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Chromogranin A and neuron-specific enolase in neuroblastoma: Correlation to stage and prognostic factors

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ABSTRACT
Chromogranin A (CgA) and neuron specific enolase (NSE) are important markers in adult neuroendocrine tumors (NET). Neuroblastoma (NB) has certain neuroendocrine properties. The aim of this study was to correlate blood concentrations of CgA, chromogranin B (CgB), and NSE to prognostic factors and outcome in children with NB. Blood samples from 92 patients with NB, 12 patients with benign ganglioneuroma (GN), 21 patients with non-NB solid tumors, 10 patients with acute leukemias, and 69 healthy children, were analyzed. CgA concentrations were higher in neonates vs. children older than one month in the control group ($p < 0.0001$), and in neonates with NB vs. the control group ($p < 0.01$). CgA and NSE concentrations were higher in patients with stages 3 and 4 disease ($p < 0.05$ and $p < 0.05$), in patients having tumors with amplification of MYCN ($p < 0.05$ and $p < 0.001$), or chromosome 1p deletion ($p < 0.05$ and $p < 0.05$). NSE correlated to the tumor size at diagnosis ($p < 0.001$) and to tumor related death ($p < 0.01$) in NB. CgA and NSE concentrations were elevated in patients with NB and especially in those with advanced disease. Both CgA and NSE correlated to genetic markers, while only NSE correlated to primary tumor size and outcome in NB. We found that CgA and NSE are clinically valuable tumor markers in NB and they merit prospective clinical evaluations as such.

Introduction
Neuroblastoma (NB) is a childhood cancer emerging from immature neuronal cells of the sympathetic nervous system. NB exhibits a unique biological and clinical heterogeneity among human cancers, which ranges from tumors with spontaneous differentiation to benign ganglioneuroma (GN) and even complete regression, to aggressive tumors with high mortality. Negative prognostic markers comprise age over 18 months, advanced tumor stage, amplification of the MYCN-oncogene \cite{1}, loss of heterozygosity on the short arm of chromosome one (1p LOH) \cite{2}, and DNA index $\leq 1$ \cite{1}.

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Chromogranin A (CgA) is an acidic, monomeric protein, co-stored and co-released with hormones and neuropeptides from storage vesicles in a variety of neural, endocrine, and neuroendocrine cells [3]. CgA serves as a clinically relevant tumor marker in several adult neuroendocrine tumors (NETs), where increasing concentrations suggest disease progression and need for intensified therapy [4]. CgA has been reported to be elevated in NB and to correlate with tumor stage and prognosis [5]. CgA is co-localized with another member of the chromogranin family, chromogranin B (CgB), a peptide less studied in tumors derived from the nervous system such as NB and GN. Neuron-specific enolase (NSE) is on the other hand a well-established tumor marker in both NET and NB, and is known to be elevated in advanced tumor stages and a prognostic marker of poor outcome [6, 7]. It has been previously reported a linear correlation between CgA concentrations in plasma and tumor volume in a murine xenograft NB model [8], and it could also be speculated that CgA might be a prognostic marker also in clinical NB, and if so, to compare this property with that of NSE.

This study was undertaken to measure concentrations of CgA, CgB, and NSE in a multi-center, retrospective material of serum and plasma samples taken at diagnosis in children with NB, and to correlate the above concentrations to prognostic factors and clinical outcome.

**Materials and methods**

**Patients and controls**

**Group 1** consisted of patient samples from a frozen (−70°C) bank of plasma and serum samples from 62 children with NB referred to one of the four regional Swedish pediatric oncology centers in Stockholm, Uppsala, Gothenburg, and Lund, between 1986 and 1995. Thirty additional patients with NB were admitted to the pediatric oncology department at Uppsala University Hospital between 1995 and 2010 (NB, n = 62 + 30 = 92). **Group 2** consisted of blood samples from 12 patients with untreated GN. The demographic, clinical, and biological features of groups 1 and 2 are shown in Table 1.

**Group 3** consisted of blood samples from patients with non-neural crest-derived solid tumors (Hodgkin’s disease, n = 5; non-Hodgkin’s lymphoma, n = 3; Wilms’ tumor, n = 3; non-Wilms’ renal tumor, n = 3; rhabdomyosarcoma, n = 3; miscellaneous solid malignant tumors, n = 4) or leukemias (acute lymphatic leukemia, n = 9; acute myeloid leukemia, n = 1), n = 21 + 10 = 31. The median age in this group was 7.5 and the 25th/75th percentile was 2.3/12.8 years (range 0.1–17.2; female/male: 11/20).

