td>
<td>52 (80%)</td>
<td>11 (21.6%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>total: 167</td>
<td></td>
<td>total: 150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>INSS stage 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pt number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>not known</td>
<td>0 (0%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>no risk</td>
<td>11 (91.7%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>199</td>
<td>risk</td>
<td>19 (9.5%)</td>
<td>6 (31.6%)</td>
<td>0.068</td>
</tr>
<tr>
<td>total: 241</td>
<td></td>
<td>total: 30</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primary operation was performed in 65/65 (100%) of stage 1 patients who had risk factors, in 52/65 (80%) stage 2 patients who had risk factors, and in 19/199 (9.5%) stage 3 patients who had risk factors. Thus a majority of patients with stage 1 and 2 disease and known pre-operative risk factors were operated on contrary to the criteria drawn up in the protocol. This applied to a minority of stage 3 patients.

In all stages, the complication rate was higher when the operation was performed in spite of pre-operative risk factors. In stage 2 patients this difference was statistically significant (p<0.01). The main complications were serious bleeding, renal ischemia and chylous leakage.

Concerning the site of the tumour, operative complications were also more frequent if risk factors, including tumour size and tumour fragility, were present. This was statistically significant in patients with abdominal primary disease (table 7). In other sites, complications were more frequent, but did not reach statistical significance probably due to smaller numbers.
Table 7. Operative complications related to the presence of risk factors (RF) and tumour site

<table>
<thead>
<tr>
<th>site/#</th>
<th>RF: known</th>
<th>RF: yes complications</th>
<th>RF: no Complications</th>
<th>Significance (Fisher)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#</td>
<td>%</td>
<td>#</td>
<td>%</td>
</tr>
<tr>
<td>cervical/23</td>
<td>21</td>
<td>3/5</td>
<td>2/16</td>
<td>12.5</td>
</tr>
<tr>
<td>thorax/123</td>
<td>112</td>
<td>4/28</td>
<td>7/84</td>
<td>8.3</td>
</tr>
<tr>
<td>Abdomen/298</td>
<td>284</td>
<td>11/75</td>
<td>7/209</td>
<td>3.4</td>
</tr>
<tr>
<td>pelvis/34</td>
<td>32</td>
<td>2/15</td>
<td>1/17</td>
<td>5.9</td>
</tr>
</tbody>
</table>

1.2.4.2. Results of excision: our data show that patients with thoracic and abdominal tumours had a statistically higher rate of macroscopic residual disease when operated on in the presence of risk factors (table 8). The same applied to patients with pelvic tumours, but without statistical significance. There were insufficient data available for patients with cervical tumours.

Table 8. Macroscopic residuals after initial surgery and according to site and surgical risk factors (RF)

<table>
<thead>
<tr>
<th>site/#</th>
<th>RF</th>
<th>macroscopic residual</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>#</td>
<td>%</td>
</tr>
<tr>
<td>cervical/21</td>
<td>no</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>thorax/112</td>
<td>no</td>
<td>84</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>28</td>
<td>14</td>
</tr>
<tr>
<td>abdomen/284</td>
<td>no</td>
<td>209</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>75</td>
<td>13</td>
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<tr>
<td>pelvis/32</td>
<td>no</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>yes</td>
<td>15</td>
<td>6</td>
</tr>
</tbody>
</table>

1.2.4.3. Effect of residual tumour on outcome: Overall and relapse free survival were lower in patients who were left with macroscopic residual disease after excision, but the results were statistically significant only in patients with abdominal primary disease (Table 9). We emphasise that 13 out of 17 patients with abdominal primary who underwent resection with macroscopic residual disease, were operated on in spite of the presence of risk factors.

Table 9. Overall and event-free survival at 3 years relative to tumour volume after excision in 289/298 patients with abdominal primary, for whom follow-up is known

<table>
<thead>
<tr>
<th>Resection</th>
<th>total</th>
<th>Deaths</th>
<th>OS %</th>
<th>p</th>
<th>Relapses</th>
<th>EFS* %</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>222</td>
<td>10</td>
<td>95.8</td>
<td>23</td>
<td>89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>micro residual</td>
<td>50</td>
<td>4</td>
<td>91.8</td>
<td>4</td>
<td>93.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>macro residual</td>
<td>17</td>
<td>3</td>
<td>81.9</td>
<td>&lt; 0.05</td>
<td>5</td>
<td>70.6</td>
<td>&lt; 0.025</td>
</tr>
<tr>
<td>Totals</td>
<td>289</td>
<td>17</td>
<td></td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* means relapse

Data on all patients with macroscopic residual disease were examined to determine whether the actual volume of disease remaining after attempted excision had an effect on outcome. Two cut-off points were chosen for the post-operative volume(9 and 20 ml) and compared. Among all patients with macroscopic residual disease for whom data were available (n = 50), there was no difference in outcome with regard to the tumour volume remaining after excision.
In conclusion:

1. Surgery alone proved to be an efficient treatment for localized neuroblastoma without MYCN amplification.
2. The presurgical risk factors examined in LNESG1 were validated as predicting a good outcome from primary operation. Patients with established pre-operative risk factors, especially with stage 2 and with abdominal primary disease, who were operated on in spite of the estimated risk, had more postoperative complications, a greater chance of postoperative macroscopic residual disease and a statistically less good overall and event-free survival. The presurgical evaluation of risk factors should therefore be followed carefully in LNESG2.
3. More data are needed to verify these results in other tumour sites and to evaluate the effect of the volume of residual disease on outcome.

1.2.5. Summary

The preliminary results of LNESG1 confirm that the majority of patients with localized, resectable, MYCN negative NB are cured with surgery alone. However, the results also show that a high number of patients, especially with stage 1 and 2 disease, underwent primary operation in spite of risk factors, and that they had more frequent postoperative complications and macroscopic residual disease. This was statistically significant for patients with abdominal primaries, and had a negative influence on OS and EFS. It is not clear, if this patient population with an overall cumulative 3-yrs risk of death below 5%, contains a subgroup of patients at much higher risk. If they can be identified, some type of adjuvant treatment after surgery may be warranted to improve outcome.

The main factors that have been investigated to this end include Shimada pathological classification, LDH at diagnosis and 1p deletion. The data from LNESG1 do not provide sufficient information to definitely assess the prognostic importance of these 3 factors, alone or combined, because of a high amount of missing data (only 71 out of the 124 trial patients had information on all 3 factors) and the low number of events with regard to the sample size which did not allow firm statistical conclusions to be reached.

The present study is aimed at:

- carefully regarding the established presurgical risk factor analysis before excision of the primary,
- evaluating the prognostic role of the above mentioned and other prognostic factors in a larger prospective series of patients with localized, resectable neuroblastoma treated with surgery alone at diagnosis,
- applying a uniform treatment for relapsed patients.
1.3. OBJECTIVES FOR LNESG 2

1.3.1. Primary objective
- To expand the information provided by LNESG1 on factors associated with clinical prognosis in localized neuroblastoma, especially preoperative LDH, 1pdel and histology

1.3.2. Secondary objectives
- To maintain or improve EFS and OS when compared to LNESG1
- To improve the quality of management and data collection in patients with resectable localized neuroblastoma without MYCN amplification by
  - A nationally centralized evaluation of the pathological and biological data with secure banking of material
  - Improved data collection, with particular regard to LDH, 1p deletion
- To establish a uniform treatment for relapsed patients

2. ELIGIBILITY

2.1. INCLUSION CRITERIA
- age between 0 and 18 years included
- all patients with resectable INSS stage 1 to 3 NB, without MYCN amplification
- all patients with resectable INSS stage 1 NB, with MYCN amplification
- no previous chemotherapy (except steroids)
- mandatory biological assessment: MYCN amplification, 1p deletion
- mandatory histological assessment: locally and nationally reviewed grading according to INPC classification
- mandatory biochemical assessment: preoperative LDH
- mandatory full metastatic work-up (MIBG at diagnosis included)
- registration within 6 weeks of surgery
- provision of follow-up for up to 3 years
- secure local or national banking of tumour material
- national/local ethical committee approval
- informed consent obtained from parent or guardian.

2.2 EXCLUSION CRITERIA
- age above 18 years
- resectable INSS stage 2 and 3 NB with MYCN amplification
- unresectable localized tumour
- metastatic disease
- no clear histological documentation of NB, no histological grade according to INPC
- lack of minimal mandatory biological (MYCN, 1p deletion) or biochemical (LDH) assessment
- lack of metastatic work-up
- registration more than 6 weeks since surgery
- parent/patient refusal
3. DIAGNOSTIC PROCEDURE

3.1. PATIENT STAGING

The International NB Staging System (INSS) will be used (Appendix I). For dumbbell tumours see Appendix VI.

3.2. PATIENT ACCRUAL

Figure 1. Guidelines for the management of localized NB (LNESG2)

```
suspected localized NB ↓
diagnostic imaging (including MIBG )
preoperative LDH ↓
surgery + BM : 2 aspirates + 2 trephine biopsies ↓
biological and histological work-up : MYCN
1p deletion
Histology (INPC) within 6 weeks (national review included)
Secure banking of tumour material and reference DNA ↓
post-surgical evaluation + MIBG + other ↓
age, stage, results of MYCN, histology ↓
therapeutic decision as per figure 2, page 15
```

3.3. DIAGNOSIS

The diagnosis of NB or ganglioneuroblastoma will be based on histological examination of the tumour. The primary diagnosis will be made by the local pathologist (for histological classification, see Appendix II). An on-line histology review has to be performed within 6 weeks from surgery:

```
Diagnosis by the local pathologist according to Appendix II ➔ sends material to the national pathologist for an independent diagnosis within 2 weeks (without giving his result). If the national pathologist is not a member of the pathology subcommittee, he sends the material for review to the pathology subcommittee, after having written his report (but without sending his report). The national or subcommittee pathologist makes a diagnosis and discusses the result by phone with the local or national pathologist.
```
3.4. WORK-UP

Pre-operative:
- physical examination, height, weight, blood pressure
- Full blood count, AST, ALT, bilirubin, creatinine, urea, coagulation profile, liver and renal function, urine catecholamines and metabolites (HVA, VMA, dopamine, noradrenaline)
- LDH
- radiological investigations of the primary (CT or MRI). N.B. check both sides of the diaphragm.
- MIBG scan (not mandatory, but highly recommended; delaying surgery for MIBG scan will not jeopardise the patient’s outcome)

Post-operative, to be performed within 6 weeks of surgery:
- Bone marrow aspiration and trephine biopsy (performed at surgery)\(^1\)
- MIBG scan (but not required again, if negative preoperatively)
- Bone scan (MDP 99Tc), if no MIBG scan or negative MIBG scan preoperatively\(^2\)
- CT or MRI of the primary at 1 month after surgery for residual tumour
- Blood for constitutional DNA

\(^1\) **Bone marrow**: four adequate negative specimens (bone marrow aspirates and trephine core biopsies from two sites obtained either during or after surgery) are requested.
If immunostaining is performed results will not influence staging.

\(^2\) **Bone**: pre- and/or post-operative MIBG scans are required (the post-operative scan will not be performed if the pre-operative one was negative). Post-operative MIBG must be negative at the level of the skeleton.
If pre-operative MIBG scan was negative on the primary tumour (this occurs in approximately 5% of the cases), an MDP 99Tc bone should be performed post-operatively.
If MIBG scan was not performed pre-operatively, both MIBG and MDP 99Tc scans should be performed post-operatively.

3.5. DECISION MAKING PROCEDURE

3.5.1. Patients at diagnosis

Based on the results of LNESG1, the majority of patients presenting with localized neuroblastoma without *MYCN* amplification can be cured by surgery alone. However, as previously stated, a small subgroup of patients might be at higher risk of relapse, that is patients with elevated LDH at diagnosis and/or unfavourable histology and/or 1p deletion and/or 17q gain ( = risk factors). The amount of missing data, however, does not permit changes to therapeutic decisions for the primary treatment based on the existing results. Therefore, patients with resectable stage 1-3 neuroblastoma without *MYCN* amplification will continue to be treated by surgery alone according to surgical guidelines. Special care will be given to the evaluation of preoperative risk factors in order to decrease postoperative...
complications, especially in the INSS stage 2 patients.

All patients with stage 1 disease and MYCN amplification will also be included into the study and treated by surgery alone. The patient number in LNESG1 was too small to draw definite conclusions, although the results showing a high mortality among those who relapsed (4/5 relapsed patients died of disease) are of concern. They need to be confirmed in a larger cohort.

Figure 2. Treatment guidelines for resectable stage 1-3 neuroblastoma

3.5.2. Patients at relapse

As discussed in 1.2.2., UH seems to be the most important predictive factor of relapse and especially adverse outcome. Therefore it seems reasonable to consider histology for further therapeutic decision in patients who will relapse after primary treatment with surgery alone.

As depicted earlier, the treatment of relapsed patients after primary surgery was very heterogeneous. The secondary objective of the study is to consider a uniform way of treating relapses after a complete work-up.

Figure 3. Treatment guidelines for relapsed stage 1-3 neuroblastoma (MYCN negative) patients after primary surgery
4. TREATMENT PLAN

4.1. SURGERY

The surgical procedure will be decided on the basis of the surgical guidelines (for details see Appendix V).

a) Pre-Operative Criteria of Resectability

Complete excision is the overriding aim of operation. The imaging techniques presently available should allow evaluation of resectability in most cases. LNESG1 analysis demonstrated that vascular encasement increases the risk of surgical complications. If it is clear from the preoperative imaging that surgical excision would be dangerous, then the child should be treated with chemotherapy according to the unresectable localized neuroblastoma protocol. Spiral CT or Digital Subtraction Angiography may provide accurate information on vascular anatomy. It is of note that the estimation of risk factors is not always precise and that chemotherapy has its own complications. In this situation, it is mandatory that the relative risks of operation be compared with those of chemotherapy in consultation with the oncologist.

b) Risk factors for Primary or Immediate excision.

Detailed clinical examination and imaging studies can assist the surgeon in deciding whether a localised tumour is amenable to primary surgical resection. **The presence of any of the risk factors listed below may well contra-indicate primary surgery.** This is true even if the risk factors were not recognised prior to the operation. In this situation serious consideration should be given to abandoning the operation. These decisions must involve the oncologist in charge of the patient.
Risk Factors Related to Localisation:

Neck

• Tumour encasing vertebral and/or carotid artery
• Tumour encasing brachial plexus roots
• Tumour crossing the midline

Thorax

• Tumour encasing the trachea or principal bronchus
• Tumour encasing the origin and branches of the subclavian vessels
• Thoraco-abdominal tumour, peri-aortic fusiform tumour
• Lower left mediastinal tumour, infiltrating the costo-vertebral junction between T9 and T12

Abdomen

• Adrenal tumour infiltrating the porta hepatis
• Suprarenal tumour infiltrating the branches of the superior mesenteric artery at the mesenteric root
• Suprarenal tumour surrounding the origin of the coeliac axis, and of the superior mesenteric artery
• Tumour invading one or both renal pedicles
• Fusiform tumour surrounding the infrarenal aorta
• Tumour encasing the iliac vessels
• Pelvic tumour crossing the sciatic notch

c) Timing of Surgery

Primary or Immediate excision - should precede any other treatment provided that there are no risk factors on imaging.

d) Surgical guidelines: see Appendix V

4.2. PATHOLOGY, BIOLOGY

Results regarding MYCN and histological INPC classification must be available within 6 weeks of surgery.

a - Before handling the surgical specimen, please read the Pathology Guidelines carefully (Appendix II).

b - The pathologist is essential in the process of harvesting sufficient material for the important genetic and biological studies which are mandatory for this study as well as the INPC classification, and to secure material for biological studies in the future.
c- Tumour tissue must be sent to the reference laboratory to assay MYCN copy number. Assessment of 1p deletion is mandatory - I thought it was?

| Tumour and reference DNA should also be collected and stored in proper conditions (Appendix III). |

4.3. TREATMENT GUIDELINES FOR RELAPSED INSS STAGE 1- 3 NB PATIENTS WITHOUT MYCN AMPLIFICATION (Figure 3)

At relapse, a biopsy of the relapsed tumour and a complete work-up are mandatory including imaging (CT or MRI), biochemical factors (LDH), urine catecholamines and metabolites, biological factors (MYCN, 1p del, 17q gain etc) and search for metastatic sites (MIBG and/or Tc scan, bone marrow aspiration and trephine biopsy).

4.3.1. Local relapse (any age)
- FH before: the patient will be operated on only and the tumour completely analysed (histologically and biologically).
- UH before: the patient will be treated on the ongoing HR protocol.

4.3.2. Metastatic relapse (= stage 4), any histology and any age: the patient will be treated on the ongoing HR protocol.

N.B. any change in parameters occurs at relapse biopsy has to be discussed with the national coordinator

4.4. TREATMENT GUIDELINES FOR RELAPSED INSS STAGE 1 PATIENTS WITH MYCN AMPLIFICATION (Figure 4)

At relapse, a biopsy of the relapsed tumour and a complete work-up are mandatory including imaging (CT or MRI), biochemical factors (LDH), urine catecholamines and metabolites, biological factors (MYCN, 1p del, 17q gain etc) and search for metastatic sites (MIBG and/or Tc scan, bone marrow aspiration and trephine biopsy).

Local or metastatic relapse: the patient will be treated on the ongoing HR protocol.

N.B. any change in parameters occurs at relapse biopsy has to be discussed with the national coordinator
5. FOLLOW-UP OF PATIENTS TREATED BY SURGERY ALONE

m1  Post-surgery imaging

Table 10. Follow-up after surgery

<table>
<thead>
<tr>
<th>months</th>
<th>Clinical evaluation</th>
<th>US or CT for abdominal NB</th>
<th>CXR or CT/MRI for thoracic NB</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m3</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m6</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m9</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m12</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m18</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m24</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m30</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m36</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m48</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>m60</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

m = months from surgery

•  CT has to be performed if US appears not adequately informative.

•• choose the most appropriate technique

6. PATIENT REGISTRATION

6.1. At diagnosis

The patients with suspected localized neuroblastoma have to be pre-registered by sending the F1A-PRE-REGISTRATION form to the national coordinator who faxes it to the international coordinator: 41/21/314 33 32. The international coordinator confirms the pre-registration.

As soon as the diagnosis is confirmed and the patient meets the eligibility criteria, the patient will be registered by sending the F1B-REGISTRATION-DIAGNOSIS form to the national coordinator who faxes it to the international coordinator: 41/21/314 33 32. The international coordinator confirms the registration by sending back a patient registration number.

The following forms have to be completed subsequently and sent to the national coordinator: F2-, F3-, F4-, F5-forms.

6.2. At relapse

For patients who relapse after primary surgery, the F1C-REGISTRATION-RELAPSE form will be filled out and sent to the national coordinator who faxes it to the international coordinator: 41/21/314 33 32. The international coordinator confirms the registration.

The following forms have to be filled out subsequently and sent to the national coordinator: F6-forms.

6.3. During follow-up

An annual follow-up will be recorded by means of the F7-forms and sending them to the national coordinator.
7. ADVERSE EVENTS

Any serious adverse event (death from any cause, relapse or grade 4 life-threatening toxicity), will be notified by fax within 24 hrs to the National Study Coordinator, who will transfer the information to the International Coordinator: +41/21/314 33 32 (F8-form).

8. STATISTICAL CONSIDERATIONS

The following statistical projections refer to INSS 2-3 patients eligible for this study, because the contribution of stage 1 patients, due to the very small number of events is going to be negligible. The new study, in a 3 year period, should be able to enroll between 100 and 150 stage 2-3 patients. This group of patients will be merged with the group of 71 patients from LNESG1 with all risk factors available, for a total of 180-220 patients. Assuming a hazard rate of relapse of 6/100 patient/years, and a hazard rate of death of 2/100 pt/years, approximately 20-30 relapses and 8-12 deaths should be observed in the LNESG2 cohort by the end of the 4th year from start of the accrual, for a total of 35-40 relapses and 12-15 deaths in both the 2 patients populations combined. (LNESG1 + LNESG2).

Considering that these factors are present in 10 to 25% of the patients, the study should have an 80% power to detect, in univariate analyses, factors (or combination of factors) associated with a 3-fold increase in relapse rate and a 4-fold increase in mortality. Estimated hazard ratios will have a 95% CL approximately equivalent to:

\[
\text{Observed hazard ratio} \times e^{\frac{1}{2.7}}
\]

that is, for relapse HR $x/\div 2.7$, and for mortality HR $x/\div 3.3$.

It is clear that an uncertainty of this order of magnitude is too large for clinical decisions. However, combined data from these 2 studies will be interpreted in the light of all the available evidence to formulate reasonable treatment guidelines for patients with resectable neuroblastoma who are presumably at increased risk of relapse and death.

Furthermore, the overall results of the 2 studies will be interpreted in the light of the current attitude aimed at reducing unnecessary exposures to toxic treatments in patients with localized NB. Should the results of the LNESG2 confirm the results of LNESG1 and indicate that patients with resectable neuroblastoma have an overall probability of death within 5 years below 10%, the policy of postponing medical treatments in the large majority of these patients, reserving these treatments for those patients who will eventually relapse (15-25%) and, in case, for a small subset of patients that might be consistently found to bear a markedly increased risk of relapse and death, would receive a strong support. With 150 patients, the study should have an 80% power to reject the hypothesis of a 5 yrs survival <90% if the present treatment strategy is associated with a 5yrs survival of at least 96%.

Since this protocol is mainly made of guidelines for the correct treatment of patients with localized resectable NB, with the objective to improve the quality of data collection, this study will be open for 4 years in order to accrue at least 150 new stage 2 patients with no MYCN amplification. This will allow the evaluation of an improved quality of management (> 95% evaluation of prognostic factors such as LDH, tumour banking). If this is achieved, the length of the accrual will be prolonged to an additional 4 years.
9. PUBLICATION POLICY

Eligible patients will be registered over 4 years. According to the statistical evaluation, this will allow observation of sufficient events for the required statistical analyses which will be performed at the earliest at the end of the 4th year from the start of accrual. No publications on the study objectives will be performed until the elapsed time.
10. REFERENCES

9. Seeger RC et al. Association of multiple copies of the N-myc oncogene with rapid progression of NBs. NEJM 1985; 313: 1111-1116
14. Gehring M et al. The 1p deletion is not a reliable marker for the prognosis of pts with NB. Cancer Res 1995; 55: 5366-5369
15. Caron et al. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in pts with NB. NEJM 1996; 334: 225-230
## INSS Classification

<table>
<thead>
<tr>
<th>Stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Localized tumour with complete gross excision, with or without microscopic residual disease; representative ipsilateral lymph nodes negative for tumour microscopically (nodes attached to and removed with the primary tumour may be positive).</td>
</tr>
<tr>
<td>2A</td>
<td>Localized tumour with incomplete gross excision; representative ipsilateral nonadherent lymph nodes negative for tumour microscopically.</td>
</tr>
<tr>
<td>2B</td>
<td>Localized tumour with or without complete gross excision, with ipsilateral nonadherent lymph nodes positive for tumour. Enlarged controlateral lymph nodes must be negative microscopically.</td>
</tr>
<tr>
<td>3</td>
<td>Unresectable unilateral tumour infiltrating across the midline*, with or without regional lymph node involvement; or localized unilateral tumour with controlateral regional lymph node involvement; or midline tumour with bilateral extension by infiltration (unresectable) or by lymph node involvement.</td>
</tr>
<tr>
<td>4</td>
<td>Any primary tumour with dissemination to distant lymph nodes, bone, bone marrow, liver, skin and/or other organs (except as defined for stage 4S).</td>
</tr>
<tr>
<td>4S</td>
<td>Localized primary tumour (as defined for stage 1, 2A or 2B), with dissemination limited to skin, liver, and/or bone marrow^ (limited to infants &lt; 1 year of age).</td>
</tr>
</tbody>
</table>

**Note**

a) Multifocal primary tumour (i.e., bilateral adrenal primary tumours) should be staged according to the greatest disease extent, and followed by a subscript letter M (i.e., 3_M).