**Table 1.** Clinical data on neuroblastoma and ganglioneuroma patients.

<table>
<thead>
<tr>
<th></th>
<th>NB (n = 92)</th>
<th>GN (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years:</td>
<td>1.7 (0.7–3.5)</td>
<td>5.7 (4.1–7.2)</td>
</tr>
<tr>
<td>Age range, years:</td>
<td>0–18</td>
<td>2.6–11</td>
</tr>
<tr>
<td>Gender: female/male</td>
<td>44/48</td>
<td>2/10</td>
</tr>
<tr>
<td>Stage* (NB): 1/2A/3/4/4S</td>
<td>12/18/51/15/6</td>
<td>—</td>
</tr>
<tr>
<td>MYCN-amplification: present/absent/N.D.</td>
<td>24/59/9</td>
<td>0/9/3</td>
</tr>
<tr>
<td>1p LOH: present/absent/N.D.</td>
<td>19/43/30</td>
<td>0/5/7</td>
</tr>
<tr>
<td>Ploidy: diploid-tetraploid/aneuploid/N.D.</td>
<td>26/30/36</td>
<td>—</td>
</tr>
<tr>
<td>Follow-up, years: mean (range)†</td>
<td>20 (3–27)</td>
<td>13.4 (10–24)</td>
</tr>
<tr>
<td>Deaths</td>
<td>40 (43%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Age is presented as median (25th–75th percentile).

*Stage classification according to the INSS.

†Survivors. N.D. – not determined.
The control group consisted of 1) healthy neonates where blood was collected after birth from the umbilical vein \((n = 10)\); 2) healthy neonates subjected to routine metabolic screening on day 3–7 \((n = 32)\), and 3) infants and children prior to a minor surgical or diagnostic procedure (e.g., inguinal hernia repair, urethrocystoscopy, circumcision, extraction of osteosynthesis material) at our outpatient surgery clinic \((n = 27)\), \(n = 10 + 32 + 27 = 69\). The median age in this group was 0.63 and the 25th/75th percentile was 0.0/5.4 (range 0.0–14.3; female/male 29/40). Analyses of CgA, CgB and NSE in patients, and healthy controls, were approved by the relevant ethical committees.

Clinical data extracted from the hospital records included age at diagnosis, gender, NB stage according to the International Neuroblastoma Staging System (INSS) [9], prognostic factors, treatment, outcome, and individual CgA, NSE and CgB. Most patients admitted for a suspected solid tumor after 1995 were screened for CgA, NSE and CgB. In our Department of Clinical Chemistry the upper normal concentration for CgA until 2010 was 4 nmol/L. In 2010 it was adjusted to 6 nmol/L, and in 2013 to 3 nmol/L. The upper limit of normal concentration for CgB was 1.8 nmol/L and for NSE 18 µg/L. MYCN-amplification was detected using Southern blot analysis [10] between 1987 and 1992, followed by interphase fluorescent in situ-hybridization after 1992 [11]. 1p LOH was detected using polymerase chain reaction [12] and ploidy was determined by image cytometry [13]. Patients were treated according the European (SIOP) protocol currently in use at the time.

**Sample handling**

Peripheral venous blood samples were collected either using sterile Vacutainer® 2 or 5 mL serum tubes without additives (BD, Franklin Lakes, NJ), or using 2 or 5 mL sodium-heparin tubes (BD). The samples were sent to the Department of Clinical Chemistry, spun at 1,300 g for 10 min at 4°C, and the supernatant divided into aliquots. Samples arriving from other hospitals than the University Children's Hospital were transported on dry ice. The samples were stored at −70°C until further processing.

**Analysis of samples**

The samples were analyzed for CgA and CgB as described previously [14, 15] and the results were expressed in nmol/L. NSE was analyzed using a commercial kit (Delfia NSE, Wallac Oy, Turku, Finland) according to the manufacturer’s instructions and the results were expressed in µg/L. Since the sample volume in some cases was small, a priority was given to firstly CgA, then CgB, and finally NSE. Thus, in some cases, only one or two of the three variables were analyzed. Neither CgA nor NSE concentrations seem to differ in simultaneous heparin plasma and serum (M. Stridsberg, unpublished observation). This was confirmed in eight patients were both plasma and serum samples were available (CgA: \(r = 0.90, p < 0.01\); NSE \(r = 0.95, p < 0.001\), respectively; Spearman rank order test). Therefore, the serum and plasma samples were pooled during analysis. CgB is not suitable for analysis in serum due to poor reproducibility (M. Stridsberg, unpublished observation), thus only plasma samples were analyzed.