*) The midline is defined as the vertebral column. Tumours originating on one side and crossing the midline must infiltrate to or beyond the opposite side of the vertebral column.

^) Marrow involvement in stage 4S should be minimal, ie, < 10% of total nucleated cells identified as malignant on bone marrow biopsy or on marrow aspirate. More extensive marrow involvement would be considered to be stage 4. The MIBG scan (if performed) should be negative in the marrow.
APPENDIX II

PATHOLOGY GUIDELINES

Pathology Guidelines for resectable Peripheral Neuroblastic Tumours / LNESG2

The NB Pathology Guidelines for resectable Peripheral Neuroblastic Tumours have been produced for SIOP/Europe NB Group and were accepted as such by the board as common rules and should serve as reference. Since the pathology review affects eligibility, all cases shall be rapidly reviewed within two to four weeks by the national pathology co-ordinator (list and address at end of Appendix).

1. Pathology guidelines. General remarks and Recommendations

- The paediatric oncologist in charge and/or the surgeon has to inform the local Pathologist and Biologist (local and/or national) in a timely manner about the new patient and the material to be expected.

- The tumour should be transferred from the operating room to the local pathology department under sterile conditions as quickly as possible. Please indicate time until freezing the biological material.

- The local pathologist must know the protocol and must be able to handle the material appropriately both for histological and biological purposes.

- The handling of the tumour tissue should always be performed by the pathologist who, besides the important task of making morphologic diagnoses and giving prognoses based on histopathologic findings, should choose the relevant tumour areas for molecular-genetic/biological analyses as another major task. This procedure is a sine qua non to enable reliable interpretation of the molecular-genetic results for which the exact tumour cell content of the specimen used for these investigations has to be determined. This is possible only if the pathologist evaluates the specimens adjacent to those used for molecular-genetic/biologic analyses.

- At least two macroscopically different tumour areas (if present, nodules are of special interest!) should be chosen for molecular-genetic/biologic analyses. The material selected for molecular-genetic/biologic investigations should be sent as quickly as possible to the National Reference Biology Laboratory. In all instances, tumour material from different tumour areas (nodules are of special interest!) ought to be taken for histologic and molecular-genetic/biologic examination. The reason for this recommendation is based on the observation of tumour heterogeneities at the genetic level (e.g. for the MYCN and/or the chromosome 1p status) and/or at the histologic level (ganglioNB, nodular subtype according to the International NB Classification, INPC, (Shimada et al., Cancer 1999;86:349-363 and 1999;86:364-372) both of which have prognostic implications.

- A close co-operation between pathologists and biologists is essential. Pathologists should inform the biologists if paraffin-embedded samples present a morphologically
• different appearance from material transmitted for molecular-genetic/biologic investigations.

• To enable reliable interpretation of the molecular-genetic results, the **exact tumour cell content of the specimen used for these investigations has to be determined**. This is possible only if the pathologist evaluates the specimens adjacent to those used for molecular-genetic/biologic analyses. The tumour cell content should be mentioned in the Pathology report.

• In addition it is important that material for biological studies is **snap frozen as soon as possible** in separate vials in liquid nitrogen.

• If the case fits the criteria for LNESG II, the national clinical coordinator asks the local pathologist within two weeks to send a HE-stained slide from each block to the national pathologist coordinator.

2. **Sectioning and Securing Tumour Material**

The following procedure is recommended:

• Cut the tumour along the largest diameter and take at least two samples from morphologically different-appearing areas (1x1x1cm) if such are present. Check carefully for the presence of nodules! Tissue from a suspected nodule must always be sampled. Identify the samples specifically (e.g: A, B, etc.), or whatever system accordingly with the practice of each laboratory, and cut each of them into four pieces which are marked with numbers (e.g. tumour specimen A 1-4, specimen B 1-4). More material can be processed in the same way (C, D, etc.), but material from two different areas is the minimum.

• Samples A2,3 and B2,3: **snap freeze as soon as possible** in separate vials in liquid nitrogen or at –70°C carbon dioxide. Before using these for further analyses, making cryosections for the determination of the tumour cell content is mandatory.

• Samples A4 and B4: put in sterile culture medium (RPMI 1640) for preparation of tumour cell suspensions.

• Samples A1 and B1: make **10 touch preparations** (at least 5). The slides are air-dried and unfixed and sent at room temperature to the biologist for fluorescence based in situ hybridisation (FISH) and image cytometry (ICM). For image cytometry a rapid fixation of slides in MFA is advisable.

• After making the touch preparations, these pieces should be fixed in formalin for routine histologic examination. This also should include the determination and indication of the tumour cell content versus content of normal cells, such as Schwann cells, lymphocytes, fibrovascular stroma etc.; amount of necrosis should be indicated as well. This information is crucial for the interpretation of the FISH, PCR, and ICM and results!

• The samples should be forwarded as soon as possible! After this procedure, the rest of the surgical specimen can be fixed in formalin and worked-up according to standard guidelines. The whole central 4 mm section of the tumour at the plane of the largest diameter should be embedded. A minimum of one tumour section per cm should be
embedded from the whole specimen including central and peripheral areas of the tumour. Surgical margins must be reliably and reproducibly identifiable.

- **Storage of Tumour Material**

  In addition to material necessary for the mandatory biological analyses of the protocol it is **essential to store suitable material** to conduct further biological and genetic analyses and to allow review and quality assessment studies. Therefore, in collaboration with the biologist **make sure adequately labeled snap frozen tumour material in liquid nitrogen can be stored and secured, by the biologist** and ideally by both the biologist and the pathologist.

### 3 Histology Report

**International NB Pathology Classification**

The tumour should be classified according to the International NB Pathology Classification (Shimada et al. Cancer 1999;86:349-363 and 1999;86:364-372). Morphologic features including differentiation, mitotic and karyorrhectic index, mitotic rate and calcifications should be indicated along with the age allowing the prognostic categorization of the tumour. For details of how to use the system, see: Joshi VJ. Peripheral Neuroblastic Tumours: Pathologic classification based on recommendations of International NB Pathology Committee (Modification of Shimada Classification). Pediatr Devel Pathol 2000;3:184-199.

Pathologist must be aware of a recent alteration concerning nodular ganglioNBs which could be of favourable or unfavourable histology depending on the same criteria used for NBs, stroma poor.

e.g: for nodular ganglioNBs, classic and variant:

<table>
<thead>
<tr>
<th>Prognostic Category</th>
<th>Criteria</th>
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<tr>
<td><strong>Favourable Subset</strong></td>
<td>NB component shows degree of differentiation and Mitosis Karyorrhexis Index appropriate for Favourable Histology for the age of the patient</td>
</tr>
<tr>
<td><strong>Example:</strong> poorly differentiated NBtous component with low MKI in a 1-year-old child.</td>
<td></td>
</tr>
<tr>
<td><strong>Unfavourable Subset</strong></td>
<td>NBtous component shows degree of differentiation and Mitosis Karyorrhexis Index appropriate for Unfavourable Histology for the age of the patient</td>
</tr>
<tr>
<td><strong>Example:</strong> Differentiating NBtous component with low MKI in a 6-year-old child.</td>
<td></td>
</tr>
</tbody>
</table>


Moreover, according to the INPC, the term “peripheral neuroblastic tumour, not classifiable” is recommended for a tumour which belongs unequivocally to the peripheral neuroblastic tumour entity, but which cannot be allocated with certainty into one of the four basic categories which are NB (Schwann cell stroma-poor), ganglioNB intermixed (Schwann cell stroma-rich), ganglioNB nodular (Schwann cell stroma-rich/-dominant and stroma-poor), ganglioneuroma (Schwann cell stroma-dominant). Other terms recommended by the INPC to be used for tumours giving rise to problems in classification, are: “NB (Schwann cell stroma-poor), NOS”. This term is used for tumours with an unequivocal categorisation, but the subtype, i.e. undifferentiated, poorly differentiated, differentiating, cannot be assessed due to
poor quality of the sections, extensive haemorrhage, necrosis, crush artefacts, etc. (see INPC). “GanglioNB, NOS” is used for a tumour with a stroma-rich/-dominant appearance containing areas of extensive calcification which may obscure a stroma-poor nodule.

**Surgical margins of resection**
There should be a comment regarding whether there are tumour cells infiltrating the resection margins or not, without making any conclusion as to whether the tumour residual is microscopic or macroscopic.

**Histologic report on the specimens A1, B1 etc.**
This report must clearly indicate the estimated percentage of tumour cells, i.e. neuroblastic/ganglionic cells, versus Schwann cells and other normal cells contained in the samples used for the biologic studies. *A copy of the report should then be submitted to the molecular biologist.*

**Regional Lymph Node Examination**
Biopsy of regional nodes is highly recommended whenever feasible despite their appearance. The histology report should include information on site and number of positive nodes, type of metastatic spread, i.e. presence of micrometastases (less than 2 mm), intranodal parcellled metastases, intranodal massive metastases, nodal metastases with extracapsular extension in localisations not adherent to the resected tumour specimen, and morphologic description of the tumour infiltrate.

**Immunohistochemistry techniques used for differential diagnosis.**
In some cases, i.e. NBs, undifferentiated subtype according to the INPC, the differential diagnosis can present difficulties. In these instances, the use of the following antibodies is recommended: MIC2 (CD99), actin, desmin, myogenin, cytokeratin, leukocyte common antigen (CD45). These antibodies are usually negative in neuroblastic tumours. Positive markers are: vimentin, CD56 (N-CAM), NB84a, neuron specific enolase (NSE), neurofilament triplet protein (NF), synaptophysin, tyrosine hydroxylase (TH), and the protein gene product 9.5. However, it has to be kept in mind that these markers, although often positive, may also be negative in undifferentiated NBs. In addition, NB84a does also react with epithelial cell and endothelial cells. Although GD2 is positive in virtually all cases of neuroblastic tumours and very useful in detection of neuroblastic cells in the bone marrow, anti GD2 staining cannot be recommended for use on paraffin sections (due to very high background staining). Moreover, GD2 expression is not restricted to neuroblastic tumours!

Lymph nodes. For differential diagnosis see above. In addition, the morphological result can be controlled by immunohistology using anti CD56 and anti TH antibodies. If the NB84a antibody is applied, its reaction with endothelial cells should be kept in mind.

**Exact Determination of the Tumour Cell Content**
*It is mandatory that the tumour cell content is evaluated in all samples used for molecular-genetic/biologic investigations and DNA analyses.* FISH/ICM is performed on touch preparations of piece 1. In this case, the tumour cell content is determined on the formalin-fixed paraffin-embedded material of piece 1 and is included in the pathology report.

Pieces 2 and 3 are snap frozen by the pathologist and are used for immunohistological investigations and DNA extraction. For interpretation of the PCR results, the knowledge of the exact tumour cell content is especially important. The following procedure is recommended: frozen sections, ideally cut from two sides, i.e. from the top and from the
bottom, are made before DNA extraction for PCR, FISH or other investigations.

4. Pathology Review

The SIOP Europe NB Board and Pathology Committee decided that tumours are to be reviewed if the case fits the criteria for LNESG II. The national clinical coordinator in each country supervises and follows the slide logistics between the local and the national pathologist coordinator, because he/she is the one to decide whether any tumour meets the criteria for any protocol, and he/she is the one who receives our evaluation form and notifies the data centre.