**Statistical analysis**

Descriptive statistics are given in Table 1. Comparisons between groups were made using the Mann-Whitney U-test and Spearman’s rank correlation was used to assess the correlations between variables. Cox proportional hazards regression was used to associate the prognostic
variables to the outcome. To avoid losing data due to missing analyses in the multivariable model, we performed multiple imputation of the baseline variables. We created 20 imputed data sets and performed the regression analysis in each imputed data set. The results were then combined using Rubin’s rules.

The number of cases per variable in the multivariable model was low. Confounder adjustment was done using ridge regression to reduce the effective degrees of freedom. Ridge regression shrinks the coefficients towards zero by introducing bias while reducing the overfitting. A drawback is that inference for ridged coefficients is not straightforward but it is an effective way of adjusting for confounders when the data set is too small for a full adjustment [16]. The amount of shrinkage was determined by maximizing a corrected Akaike Information Criterion (AIC).

Data were analyzed in IBM SPSS Statistics 20 (Armonk, NY), and R version 3.2.4 (Vienna, Austria).

Results

Concentrations of CgA, CgB, and NSE

One newborn girl with a stage 4S tumor was excluded from all parametric statistics due to excessive levels of CgA (2746 nmol/L), but was included in non-parametric calculations. Only six NSE concentrations were available from the group of 32 neonates arriving for a post-partum health control. All six concentrations differed substantially from the other controls (mean > $\times 10$ higher), interpreted as a consequence of the capillary sampling and hence these six observations were excluded. Abbasoglu et al. [17] have reported high NSE levels in healthy preterm and term newborns.

The median concentrations and 25th–75th percentile of CgA, CgB and NSE are shown in Table 2. CgA plasma concentrations were significantly higher in samples obtained from neonates compared with older children ($p < 0.0001$). No difference in concentrations of CgB with age could be found. Significantly higher NSE concentrations were found in umbilical vein blood samples from neonates compared to peripheral venous blood samples in older children; 20 (15–29) and 8 (5–14) $\mu$g/L, respectively, $p < 0.001$. However, due to the small sample size in the neonates, we decided that all the NSE concentrations should be pooled.

In our material CgA as a tumor marker in NB had a specificity of 72% and a sensitivity of 96% whereas NSE 72% and 68%, respectively.

There was no significant difference in the NSE concentrations in the non-NB or leukemia group nor in the patients with GN compared to healthy controls. NSE concentrations were higher in NB patients compared to healthy controls ($p < 0.0001$) and to patients with other malignancies ($p < 0.0001$).

Correlation to stage and genetic markers

The median CgA and NSE concentrations were higher in high stage (stage 3–4) disease, [CgA 10 (5–38) nmol/l, and NSE 41 (15–319) $\mu$g/L, respectively] as compared to low stage (stage 1–2) disease [5 (4.8–6) nmol/L, 16 (12.5–31) $\mu$g/L; $p < 0.05$ and $p < 0.05$, respectively, Mann-Whitney U test]. Concentrations of CgB were not different in low vs. high stage disease.

CgA and NSE were elevated in patients with 1p LOH ($p < 0.05$ and $p < 0.05$, respectively) and MYCN-amplification ($p < 0.05$ and $p < 0.001$) but not in diploid or tetraploid tumors ($p > 0.05$). CgB concentrations did not differ between groups (Table 3).
**Table 2.** Concentrations of CgA, CgB, and NSE in patients compared with healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Malignant Solid Tumors, Leukemias</th>
<th>GN</th>
<th>NB</th>
<th>All ages and stages</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;1 month</td>
<td>≥ 1 month</td>
<td>All ages</td>
<td>p</td>
<td>All ages</td>
<td>P</td>
</tr>
<tr>
<td>CgA (nmol/L)</td>
<td>5 (4.7–6)</td>
<td>3 (2.7–4)</td>
<td>3 (3–4)</td>
<td>N.S.</td>
<td>3.5 (3–4)</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>n = 42</td>
<td>n = 26</td>
<td>n = 31</td>
<td>n = 12</td>
<td>n = 12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CgB&lt;sup&gt;1&lt;/sup&gt;</td>
<td>2 (2–3)</td>
<td>2 (2–3)</td>
<td>N.S.</td>
<td>2 (1–2)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>(nmol/L)</td>
<td>n = 63</td>
<td>n = 31</td>
<td>n = 31</td>
<td>n = 31</td>
<td>n = 31</td>
<td>n = 69</td>
</tr>
<tr>
<td>NSE&lt;sup&gt;2&lt;/sup&gt;</td>
<td>11 (5–23)</td>
<td>7 (4–19)</td>
<td>N.S.</td>
<td>8.5 (6.2–14)</td>
<td>N.S.</td>
<td>31 (14–177)</td>
</tr>
<tr>
<td></td>
<td>n = 35</td>
<td>n = 31</td>
<td>n = 31</td>
<td>n = 12</td>
<td>n = 12</td>
<td>n = 89</td>
</tr>
</tbody>
</table>