Quality control for slide review of LNESG II cases

• The local pathologist must know the protocol and must be able to handle the material appropriately both for histological and biological purposes.
• The local clinician receives the diagnosis from his pathologist and discusses the case with the national clinical coordinator who, on the basis of all clinical and laboratory data, makes the decision of the type of protocol to be chosen.
• If the case fits the criteria for LNESG II, the national clinical coordinator asks the local pathologist within two weeks to send a HE-stained slide from each block (and possibly one or two representative paraffin embedded blocks) to the national pathologist coordinator.
• If the national pathologist coordinator agrees to the local pathologist's conclusion, he/she fills the evaluation form and sends within one week one copy to the national clinical coordinator who notifies the data centre, one to the local clinician who is responsible for the treatment, and one to the local pathologist.
• If the local pathologist disagrees with the reference pathologist, both should discuss the case and try to find a consensus.
• If no consensus can be obtained, the reference pathologist sends the slides to another expert pathologist/member of the review group who is available for immediate review and discussion on phone (make sure of this before you send the case!).
• The decision on prognosis given in the pathology evaluation form, must always be based on at least two pathologist's opinions (local+expert from the same country or expert from country X + expert from country Y), and their initials should be added on the final form.

NB: Regular meetings are organized by the panel to retrospectively review all the cases enrolled in the trial.
APPENDIX IIa

BONE MARROW EXAMINATION GUIDELINES

General Remarks on Bone Marrow Aspirations
Guidelines for the evaluation of minimal residual disease in bone marrow have been developed for the purpose of improving initial staging accuracy, treatment response evaluation, and, ultimately, patient care. These well established guidelines have been published and should be followed here (Sverts et al, Journal of Histochemistry and Cytochemistry, in press).

In brief:
Bone marrow (BM) aspirates from two sites (left and right) have to be performed. In addition, two trephine biopsies are mandatory. Three sequential aspirations for each puncture site are necessary: one for bone marrow smears, the second for immunocytology, classical cytogenetics and FISH, and the third which should not contain heparin for PCR or other techniques. The aspirations from the different sites should not be pooled together. Two to four syringes with plugs and 10 to 20 glass slides for the bone marrow smears and one polished cover glass should be prepared.

- **First aspiration and preparation of bone marrow smears**
  Half a milliliter of BM is aspirated into the syringe and immediately dropped on a glass slide.

- **Second aspiration for immunocytology**
  The appropriate amount of anticoagulant [e.g. 200µl heparin (5000IE/ml) in 10 ml peripheral blood or 5 ml BM] is aspirated into the syringe. To avoid excessive dilution with peripheral blood, each aspirate should be ideally 2-3 ml.

- **Third aspiration for PCR**
  To enable a highly sensitive technique for detecting rare neuroblastoma cells with the PCR technology, an additional BM sample is needed. Aspirate 2 to 3 ml BM in a separate syringe containing the appropriate amount of EDTA or ACD or another anticoagulant specified by the laboratory.
APPENDIX IIb

The International NB Pathology Classification (the Shimada system)

A/ Classification of Peripheral neuroblastic tumours

<table>
<thead>
<tr>
<th>Categories</th>
<th>Stroma</th>
<th>subtype</th>
<th>Other items</th>
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<tr>
<td>NB</td>
<td>stroma poor</td>
<td>1. undifferentiated</td>
<td>MKI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. poorly differentiated</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. differentiating</td>
<td>Mitotic rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Calcification</td>
</tr>
<tr>
<td>GanglioNB</td>
<td>composite, stroma rich and</td>
<td>1. intermixed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>poor</td>
<td>2. nodular</td>
<td>MKI</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mitotic rate</td>
</tr>
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<td></td>
<td></td>
<td>Calcification</td>
</tr>
<tr>
<td>Ganglioneuroma</td>
<td>stroma dominant</td>
<td>1. maturing</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. mature</td>
<td></td>
</tr>
</tbody>
</table>

B/ Prognostic categories of Peripheral neuroblastic tumours

1. NBs and ganglioNBs, nodular (classic and variants: morphologic features determined in the nodule(s)).

• **Favorable**
  
  < 1.5 yrs Poorly differentiated or differentiating and low or intermediate MKI tumour
  
  1.5-5 yrs Differentiating and low MKI tumour

• **Unfavorable**
  
  < 1.5 yrs a) undifferentiated tumour
             b) high MKI tumour
  
  1.5-5 yrs a) undifferentiated or poorly differentiated tumour
             b) intermediate or high MKI tumour
  
  ≥ 5 yrs All tumours

2. Ganglioneuromas and ganglioNB intermixed: favorable
The same prognostic categorization in a graphical and easy to remember form:

**PROGNOSTIC CATEGORIZATION OF NBS AND NODULAR GANGLIONBS**  
*CLASSIC AND VARIANTS, PROGNOSIS DETERMINED BY THE NODULE*  
*U and red: unfavourable.*  
*F and green: favourable*

### Age <1.5 yrs

<table>
<thead>
<tr>
<th></th>
<th>Undifferentiated</th>
<th>Poorly differentiated</th>
<th>Differentiating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low MKI</td>
<td>U</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>Intermediate MKI</td>
<td>U</td>
<td>F</td>
<td>F</td>
</tr>
<tr>
<td>High MKI</td>
<td>U</td>
<td>U</td>
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### Age 1.5-6 yrs

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<tr>
<td>Low MKI</td>
<td>U</td>
<td>U</td>
<td>F</td>
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<tr>
<td>Intermediate MKI</td>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>High MKI</td>
<td>U</td>
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### Age > 6 yrs

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<th>Undifferentiated</th>
<th>Poorly differentiated</th>
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</tr>
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<tbody>
<tr>
<td>Low MKI</td>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>Intermediate MKI</td>
<td>U</td>
<td>U</td>
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</tr>
<tr>
<td>High MKI</td>
<td>U</td>
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**PROGNOSTIC CATEGORIZATION OF GANGLIONEUROBLASTOMAS, INTERMIXED AND GANGLIONEUROMAS.**

### Any age

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<td>F</td>
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APPENDIX IIc

Recommandation for the sampling of tumours

Cut the tumour along the largest diameter and take at least two samples from morphologically different-appearing areas (1x1x1cm) if such are present. Tissue from a suspected nodule must always be sampled.

![Diagram of tumour sampling]

Figure 1

Tumour samples (at least 1x1x1cm) are designated with the letters A, B, etc. There are two methods of sectioning. From piece 1 touch preparations are made before formalin fixation; in the variant on the right hand side, one of the central sections is designated as piece 1. Pieces 2, 3 (and 4) are snap frozen. Piece 4 is put in RPMI 1640 for classic cytogenetic investigation and/or making cytospin preparations. In case of small specimens, touch preparations and snap freezing are the first priorities.

APPENDIX IIId

Immunohistochemistry techniques used for differential diagnosis.

<table>
<thead>
<tr>
<th>Peripheral Neuroblastic Tumours, differential diagnosis</th>
<th>TH*</th>
<th>Vimentin</th>
<th>Pgp 9.5</th>
<th>CD 45</th>
<th>Desmin</th>
<th>MyoD1/Myogenin</th>
<th>CD99</th>
</tr>
</thead>
<tbody>
<tr>
<td>NB (Schwannian stroma-poor)</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ewing’s/pPNET</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Non-Hodgkin Lymphoma (lymphoblastic type)</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

Notes:
1- Antibodies directed against NSE (Neuron Specific Enolase) find a great interest in the identification of peripheral neuroblastic tumours and neuroendocrine tumours. However, they have a limited value in the diagnosis of poorly or undifferentiated tumours because NSE expression is documented in a wide spectrum of tumours. NSE, present in a high concentration in the brain, corresponds to the gamma subunit of...
enolase and is also known to be expressed by other tissues than nervous system. By immunohistochemical techniques performed on fixed tissue sections, NSE can be found not only in neuronal or neuroendocrine cells but also in epithelial cells, mesenchymal cells, lymphoid cells, and other hematopoietic cells.

2- Chromogranin, synaptophysin and neurofilaments are frequently expressed by peripheral neuroblastic tumours. The more differentiated NBs also could express vaso-active intestinal peptide (VIP) and other neuronal peptides.

3- Vimentin is expressed by the neuroblastic cells of many tumours in the undifferentiated subtype and of some tumours in the poorly differentiated subtype.

4- Tables describe immunohistochemical data in typical cases. Variant or aberrant antigenic expressions could be found in some individual cases. This fact highlights the recommendation to use a battery of selected antibodies with appropriate controls. The results of the immunostaining has to be interpreted along with the histopathological findings.

References


<table>
<thead>
<tr>
<th>Name</th>
<th>Address</th>
<th>Country</th>
</tr>
</thead>
<tbody>
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<td>Email: <a href="mailto:gabriele.amann@akh-wien.ac.at">gabriele.amann@akh-wien.ac.at</a></td>
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<td>Phone: 47-23-071400 office: 47-23-074076</td>
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<td>Fax: 39 010 377 6590 Email: <a href="mailto:claudio.gambini@ospedale-gaslini.ge.it">claudio.gambini@ospedale-gaslini.ge.it</a></td>
<td>Italy</td>
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<td>Phone: 34 96 3864 146 Fax: 34 96 3864 173 Email: <a href="mailto:Samuel.navarro@uv.es">Samuel.navarro@uv.es</a></td>
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<td>France</td>
</tr>
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APPENDIX III

BIOLOGY GUIDELINES

General Remarks and Recommendations for Biology

In NBs, investigation of the MYCN gene status, of the chromosomal region 1p36.3 and of the DNA content gives critically important information. More recently identification of several other chromosomal abnormalities on 17q, 2p, 3p, 11q, 14q, have improved neuroblastoma classification into different risk groups. (Lastowska, 2001, Vandesompele 2001, Brodeur 2003). The recommended methods to analyse these individual parameters are summarised below. Notice that besides classical molecular-genetic/biological methods, powerful genomic-wide assays are available.

As already pointed out in the Pathology Guidelines, a reliable interpretation of the molecular-genetic results is possible only if the exact tumour cell content of the specimen used for these investigations is determined. This is possible only if the biologist performs molecular genetic/biological analyses on the specimens adjacent to those evaluated by the pathologist.

Procedures for the Determination of the Tumour Cell Content

In case of resected tumours (see also Pathology Guidelines), FISH/ICM is performed on touch preparations of piece 1 (see Figure 1, appendix IIb). In this case, the tumour cell content is determined on the formalin-fixed paraffin-embedded material of piece 1 and is included in the pathology report. The cellularity of the prints must be checked by phase contrast microscopy and at least 500 nuclei should be present on the slide to be analysed. In case of low cellularity, or if only frozen samples are available, MYCN status will be established from frozen sections.

As a general rule, before performing biological/genetic analyses, be certain you have received the piece/touch prints/section used to evaluate the exact tumor cell content by the pathologist (see Pathology Guidelines) or alternatively, ask the pathologist to give percentage of tumour cells of the fragments received, on frozen sections. In case unambiguous genetic aberrations are present, also FISH results can be used to determine the tumor cell content.

Genetic Parameters to be analysed

General remark
Therapy stratification based on genetic markers is becoming increasingly important. This makes commitment to the highest possible reliability of the involved markers mandatory. To guarantee reliable and standardized quality of genetic features, a quality-assessment study was initiated by the European Neuroblastoma Quality Assessment Group (ENQUA). This study led to the formulation of guidelines that are applicable to all kinds of tumors and that contain the standardization of techniques, including the exact determination of the tumor cell content. A common terminology has been developed for molecular genetic results that will be used in this study. (J Clin Oncol 21:2077-2084. 2003)
1. MYCN Oncogene

Methods and general remarks.
The MYCN copy number can be determined by:

1. Fluorescence based in situ hybridisation (FISH) as standard and first priority.
2. PCR. In case of PCR used for MYCN evaluation these data should be confirmed with a second method, preferably FISH.