Median (25th percentile–75th percentile), Mann-Whitney U-test.

<sup>1</sup>Plasma samples only—see text for details.

<sup>2</sup>All controls, both < 1 mo and ≥ 1 mo.

All controls, both < 1 mo and ≥ 1 mo.

The p value is from the comparison between the control group and the different patient groups. N.S. – not significant.
Table 3. CgA, CgB, NSE, and genetic markers in neuroblastoma.

<table>
<thead>
<tr>
<th></th>
<th>1p LOH</th>
<th>MYCN-amplification</th>
<th>Ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>CgA (nmol/L)</td>
<td>n = 37</td>
<td>n = 16</td>
<td>p = 0.029</td>
</tr>
<tr>
<td>CgB (1–3.1)</td>
<td>n = 30</td>
<td>n = 14</td>
<td>N.S.</td>
</tr>
<tr>
<td>NSE (12–52)</td>
<td>n = 41</td>
<td>n = 19</td>
<td>0.009</td>
</tr>
</tbody>
</table>
| (µg/L)           |              |                    | Median (25th percentile-75th percentile), Mann-Whitney U-test. If n is lower than stated in text, it is due to lack of data in some patients. N.S. – not significant.

Figure 1. NSE correlation to tumor size at diagnosis. $R = 0.78$. Spearman’s rank correlation.

NSE had a linear correlation to the maximal tumor diameter at diagnosis (Figure 1). The NB patients who died of disease had a larger maximal tumor diameter at diagnosis than the survivors (Figure 2). No correlation to tumor size was seen for CgA or CgB.

**Correlation to outcome**

NSE concentrations were higher in NB patients who died of disease compared with those of survivors ($p < 0.01$). There was no such correlation to outcome for CgA or CgB. Hazard Ratios and 95% confidence intervals adjusted for known prognostic markers (age over 18 months, metastatic disease, MYCN-amplification, 1p LOH and ploidy) are given in Table 4. Higher stage disease (stages 3–4) and metastatic disease were also correlated to risk of death.

Table 4. Multivariable adjusted Hazard Ratios for death for an inter-quartile range increase in each marker.

<table>
<thead>
<tr>
<th></th>
<th>Hazard ratio</th>
<th>95% lower</th>
<th>95% upper</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSE</td>
<td>1.47</td>
<td>1.14</td>
<td>1.89</td>
<td>0.003</td>
</tr>
<tr>
<td>CgA</td>
<td>1.05</td>
<td>0.98</td>
<td>1.13</td>
<td>N.S.</td>
</tr>
<tr>
<td>CgB</td>
<td>1.00</td>
<td>0.72</td>
<td>1.39</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Adjusted for age at diagnosis, sex, MYCN amplification and 1p LOH. N.S. – not significant.
Discussion

CgA is a member of a family of water-soluble glycoproteins, which besides CgA also comprises CgB (i.e. secretogranin I). CgA is stored in vesicles in neuroendocrine cells such as sympathetic ganglion cells and peptide hormone-releasing cells and was first identified in the adrenal medulla [18]. Kim et al. [19] showed evidence that regulation of dense-core secretory granule biogenesis and hormone secretion in endocrine cells is dependent on CgA. Its release increases at autonomic nervous system activation, for example during stress (e.g., birth), and hence are CgA concentrations high in newborns [20], an observation our study confirms. CgA concentrations were almost twice as high in neonates as compared to infants and children. Therefore, separate reference intervals for neonates and infants had to be used.