- **For FISH**, all slides and all areas of the individual slides have to be screened and analysed carefully. FISH for MYCN status can be performed on frozen sections as well. An analysis by FISH on frozen (or paraffin, if not available) section, is mandatory in case of suspicion of heterogeneous amplification. At least 200 cells from different areas of the slides should be counted including all cells (different hybridisation patterns and also cell without signals which give valuable information about the hybridisation efficiency). At least 3 representative images should be archived, for review sessions.

- **Recommendation of DNA probes.** For FISH either the MPbio or Vysis (or other validated commercial probe) MYCN probe or the pNb9/pNb101 (Dr. Rocchi, Italy) and a chromosome 2 specific probe either specific to the centromere (D2Z, MPbio) or to chromosomal region 2q or 2p (to avoid misinterpretation by centromeric associations) are recommended. PCR results are only accepted when the PCR data are compared with FISH results.

- **MYCN copy number.** Irrespective of the method used, the copy number has to be indicated together with the number of chromosomes 2. The MYCN and chromosome 2 copy numbers have both to be indicated in the internal reports.

- **For MYCN definitions and report of results see Table 1.** The terms given in this table should be used for reporting the results to the clinicians and for documentation (data base).
Table 1. Definitions and report of the results for *MYCN* status determined by fluorescence in situ hybridisation and by polymerase chain reaction.

**FISH - MYCN**

*MYCN amplification* = Greater than four-fold increase of the signal number as compared with the reference probe located on the same chromosome.

*MYCN amplification heterogeneous: focal* = Group(s) of cells (at least 20 cells) showing gene amplification surrounded by non-amplified tumor cells. *Scattered* = The number of cells seen as amplified should be indicated.

*MYCN gain* = An up to four-fold excess of gene copies or chromosomal regions in relation to the reference probe on the same chromosome.

No *MYCN amplification* = Equal number of signals of the *MYCN* probe and the reference probe.

No result (please specify) = Unclear or not interpretable result

  - Not enough tumour cells contained in the sample
  - No tumour
  - Not done

**PCR - MYCN**

*MYCN amplification* = Greater than four fold increase of the signal intensity relative to the internal reference located on the same chromosome.

  Needs further clarification by FISH!

*MYCN gain (=inconclusive)* = An up to four-fold increase of the band intensity in relation to the internal reference. Needs further clarification by FISH!

No *MYCN amplification*

No result (please specify) = Unclear or not interpretable result

  - Sample contains less than 60% of tumour cells
  - No DNA
  - No tumour
  - Not done
2. Chromosome 1p36.3 status

- Methods and general remarks

The integrity of the short arm of chromosome 1 (1p36.3) can be determined by 3 methods, i.e.

1. FISH
2. PCR
3. CGH/arrayCGH

**FISH is mandatory.** PCR for 1p36.3 status has to be performed only for cases showing a predominant population of 2/2 ratio and FISH imbalance. In contrast, analyses done by FISH or CGH give information on the chromosomal level, i.e. on the relation between the numbers of centromeres and subtelomeric regions of chromosome 1. If two centromeres to one subtelomeric region is observed, it most likely corresponds to a loss of heterozygosity (LOH) found by PCR. However, every other disproportion of centromeres and subtelomeric regions but with more than one 1p36.3 signals (i.e. a 3 to 2, 4 to 3, 5 to 3 ratio etc.) found by FISH can but does not necessarily reflect the presence of an LOH. Vice versa, lack of an LOH does not necessarily reflect lack of cytogenetic aberrations on chromosome 1p36.3. Therefore, it is strongly recommended to use at least 2 methods (PCR and FISH or CGH) in the detection of chromosome 1p36.3 aberrations in order to obtain sufficient information to address all possible changes in this important chromosomal region! For definition and interpretation of a so-called allelic imbalance (PCR) see Table 2.

- For determination of the 1p status, the tumour cell content has to be at least 60 per cent

For FISH, all slides used and all areas of the individual slides have to be screened and analysed carefully. In general, 500 tumour cells is the minimum cell number which should be available for analysis and at least 200 cells should be counted including all cells (different hybridisation patterns including cells without signals). In case of an unequivocal aberrant result, 50 cells can be regarded as sufficient. At least 3 representative images should be archived, for review sessions.

- For array-CGH, as initial basis we will consider a fluorescence ratio of tumour/normal DNA < 0.8 as deleted. Genomic profiles will be shown at review sessions.

- For chromosome-CGH, a segment of 1p is considered as deleted, when showing a ratio of fluorescence of tumour/normal DNA < 0.8. The interpretation should be sometimes cautious for 1p representing a highly GC-rich region., especially if other GC-rich chromosomes, as 17, 19, and 22, appear as lost. Genomic profiles will be shown at review sessions.

- Recommendation of DNA probes

For PCR e.g. the primers specific for D1S76 and D1S80 or others within the region 1p36.33 are advisable. FISH investigations are recommended to be performed with the probe D1Z2 (or 1p36 probe MPbio) together with a centromeric probe (D1Z1) or a probe located on the long arm of chromosome 1 (to avoid misinterpretation caused by centromeric associations) in a double colour FISH approach.
• **For Chromosome 1p36.3 definitions and report of results** see Table 2. The terms given in this table should be used for giving the results to the clinicians and for documentation (data base).

• **Recommendation**
  If less than 50% of the tumour cells show deletion or imbalance by FISH, more slides, samples or nuclei isolated from paraffin material shall be analysed; paraffin material should also be used when FISH on touch slides was not successful. To exclude methodical reasons, the number of cells with less 1p36.3 signals as compared to the number of centromeric signals has to be compared always with the percentage of cells with more 1p36.3 signals.

• **Report of the molecular-genetic results**
  The molecular-genetic results should be reported in the following manner: A detailed description of the results and the methods used including percentages of tumour cells versus normal cells, hybridisation pattern etc. should be described in a report section. In addition, PCR and FISH results should be discussed together with an interpretation of their meaning. In the conclusion section, the result should be given as briefly and precisely as possible using the terminology given in Tables 1 and 2.

**Table 2.** Definitions and report of the results for chromosome 1p36.3 status determined by by fluorescence in situ hybridisation or Polymerase chain reaction

<table>
<thead>
<tr>
<th><strong>FISH – Chromosome 1p36.3</strong></th>
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<td><strong>Deletion</strong> = Presence of only one signal for the concerned chromosomal region (2/1 ratio possibly combined with 4/2 in the same tumor; 3/1, 4/1)</td>
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<td><strong>FISH imbalance (=inconclusive)</strong> = Disproportion of the ratio of signals of the reference probe to the signals of the concerned chromosomal region with more than one signal (3/2, 4/3, 4/2, 5/3, and so on); needs further clarification by polymerase chain reaction.</td>
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<td><strong>Focal deletion/imbalance</strong> Focal occurrence of cells (at least 20) showing a deletion or imbalance surrounded by tumor cells with a balanced hybridization pattern.</td>
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<td><strong>No deletion, no FISH imbalance</strong> Equal number of signals of the concerned chromosomal region and the reference probe.</td>
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<td><strong>No result (please specify)</strong> = Unclear or not interpretable result</td>
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<td>Not enough tumour cells contained in the sample</td>
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**PCR-Chromosome 1p36.3**

**Allelic loss (LOH) =** Complete or almost complete disappearance of one band

**Allelic imbalance (=inconclusive) =**

One band is weaker than the other band when compared with the ratio observed with constitutional DNA; this result reflects either allele disequilibrium or LOH and). This can mean either allele disequilibrium (e.g. two paternal and one maternal chromosomes 1) or LOH and needs further clarification (FISH, re-evaluation of tumor cell content)

**No allelic loss, no allelic imbalance =** Equal intensity of both bands

**No result (please specify) =** Unclear or not interpretable
- Constitutional homozygosity
- Sample contains less than 60% of tumour cells
- No DNA
- No tumour
- Not done

### 3. Chromosome 17q gain

**Methods and general remarks**

It appears to be of importance to introduce the status of 17q for the assessment of localised neuroblastoma, especially in those without MYCN amplification, as it discriminates near-triploid tumours, with whole chromosome imbalances, of good prognosis, from near-diploid/tetraploid tumours, with partial chromosome imbalances. It is associated in the majority of the studies to an unfavourable outcome (Abel, 1999; Bown, 2001, Brinkschmidt, 2001, Vandesompele, 2005)

The status of the long arm of chromosome 17 can be determined by:

- FISH with a probe distal to 17q21, by reference to a 17p probe
- Array- or chromosome-CGH (see CGH section below)
  
  Tumour cell content has to be at least 60%.

**Recommendations for the determination of 17q status by FISH**

Any validated probe, or mixture of probes, located in 17q22-25 can be used, together with a 17p probe. Alternatively, the MPO/p53 dual-colour probe of MPbio is suitable. Counting of signals should be done as for 1p.

**Report of 17q status FISH results**

- No gain: equal number of 17q signals, in relation to 17p signals
- Gain: excess of 17q signals in relation to 17p signals.
Report of 17q status CGH results (see CGH section below)
- No gain: 17p and 17q (or clones in these two regions) show an equal ratio
- Gain: a segment of 17q (or clones in this region) shows specifically a ratio >1.2 as compared to the whole chromosome 17.

4. Other whole/partial chromosome imbalances: 2p, 3p, 11q, 14q, CGH analyses

There are an increased number of reported chromosome imbalances (e.g. on chromosomes 2p, 3p, 11q, 14q) that are additional potential prognostic markers believed to give further prognostic information (Lastovska, 2001, Vandesompele, 2005).

CGH analyses (on chromosomes or pan-genomic arrays) are best suitable for the detection of these multiple whole/partial chromosome imbalances. They allow to classify neuroblastoma into distinct genetic types with specific clinical behaviour.

Report of CGH results

The following items should be specifically reported:

- no imbalances [ ]
- gains/loss of whole chromosomes [ ]
- gains/loss partial chromosome segments [ ]
- 1p loss: [ ]
- 2p gain: [ ]
- 3p loss: [ ]
- 11q loss: [ ]
- 17q gain: [ ]
- others, please specify [ ]
- other amplifications, please specify [ ]

5. DNA content

Methods and general remarks.
Two methods, i.e. Flow Cytometry (FCM) and Image Cytometry (ICM) can be used for the assessment of the tumour cell DNA content. For both techniques reference cells derived from human tissue (peripheral blood lymphocytes) from normal individuals or the same patient should be used.

Report of the FCM/ICM results. The report should indicate the method used and specify the number of tumour cells versus normal cells contained in the sample under investigation. The results on the DNA content of the tumour cells should be given in absolute numbers.
6. Gene products of prognostic interest

The protocol intends to evaluate the expression of TRKA and CD44 expression.

- Detection of the antigen will be performed on cryosections and, if feasible, also on paraffin sections

TRKA. The primary component of the high-affinity nerve growth factor receptor (NGFR) is encoded by the trkA (p140trk) proto-oncogene. This oncogene is highly expressed in approximately two thirds of neuroblastomas; particularly in stage 1, 2 and 4s and in tumours with a normal MYCN copy number (Kogner, 1993, Nakagawara, 1993). Co-expression with the low affinity NGFR distinguishes a prognostic subset (Ryden, 1996). TRKA expression will be measured by immunohistochemistry. Adequate antibody to Trk-A must be checked.

CD44. CD44 is a cell surface glycoprotein involved in cell-cell and cell-matrix interactions. The CD44 standard molecule is expressed in all stage 1 and 2 tumors, but only in half of the more advanced cases (Favrot, 1993, Gross, 1994, Combaret and Gross, 1996). As a negative relationship between MYCN amplification and CD44H expression was reported (Gross, 1994), CD44-negative tumors represent a subset of highly aggressive and metastatic tumors. (Combaret & Gross, 1997). CD44 expression can be measured on frozen sections and paraffin embedded tumors. J173 antibody (Coulter) can be used on frozen section. Ideally paraffin sections should be used and evaluation be done together with a histopathologically trained person. Antibodies suitable for paraffin sections are under investigation.