CgA is the most important tumor marker in adult well differentiated NETs, and in metastatic disease [21]. CgA is expressed both by functioning and non-functioning NETs, and has a sensitivity and specificity of 70–95% and 70–80%, respectively [3]. Hsiao et al. [5] found a 100% specificity when comparing 34 NB with 15 non-NB patients. However, in our study we found 96% sensitivity and 72% specificity for CgA in NB but elevated concentrations of CgA in two non-NB patients. Both these patients had renal impairment (a clear cell kidney sarcoma, and an acute lymphatic leukemia with bilateral renal involvement, both having a serum creatinine concentration above 115 µmol/L), a condition known to reduce CgA elimination [22]. Wassberg et al. [8] reported that plasma levels of chromogranin A are directly proportional to tumour burden in neuroblastoma in an animal experimental model. In our study, we found higher CgA concentrations in high stage and metastatic disease. In view of its higher sensitivity, its reliability in NETs, and its theoretical correlation to viable tumor cell burden, we suggest that also CgA merits prospective evaluation as a NB prognostic marker. We conclude that CgA is a tumor marker in NB, but concentrations are unspecifically elevated in renal impairment.

NSE is an isoenzyme of the glycolytic enzyme enolase. Enolase catalyzes the dehydration of 2-phospho-δ-glycerate (PGA) to phosphoenolpyruvate (PEP) in the glycolytic pathway, and the reverse reaction, the hydration of PEP to PGA, in gluconeogenesis. NSE is found
in the cytoplasm in many human tissues. There are three isoenzymes, enolase a, enolase b and enolase g. The g isoenzyme is called NSE, and its synthesis is a late event in neuronal differentiation. NSE is a marker of neuronal tissue and neuroendocrine cells, and an important clinical tumor marker for adult, poorly differentiated NET [21]. High serum concentrations are correlated to a large tumor burden and to a poor outcome. Longitudinally sampled NSE concentrations can be used to monitor response to treatment and to detect relapse before clinical or radiological recurrence in different NETs [23]. Massaron et al. [6] reported that high NSE levels are correlated to higher disease stages and to death which we also confirmed. They also found that repeated NSE measurements during follow-up could predict relapse without any clinical symptoms. In a paper from Cangemi et al. [24] NSE levels >200 ng/ml were associated with worse outcome only in stage 4 patients without MYCN amplification. In our material NSE was correlated to NB tumor burden and death, and has a linear correlation to the maximum tumor diameter at diagnosis but that was not the case for CgA. We also suggest that NSE could be used to distinguish survivors from those in high risk of dying of NB. Thus, NSE is superior to CgA as a predictor of outcome. The sensitivity and the specificity of NSE was 68% and 71% respectively. Furthermore as it derives from our study there is a linear correlation to the maximal tumor diameter at diagnosis that can be used as a clinical tool.

It has been shown that plasma concentrations of CgB are increased in pheochromocytoma patients [25]. However, in material we did not find increased concentrations of CgB, which could indicate that NB cells, although secreting catecholamines, are biologically different from the chromaffin cells in the adrenal medulla. Thus, CgB should not be used as a tumor marker in NB.

Many negative prognostic factors are known in NB; clinical, such as age over eighteen months, high stage disease [7], as well as genetic markers, such as MYCN-amplification, 1p LOH, and di- or tetraploidy [26]. CgA and NSE concentrations were higher in MYCN-amplified and 1p LOH positive tumors. CgA has been proven to be an independent prognostic factor in, for example, small intestine NETs [27]. Hsiao et al. [5] reported that high CgA concentrations were a negative prognostic factor in NB. In our study we included almost three times more NB patients and we also included GN patients in order to investigate whether both CgA and NSE are prognostic markers. We confirmed that CgA is a tumor marker in NB whereas NSE is even a prognostic factor in NB. Furthermore, higher blood concentrations of only NSE correlate to a poor outcome. CgA concentrations were not a prognostic factor in NB in our study, but larger, prospective studies are needed to address this hypothesis.

The stage 4S-tumors represent a special subset of NB. In the present study, only five patients were included. CgA was elevated above the reference value of controls in all five patients, and CgA was exceptionally high in one newborn girl with disseminated, fatal disease. There is a clinical need to distinguish stage 4S patients with a clinically benign course and without need of therapy from those with a more aggressive NB. Determination of CgA concentrations may add information when to make such a distinction. These concerns and observations merit a more extensive analysis of 4S patients.

**Conclusion**

This study has demonstrated elevated plasma and serum concentrations of CgA and NSE in patients with NB. We have also showed higher concentrations of CgA and NSE in higher stage patients, as well as that CgA and NSE correlate to genetic markers. Furthermore only NSE is correlated to outcome and to the tumor size. The implication of our observations is that NSE
is a clinically valuable tumor marker in NB and that CgA may also be valuable and merits prospective, multicenter, clinical studies.

**Declaration of interest statement**

The authors have no conflicts of interest.

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