7. Central Review

The molecular-genetic/biological data will be reviewed by the ENQUA central review panel through the web based database and/or by exchanging tumour material. All MYCN results will be reviewed among the group. For the other biologic/genetic data, only cases showing divergent interpretations will be discussed in the central review panel.

8. Storage of Tumour Material, Slides, peripheral blood and Bone Marrow Samples

In addition to material necessary for the mandatory biological analyses of the protocol it is essential to store suitable material to conduct further biological and genetic analyses and to allow review and quality assessment studies. The mandatory tumour material to store includes:

- Snap frozen tumor material (in liquid Nitrogen). Indicate the time interval between resection and freezing.
- Touch preparations, cytopsins of tumor samples and fixed cells stored at –20°C (-80°C).
- Cell suspensions (including DMSO), if available, in liquid Nitrogen.
- Peripheral blood is needed as reference for molecular-biologic studies.
- Plasma and/or serum Samples (2 aliquots of 200ul each) for determination of tumor DNA (Combaret, 2003) should be stored at -80°C. Samples should be prepared by centrifugation at 2000 rpm g for 10 min at 4°C, followed by careful aliquoting and freezing at -80°C.
Furthermore enough FISH and PCR pictures, CGH profiles, have to be stored for documentation, review purposes and inclusion into the database.

**List and addresses of the reference biologists**

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APPENDIX IV

RADIOLOGICAL GUIDELINES

1.0 INTRODUCTION

1.1 The aim of these guidelines is to define standard procedures that will enable accurate evaluation of the resectability of a localised NB.

1.2 The different sections of the notification forms are intended to answer specific questions. They must be filled in according to the results of the whole imaging work-up.

1.3 It is not necessary to describe which investigation (i.e., US or CT) has provided a given piece of information.

2.0 METHODS OF INVESTIGATION

2.1 The radiologist will select the most convenient imaging methods according to the available equipment in his/her institution but should follow the INSS guidelines, (15).

- **Thoracic**: MRI. If not available, CT with i.v. contrast
- **Abdominal**: CT or MRI, plus US
- **Cervical**: MRI. If not available CT with i.v. contrast
- **Pelvic**: CT or MRI

2.2 The relationship between the tumour and major adjacent vessels is of critical importance for defining the resectability of a localised NB.

Different techniques could provide this information, e.g. US and CT after injection of contrast medium. For MRI the choice of vertical planes parallel to the great vessels is indicated.

2.3 Pre-operative MRI sequences should be chosen to give maximum anatomical detail. Contrast medium is not mandatory with MRI, unless doubts about tumour limits exist.

2.4 MRI is recommended to confirm the hepatic infiltration, especially if the posterior segments of the liver (which are not easily explored by US) are involved.

3.0 RADIOLOGICAL FORMS

3.1 Choice of the form

3.1.1 There is a specific form for each major anatomic region: cervical, thoracic, abdominal, pelvic.

3.1.2 If a tumour extends across one compartment (cervico-thoracic, thoraco-abdominal, abdomino-pelvic), its centre will define the primary tumour site. In this case a second form will be filled in related to the second compartment involved by the tumour.
For example: an abdominal tumour extending across the pelvic brim will be evaluated by filling in the abdominal form, while the part of the tumour extending in the pelvic area will be evaluated by filling in the form for pelvic NBs.

3.1.3 A box not filled in is considered as information not available from the diagnostic work-up.

3.2 Tumour size

3.2.1 In case of a round and well defined tumour the size will be defined by the diameter.

3.2.2 If the tumour can not be distinguished from adherent lymph nodes (in which case the term "mass" or "tumour mass" will be preferred, as described in 2.3.1.) the measurements should be approximated to the nearest half-centimetre and should be taken in the 3 orthogonal axes (x/y/z).

3.2.3 If the tumour has an irregular shape, tangents to the boundaries should define the three dimensions (see figure next page).

4.0 SOME DEFINITIONS FOR THE TERMS IN USE

4.1 TUMOUR and MASS

4.1.1 The term "tumour" indicates a well defined neoplasm, whereas the term "mass" or "tumour mass" should be used when there are lymph nodes indistinguishable from the tumour because of its irregular shape. This should be clearly indicated when filling in the forms.

4.2. LOCATION

4.2.1 For cervical, thoracic or pelvic NBs the vertebrae are identified by two boxes: the first indicates the cervical, thoracic, lumbar or sacral level; the second indicates the number of the involved vertebra

4.2.2 For abdominal NBs the definition of midline or lateral is given by the centre of the tumour. The limits of the midline are defined by INSS criteria, i.e. a tumour is crossing over the midline if it infiltrates beyond the opposite side of the vertebral column (see: JCO 1993, 11: 1466-1477). At a level of the sacrum the lateral limits are defined by a tangent to the lateral border of L5.

4.2.3 Midline tumours are classified according to their relationship with the great vessels.

For example, a perivascular tumour encompasses the aorta while a lateral tumour is described as "intervascular-renal" if it is located between one of the IVC or aorta described as and the kidney (which is usually displaced).
4.3 LOCAL EXTENSION OF THE PRIMARY TUMOUR

4.3.1 Extension to intervertebral foramina and spinal canal

This defines the tumour involvement by continuity of one or more intervertebral foramina with or without tumour extension into the spinal canal.

In most cases it is difficult to measure the intraspinal tumour proportion due to its half-moon shape. For practical purposes we may describe it by the percentage of its maximum size in relation to the diameter of the spinal canal: <25%, between 25 and 50%, >50%.

An abdominal NB has a pelvic extension if the tumour passes over the pelvic brim.

4.3.2 Relationship between tumour and vessels

To describe this important aspect, the use of the following three terms is suggested: separated, in contact and encased

- **separated** describes the situation where a clear-delineated-fat plane is found between tumour and vessels. The thickness has little relevance.

- **in contact** describes the situation where no clear-delineated-fat plane is found between tumour and vessels.

- **encased** means that one or more vessels are within the tumour mass.

4.3.3 Relationship between tumour and contiguous organs

To describe this aspect, the use of the following two terms is suggested: separated or in contact

- **separated** describes a situation where either a clear-delineated-fat plane is found between the tumour and contiguous organs or where a relative mobility is seen on dynamic US.

- **in contact** describes the situation where no clear delineated fat plane is found between the tumour and contiguous organs.

5.0 INFORMATION DERIVED BY THE IMAGING STUDIES THAT MAY HELP THE SURGEON TO DEFINE THE RESECTABILITY OF A NB

5.1 THORACIC NB

5.1.1 Relevant information includes: a) crossing of the midline, b) extension into contiguous compartments (neck or abdomen), c) intraspinal extension, d) relation to the airways. There is no practical benefit in localising the oesophagus.

5.1.2 Superior thoracic NB

The surgical access by thoracotomy does not usually allow a good view of the upper part of the tumour. Therefore the pre-surgical evaluation should concentrate on: a) the
possible cervical extension of the tumour, b) the involvement of the subclavian vessels and of their branches, c) the involvement of the roots of the brachial plexus.

5.1.3 **Inferior thoracic NB of the left side**

The medullary artery originates in 85% of the cases between T9 and T12 on the left side. However, in 15% of the cases it originates on the right side and is usually associated with a supplementary circulatory circuit. Tumour growth within this area induces the formation of collaterals.

Therefore arteriography of the medullary artery is not mandatory, since the preoperative risk is minimal, although it should be mentioned to the parents. On the other hand an invasive procedure such as arteriography in the very young ones is risky. Important elements to evaluate the risk are: a) the precise tumour extension towards the vertebrae T9-T12, b) the infiltration of the intervertebral foramina, and c) the infiltration of the posterior wall of the descending aorta.

5.2 **ABDOMINAL NBS**

5.2.1 **Vascular investigation**: the relationship of tumour to the veins is important to define, allowing better assessment of surgical risk. In fact, arterial injuries are easier and quicker to repair than injuries to veins.

5.2.2 **Porta hepatis**: the invasion can be detected if the distance between the structures is enlarged. The relationship of the tumour with this region is crucial at the spleno-mesenteric confluence duodeno-pancreatic block).

5.2.3 **Inferior mesenteric artery**: the visualisation is of modest interest, except if the celiac axis and the superior mesenteric artery are involved.

5.2.4 **Renal vessels**: evaluation is essential to measure the possibility and ease or otherwise of performing a nephrectomy. To this purpose it is especially important to define the relationship between the tumour and both renal artery and vein (separated, in contact, or encasing). If the tumour is encasing the vessels, one has to specify if this is also true for the hilar region, defined by a tangent to the upper and lower lip of the hilum (in axial plane). The stretching of the vessels by the tumour is considered as a pre-operative risk factor.

5.2.5 **Contiguous organs**: definition of the relation of the tumour to the contiguous organs relies on the evaluation of the dividing planes which are often better seen by real time US. However, the lack of demonstrable dividing planes (in contact) does not allow prediction of surgical difficulties. In perspective, the study of the information derived by the pre-operative diagnostic forms and the surgical report could help to clarify this aspect.

5.2.6 **Duodeno-pancreatic region**: this is a difficult area to examine but in most pts US with CT or MRI with i.v. contrast medium will provide adequate assessment. Imaging studies of the gastro-intestinal tract add no practical information.
5.2.7 **Renal function:** the course of the ureter should also be evaluated and this may require a late abdominal X-ray at the end of investigation by CT scan. If the examination is performed by MRI an intravenous pyelogram should be added.

It could also be of interest to define the borders between the tumour and the adrenal gland.

5.3 **PELVIC NBS**

5.3.1 The tumour extension at the level of the crossing of the iliac vessels is important. It is especially relevant to know the relationship of the tumour with the iliac veins and their branches. The involvement of the sciatic notch is considered as a risk factor.
APPENDIX V

SURGICAL GUIDELINES

1. Aims of Surgery

The aim of surgery in localised NB is to achieve complete excision of the tumour with minimal morbidity.

2. Definitions

2.1. Complete Excision

a) Complete excision is defined as the removal of all visible tumour, including the removal of abnormal lymph nodes and the sampling of normal lymph nodes.

b) It is important to assess the likelihood of microscopic residual tumour even if macroscopic complete resection has been achieved. This can be aided by pathological examination of biopsies taken from the tumour bed as well as examination of the tumour margins.

2.2. Excision with minimal residual disease (<5% of original or <5ml volume)

“Minimal” macroscopic residual disease remains after operation. The amount should be estimated by the surgeon in ml or as percentage of the original and evaluated by post-operative imaging.

2.3. Incomplete gross excision

More than 5% or 5ml of tumour remaining after operation. The residual should be evaluated by post-operative imaging.

3. Pre-Operative Criteria of Resectability

Complete excision is the overriding aim of operation. The imaging techniques presently available should allow evaluation of resectability in most cases. LNESG1 analysis demonstrated that vascular encasement increases the risk of surgical complications. If it is clear from the preoperative imaging that surgical excision would be dangerous, then the child should be treated with chemotherapy according to the unresectable localized neuroblastoma protocol. Spiral CT or Digital Subtraction Angiography may provide accurate information on vascular anatomy. It is of note that the estimation of risk factors is not always precise and that chemotherapy has its own complications. In this situation, it is mandatory that the relative risks of operation be compared with those of chemotherapy in consultation with the oncologist.
4. Risk factors for Primary or Immediate excision.

Detailed clinical examination and imaging studies can assist the surgeon in deciding whether a localised tumour is amenable to primary surgical resection. The presence of any of the risk factors listed below may well contra-indicate primary surgery. This is true even if the risk factors were not recognised prior to the operation. In this situation the serious consideration should be given to abandoning the operation. These decisions must involve the oncologist in charge of the patient.

Risk Factors Related to Localisation:

4.1. Neck

- Tumour encasing vertebral and/or carotid artery
- Tumour encasing brachial plexus roots
- Tumour crossing the midline

4.2. Thorax

- Tumour encasing the trachea or principal bronchus
- Tumour encasing the origin and branches of the subclavian vessels
- Thoraco-abdominal tumour, peri-aortic fusiform tumour
- Lower left mediastinal tumour, infiltrating the costo-vertebral junction between T9 and T12

4.3. Abdomen

- Adrenal tumour infiltrating the porta hepatis
- Suprarenal tumour infiltrating the branches of the superior mesenteric artery at the mesenteric root
- Suprarenal tumour surrounding the origin of the coeliac axis, and of the superior mesenteric artery
- Tumour invading one or both renal pedicles
- Fusiform tumour surrounding the infrarenal aorta
- Tumour encasing the iliac vessels
- Pelvic tumour crossing the sciatic notch

5. Timing of Surgery

Primary or Immediate excision - should precede any other treatment provided that there are no risk factors on imaging.

6. Surgical Procedures

6.1. Surgery of NB aims to completely remove the primary tumour with evaluation of its extension. The abdominal and/or thoracic cavity should be thoroughly explored, with biopsy of any suspicious lesion.
6.2. It may occur that macroscopic residual disease remains when an excision which was expected to be complete turns to be incomplete (2.2. and 2.3.). Operations which leave residual disease are acceptable but indicate inadequate preoperative evaluation. (Although the incompleteness of the excision seems not to affect the prognosis in absence of positivity of biological factors, every effort should be made to plan a correct excision). Patients in whom no attempt at resection was made will be treated according to the unresectable protocol.

6.3. Surgical approach

In the case of a retroperitoneal lesion, a transverse laparotomy (at times extended to a thoraco-phreno-laparotomy) is recommended.

Large thoracic lesions may require a double thoracotomy at a distance of 2-3 intercostal spaces, using the same soft tissue incision.

A 'trap-door' incision including neck, clavicle and sternum (ref) gives good access to a lesion in the dome of the thorax.

In some instances, a combined surgical approach is required and may need more than one operating session. Examples include:

a) approach through cervicotomy and thoracotomy in cervico-mediastinal or upper-mediastinal NB;

b) laparotomy and posterior sacral - coccygeal approach in some cases of pelvic NB;

c) bilateral thoracotomy in some cases of mediastinal NB extending beyond the midline (if two sessions, allow 3-4 weeks interval between them);

d) laminecotomy associated with thoracic or abdominal operation.

6.4. Lymph Node Evaluation

Depending on the site of the primary tumour, lymph nodes from the following regions should be examined and removed if they appear abnormal:

a) Lateral cervical region: jugular chain and supraclavicular area;

b) Chest: mediastinal lymph nodes above and below the tumour;

c) Abdomen: coeliac nodes (infra-diaphragmatic), mid-aortic (at renal level) and iliac region (bilaterally).

6.5. Intraspinal Extension

If feasible the extraspinal mass should be removed even though intraspinal disease remains (but see 9.6.4). Macroscopic disease may be left in the intervertebral foramina, especially when there is a risk of leakage of spinal fluid and/or jeopardising the blood supply of the spinal cord.
6.6. **Intraspinal disease with neurological symptoms**

- The urgent need in this situation is relief of pressure on the spinal cord rather than excision of the primary. This is best achieved by chemotherapy.

6.7. **Nephrectomy**

Nephrectomy is **not acceptable** as part of primary or immediate excision.

If imaging suggests that this is the only way to achieve excision, the patient should be treated according to **unresectable protocol**.

6.8. **Tumour Relation with Great Vessels**

In order to gain further information on the accuracy of the pre-operative imaging, the intra-operative findings should be described in detail. Particular attention should be given to the technical difficulties encountered when the tumour is in contact with the vessels.

6.9. **Tumour incision or rupture**

If the tumour is firm then the operation should proceed to complete excision.

If the tumour is friable and further dissection would lead to tumour spillage then the **operation should be abandoned** and the patient treated on the **unresectable protocol**.

6.10. **Clips**

Titanium or absorbable clips should be used if necessary to avoid interference with subsequent imaging.
APPENDIX VI

NEUROBLASTOMA WITH INTRASPINAL EXTENSION
(Dumbbell NB)

by Bruno De Bernardi and Dominique Plantaz

1 Background

1.1 The connections existing between the sympathetic nervous system and the spinal cord account for the ability of NB to infiltrate the intervertebral foramina with occasional involvement of the spinal canal. Despite the fact that tumour growth almost invariably remains extradural, it may still cause spinal cord compression which can progress to irreversible paraplegia. Early diagnosis and prompt treatment of spinal cord compression in NB is therefore of critical importance.

1.2 Dumbbell NB tends to present at a younger age, is commonly associated with intra-thoracic disease and is significantly more frequent amongst children with localised, rather than metastatic disease.

1.3 Evaluation of intraspinal extension has been greatly improved thanks to modern diagnostic imaging (CT scan and MRI). MRI has proven to be especially effective in demonstrating both the infiltration of the intervertebral foramina and the invasion of the spinal canal. On the bases of MRI, 10-15% of children with NB have documented foramina or intraspinal involvement, although only half of them present with neurologic signs of spinal cord compression, and few develop paraplegia.

1.4 Treatment of symptomatic spinal cord compression has evolved over the last few years. Until the mid-80s, decompressive laminectomy was performed as a rule in an attempt to avoid progression to paraplegia. Surgery was frequently carried out even when paraplegia had already developed. Radiotherapy was considered less effective, although some reports suggested that it could be as effective as surgery in metastatic disease.

1.5 Both laminectomy and radiotherapy have potential disadvantages. The former requires highly experienced hands and may carry the risk of late spinal deformities. Radiation reduces and alters the growth of irradiated vertebrae and increases the risk of late secondary malignancy.

1.6 The use of chemotherapy as an effective alternative to laminectomy or radiotherapy was first reported in 1984. Since then, several authors have confirmed that chemotherapy can be used successfully instead of surgery or radiotherapy, without compromising the chance of neurologic recovery. The concern that chemotherapy might not work quickly enough to prevent permanent neurologic damage has been contradicted by several reported cases.
2 Diagnosis and Evaluation of Spinal Cord Compression

2.1 Diagnosis: Early detection of spinal cord compression can be difficult especially in younger children, unless one thinks about this possibility in any child with a tumour. The most common symptoms are back pain, reduced mobility of the legs and/or arms, sensory and sphincteric dysfunction.

The presence of motor deficit is particularly important since children who develop complete motor loss usually experience little or no recovery. Infants with congenital dumbbell tumours have a particularly poor outcome with regards to neurologic recovery.

2.2 The extent of motor loss can be graded as follows;

0 Capable of unassisted ambulation, may have pain and/or difficulty with micturition.
1 Can only walk with assistance.
2 Antigravity strength alone.
3 Presence of trace movement alone.
4 Complete motor and sensory loss.

Note: most episodes of spinal cord compression in NB occur in infants, for whom this scale is only partially applicable.

2.3 Evaluation: As previously stated, MRI is the best method to detect infiltration of the intervertebral foramina and invasion of the spinal canal by NB. Although a CT scan may be adequate in many cases, it is recommended that MRI be used wherever possible, particularly in the diagnostic work-up of infants with cervical, thoracic, or pelvic disease.

2.3.1 It may be difficult to accurately assess the volume of the intraspinal tumour due to the half-moon configuration it commonly takes in that particular site. As indicated in the Radiologic Guidelines (Addendum 3), it is suggested that the maximum diameter of the tumour be expressed as a percentage in relation to the diameter of the spinal canal: <25%, between 25-50%, >50%. The extent of the intraspinal component is measured in relation to the vertebrae involved.

2.3.3 Myelography and lumbar puncture are of no diagnostic use, and are absolutely contraindicated.

3 Treatment of spinal cord compression

3.1 Spinal cord compression without symptoms

3.1.1 The regular use of MRI has increased the number of cases with documented infiltration of foramina (with or without invasion of the spinal canal). However, in the majority of cases, especially when the intraspinal component is modest (less than 50% of the diameter), there are no neurological symptoms.

3.1.2 There is very little, if any, evidence that an asymptomatic intraspinal tumour may grow any more after the extraspinal component of the tumour has been resected. Information related to the few, well documented cases suggests that the intraspinal NB in patients with no neurological symptoms tends to remain stable or even regress without specific treatment.
3.1.3 Administering chemotherapy to patients with unresectable tumour and asymptomatic intraspinal extension to reduce size tumour is also likely to be effective in treating the intraspinal component. No specific additional treatment is required unless neurologic symptoms develop.

3.2 Spinal cord compression with neurologic signs

3.2.1 Patients with localised NB who present with signs of spinal cord compression do require specific treatment. If neurological deficits are present and/or progress rapidly therapeutic decisions must be made in a matter of hours or at most within 1-2 days. Laminectomy, or laminotomy is preferable only in infants showing very rapid where neurologic deterioration. This however occurs infrequently. The decision to use chemotherapy, on infants with partial compromise or a stable neurological deficit, should be discussed urgently between the oncologist and the neurosurgeon.

3.2.2 Once it has been decided that chemotherapy should be given, there is no urgent indication to remove the extraspinal tumour (which is likely to be unresectable, and surgery runs the risk of worsening the neurological deficit). The tumour should be biopsied (by Tru-cut, fine needle, or open biopsy). It may be necessary to delay this for a few days, to avoid postponing initial chemotherapy.

3.2.3 Medical treatment is as follows:
Dexamethasone 0.5 mg/kg I.V. bolus followed by 0.2 mg/kg/day I.V. in 3 divided daily doses. Chemotherapy is given using "Carbo-VP16". A second course should be given 21 days after the beginning of the first course. (See section 10 of the protocol for further details about chemotherapy doses and administration).

3.2.4 A further MRI scan should be obtained following the first course of chemotherapy. If either no improvement occurs or if deterioration of the neurological signs are observed, and there is no response by the intraspinal component, laminotomy and excision of the intraspinal component should be taken in consideration.

3.2.5 If the tumour remains unresectable after 2 courses of VP-Carbo, then 2 courses of CADO should be given, and assessment carried out with MRI as above.

4 Asymptomatic intraspinal residual tumour following chemotherapy.

It is not necessary to remove asymptomatic intraspinal residual tumours after chemotherapy. The removal of any residual extraspinal component should be undertaken only after careful assessment of the intraspinal residue.

REFERENCES


APPENDIX VII

DECLARATION OF HELSINKI

WORLD MEDICAL ASSOCIATION DECLARATION OF HELSINKI

Ethical Principles for Medical Research Involving Human Subjects

Adopted by the 18th WMA General Assembly Helsinki, Finland, June 1964 and amended by the
29th WMA General Assembly, Tokyo, Japan, October 1975
35th WMA General Assembly, Venice, Italy, October 1983
41st WMA General Assembly, Hong Kong, September 1989
48th WMA General Assembly, Somerset West, Republic of South Africa, October 1996
52nd WMA General Assembly, Edinburgh, Scotland, October 2000

Introduction

The World Medical Association has developed the Declaration of Helsinki as a statement of ethical principles to provide guidance to physicians and other participants in medical research involving human subjects. Medical research involving human subjects includes research on identifiable human material or identifiable data.

It is the duty of the physician to promote and safeguard the health of the people. The physician's knowledge and conscience are dedicated to the fulfilment of this duty.

The Declaration of Geneva of the World Medical Association binds the physician with the words, "The health of my patient will be my first consideration," and the International Code of Medical Ethics declares that, "A physician shall act only in the patient's interest when providing medical care which might have the effect of weakening the physical and mental condition of the patient."

Medical progress is based on research which ultimately must rest in part on experimentation involving human subjects.

In medical research on human subjects, considerations related to the well-being of the human subject should take precedence over the interests of science and society.

The primary purpose of medical research involving human subjects is to improve prophylactic, diagnostic and therapeutic procedures and the understanding of the aetiology and pathogenesis of disease. Even the best proven prophylactic, diagnostic, and therapeutic methods must continuously be challenged through research for their effectiveness, efficiency, accessibility and quality.

In current medical practice and in medical research, most prophylactic, diagnostic and therapeutic procedures involve risks and burdens.

Medical research is subject to ethical standards that promote respect for all human beings and protect their health and rights. Some research populations are vulnerable and need special protection. The particular needs of the economically and medically disadvantaged must be recognized. Special attention is also required for those who cannot give or refuse consent for themselves, for those who may be subject to giving consent under duress, for those who will not benefit personally from the research and for those for whom the research is combined with care.

Research Investigators should be aware of the ethical, legal and regulatory requirements for research on human subjects in their own countries as well as applicable international requirements. No national ethical, legal or regulatory requirement should be allowed to reduce or eliminate any of the protections for human subjects set forth in this Declaration.

Basic Principles for All Medical Research
It is the duty of the physician in medical research to protect the life, health, privacy, and dignity of the human subject.
Medical research involving human subjects must conform to generally accepted scientific principles, be based on a thorough knowledge of the scientific literature, other relevant sources of information, and on adequate laboratory and, where appropriate, animal experimentation.

Appropriate caution must be exercised in the conduct of research which may affect the environment, and the welfare of animals used for research must be respected.

The design and performance of each experimental procedure involving human subjects should be clearly formulated in an experimental protocol. This protocol should be submitted for consideration, comment, guidance, and where appropriate, approval to a specially appointed ethical review committee, which must be independent of the investigator, the sponsor or any other kind of undue influence. This independent committee should be in conformity with the laws and regulations of the country in which the research experiment is performed. The committee has the right to monitor ongoing trials. The researcher has the obligation to provide monitoring information to the committee, especially any serious adverse events. The researcher should also submit to the committee, for review, information regarding funding, sponsors, institutional affiliations, other potential conflicts of interest and incentives for subjects.

The research protocol should always contain a statement of the ethical considerations involved and should indicate that there is compliance with the principles enunciated in this Declaration.

Medical research involving human subjects should be conducted only by scientifically qualified persons and under the supervision of a clinically competent medical person. The responsibility for the human subject must always rest with a medically qualified person and never rest on the subject of the research, even though the subject has given consent.

Every medical research project involving human subjects should be preceded by careful assessment of predictable risks and burdens in comparison with foreseeable benefits to the subject or to others. This does not preclude the participation of healthy volunteers in medical research. The design of all studies should be publicly available.

Physicians should abstain from engaging in research projects involving human subjects unless they are confident that the risks involved have been adequately assessed and can be satisfactorily managed. Physicians should cease any investigation if the risks are found to outweigh the potential benefits or if there is conclusive proof of positive and beneficial results.

Medical research involving human subjects should only be conducted if the importance of the objective outweighs the inherent risks and burdens to the subject. This is especially important when the human subjects are healthy volunteers.

Medical research is only justified if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the results of the research.

The subjects must be volunteers and informed participants in the research project.

The right of research subjects to safeguard their integrity must always be respected. Every precaution should be taken to respect the privacy of the subject, the confidentiality of the patient's information and to minimize the impact of the study on the subject's physical and mental integrity and on the personality of the subject.

In any research on human beings, each potential subject must be adequately informed of the aims, methods, sources of funding, any possible conflicts of interest, institutional affiliations of the researcher, the anticipated benefits and potential risks of the study and the discomfort it may entail. The subject should be informed of the right to abstain from participation in the study or to withdraw consent to participate at any time without reprisal. After ensuring that the subject has understood the information, the physician should then obtain the subject's freely-given informed consent, preferably in writing. If the consent cannot be obtained in writing, the non-written consent must be formally documented and witnessed.
When obtaining informed consent for the research project the physician should be particularly cautious if the subject is in a dependent relationship with the physician or may consent under duress. In that case the informed consent should be obtained by a well-informed physician who is not engaged in the investigation and who is completely independent of this relationship.

For a research subject who is legally incompetent, physically or mentally incapable of giving consent or is a legally incompetent minor, the investigator must obtain informed consent from the legally authorized representative in accordance with applicable law. These groups should not be included in research unless the research is necessary to promote the health of the population represented and this research cannot instead be performed on legally competent persons.

When a subject deemed legally incompetent, such as a minor child, is able to give assent to decisions about participation in research, the investigator must obtain that assent in addition to the consent of the legally authorized representative.

Research on individuals from whom it is not possible to obtain consent, including proxy or advance consent, should be done only if the physical/mental condition that prevents obtaining informed consent is a necessary characteristic of the research population. The specific reasons for involving research subjects with a condition that renders them unable to give informed consent should be stated in the experimental protocol for consideration and approval of the review committee. The protocol should state that consent to remain in the research should be obtained as soon as possible from the individual or a legally authorized surrogate.

Both authors and publishers have ethical obligations. In publication of the results of research, the investigators are obliged to preserve the accuracy of the results. Negative as well as positive results should be published or otherwise publicly available. Sources of funding, institutional affiliations and any possible conflicts of interest should be declared in the publication. Reports of experimentation not in accordance with the principles laid down in this Declaration should not be accepted for publication.

Additional Principles for Medical Research Combined with Medical Care

The physician may combine medical research with medical care, only to the extent that the research is justified by its potential prophylactic, diagnostic or therapeutic value. When medical research is combined with medical care, additional standards apply to protect the patients who are research subjects.

The benefits, risks, burdens and effectiveness of a new method should be tested against those of the best current prophylactic, diagnostic, and therapeutic methods. This does not exclude the use of placebo, or no treatment, in studies where no proven prophylactic, diagnostic or therapeutic method exists.

At the conclusion of the study, every patient entered into the study should be assured of access to the best proven prophylactic, diagnostic and therapeutic methods identified by the study.

The physician should fully inform the patient which aspects of the care are related to the research. The refusal of a patient to participate in a study must never interfere with the patient-physician relationship.

In the treatment of a patient, where proven prophylactic, diagnostic and therapeutic methods do not exist or have been ineffective, the physician, with informed consent from the patient, must be free to use unproven or new prophylactic, diagnostic and therapeutic measures, if in the physician's judgement it offers hope of saving life, re-establishing health or alleviating suffering. Where possible, these measures should be made the object of research, designed to evaluate their safety and efficacy. In all cases, new information should be recorded and, where appropriate, published. The other relevant guidelines of this Declaration should be followed.
GUIDELINES FOR THE TREATMENT OF PATIENTS WITH LOCALIZED RESECTABLE NEUROBLASTOMA (LNESG2 STUDY)

PARENT INFORMATION AND CONSENT FORM

You and your child are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Thank you for reading this.

What is the purpose of the study?

If your doctor gives you this letter, he or she will have explained that your child has a form of cancer called neuroblastoma which is a cancer found almost exclusively in infants and young children. Neuroblastomas arise in nerve cells, which run in a chain down the back of the chest and abdomen (tummy).

As you are aware, the tumour has already been removed by the surgeon. Other tests have confirmed that it is localized and has not spread anywhere else in the body - we call this Stage 1 or Stage 2 neuroblastoma depending on whether some of it may have been left behind (very rarely it may even be possible to remove Stage 3 tumours without any other treatment as well). As well as tumour stage, tests on the tumour itself can help us in trying to predict the outlook. The most important one of these discovered so far is a protein called MYCN oncogene (part of the genetic make-up of the tumour). If this oncogene is present in the tumour, additional treatment with chemotherapy (anti-cancer medication) is necessary with the exception of stage 1 neuroblastoma. If the oncogene is not present, surgery alone is the only treatment to be given. In the former study (LNESG1), we have given no further treatment in this situation and the majority of children do well. However, the disease may return in a small number (<10%) of children. We are trying to establish whether any particular factors to do with blood tests, how the tumour looks down the microscope and other aspects of the genetic make-up of the tumour make it more likely to come back. We would like to carefully examine all these things in your child's case. If the tumour does come back, then the tumour can be removed surgically again in most cases. For a few children, more intensive treatment may be required.

How many children will participate and for how long?

All children with this type of tumour in many European countries will be invited to take part in this study. If you decide to take part, your child will be one of 400 subjects Europe-wide. It is expected that the research study will continue for at least 4 years.
**Who is organising this study?**

Doctors in Europe are working together within a group called SIOP (International Society of Pediatric Oncology) to develop treatment strategies for neuroblastoma. Within every participating European country, a national group of paediatric oncologists is in charge of the patients with neuroblastoma.

**Do I have to take part?**

Taking part is entirely voluntary. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form, of which you will be given a copy. This is known as informed consent. If you decide to take part you are still free to withdraw at any time without giving a reason, without affecting the standard of care your child receives. Your doctor may also withdraw your child from the study if he/she considers it to be appropriate.

**What will happen to my child if I take part?**

We will want to perform a blood test (LDH level). After the tumour has been removed, we will want to perform some tests on it (what the tumour looks like down the microscope, aspects of its genetic make-up). Tumour slides will be sent to the national pathologist and/or study pathologist for review and confirmation of diagnosis. No further treatment after surgery is planned. However, we will want to monitor your child regularly in the clinic.

If the tumour does come back, we would need to establish the extent of disease, and look again at aspects of the tumour. Depending upon these results, your child will have further surgery only, or receive more intensive treatment to include chemotherapy, which will then be explained to you.

With your permission, after we have performed these tests we would like to store some tumour tissue for future scientific research to improve our understanding of neuroblastoma.

**What are the possible risks of taking part?**

There is a small chance that the tumour may recur.

**What are the possible benefits of taking part?**

The information we get from this study may help us to improve treatment for children with neuroblastoma in the future.

**What happens if something goes wrong?**

We do not expect your child to suffer any additional problems over and above those which are recognized risks associated with the previous standard treatment (in this case, surgery). If any harm arises, there are no special compensation arrangements. However if you have any cause to complain about any aspect of the way you have been approached or your child has been treated, the normal National Health Service complaints mechanisms are available to you.
Will my child’s participation in this study be kept confidential?

All information that is collected about your child during the course of the study will be kept strictly confidential. With your permission, we will inform your child’s General Practitioner of his/her participation in the study, the treatment and progress. We would also like to ask your permission to send data on your child and the tumour to a central database. All information regarding your child will be made anonymous in any publications resulting from this study.

Who can I contact to discuss this further?

Your doctor ................................................................. will be happy to answer any questions you may have and will explain the treatment in more detail. He/she can be contacted by telephone as shown in the headed details.

Thank you for taking time to read the information about this research study and for considering taking part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled “Medical Research and You”. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy of this can be made available to you.

June 2004
AF, MBP
PARENT/CHILD CONSENT FORM

Title of Project: GUIDELINES FOR THE TREATMENT OF PATIENTS WITH LOCALIZED RESECTABLE NEUROBLASTOMA (LNESG2 STUDY)

Name of Researcher: __________________________

Please initial box (delete words as necessary)

1. I confirm that I have read and understand the information sheet(s) dated …….(Final version) for the above study and have had the opportunity to ask questions.

2. I understand that my/my child’s participation is voluntary and that I am/he/she is free to withdraw at any time, without giving any reason, without my/his/her medical care or legal rights being affected.

3. I understand that sections of any of my/my child’s medical notes may be looked at by responsible individuals from the UKCCSG or from regulatory authorities where it is relevant to my/my child’s taking part in research. I give permission for these individuals to have access to my/my child’s records.

4. I understand that central review of pathology slides will be required for the above study. I give permission for responsible individuals to have access to this material

5. I agree/agree for my child to take part in the above study.

6. I agree that my/my child’s GP is notified about participation in the above study

7. I agree for samples of my/my child’s tumour to be stored and used in the biological research studies integrated into this study.

8. I agree that data about my child relating to this study may be sent to countries involved into the study (SIOP)

__________________________ __________________________ ___________________________
Name of patient Date Signature

__________________________ __________________________ ___________________________
Name of parent/guardian Date Signature

__________________________ __________________________ ___________________________
Name of person taking consent Date Signature
(if different from researcher)

__________________________ __________________________ ___________________________
Researcher Date Signature

1 for patient;  1 for researcher;  1 to be kept with hospital notes