Prognostic value of ferritin, neuron-specific enolase, lactate dehydrogenase, and urinary and plasmatic catecholamine metabolites in children with neuroblastoma

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Abstract: Different plasma and urinary parameters have been tested as valuable prognostic markers for children with neuroblastoma (NB), but conclusive results from multivariate analyses are still lacking. Samples collected at diagnosis from 505 patients diagnosed in Italy between June 1994 and November 2010 were analyzed at the Italian reference laboratory according to standard methodologies. Patient clinical data were retrieved from the Italian NB Registry. For statistical analysis, patients were grouped according to stage, age, MYCN status, and outcome. Cumulative survival was calculated by the Kaplan–Meier procedure using the first quartile of the marker distribution as a cut-off value to stratify the patients. Multivariate analysis was performed by the Cox regression model by considering only the significant variables. When the entire cohort of patients was considered, none of the different parameters had an independent prognostic value. However, in patients with localized disease without MYCN amplification the significant positive associations between urinary and plasmatic vanillylmandelic acid (VMA)/homovanillic acid (HVA) ratio and a better prognosis remained significant (P < 0.05 and P < 0.01, respectively), as well as, the positive association between high lactate dehydrogenase (LDH) values and a worse prognosis (P < 0.001). Moreover, in stage 4 patients without MYCN amplification, neuron-specific enolase levels above 200 ng/mL and LDH levels above 2500 IU/mL maintained their significant association with a worse outcome (P = 0.01 and P = 0.0001, respectively). In conclusion, LDH had an independent prognostic value in patients of all stages without MYCN amplification. Moreover, the urinary and plasmatic VMA/HVA ratio was an independent predictor of prognosis in patients with localized disease without MYCN amplification. Since LDH and catecholamine metabolites are measured in all patients at diagnosis, these findings may be helpful for an easy, cost-effective, patient risk stratification.

Keywords: neuroblastoma, markers, prognosis

Introduction

Ferritin, neuron-specific enolase (NSE), lactate dehydrogenase (LDH), and the catecholamine metabolites, vanillylmandelic acid (VMA) and homovanillic acid (HVA), are commonly assessed in children suspected to have neuroblastoma (NB), and the levels of these markers are commonly used for differential diagnosis.⁶ Several studies performed over more than 3 decades²–⁷ have suggested that they may have a prognostic value, but the small numbers of patients studied in each cohort have hampered the possibility to assess their role independent from stage, age, and MYCN status, which are the established parameters to stratify patients.¹ Only Berthold et al⁶ demonstrated an
independent prognostic role of plasma VMA/HVA ratio in patients with localized NB, but this finding was of limited interest since catecholamine metabolites are commonly measured in urine rather than in plasma. Methodological issues further limited the power of previous reports. Analyses were often performed at the home institutions rather than at a national reference laboratory, and unavoidable methodological changes occurred over the years.

Recently, it has been shown that the presence of either structural or numerical aberrations in the genome of primary NB tumor cells can predict outcome of low- and intermediate-risk NB patients. Thus, multicenter studies are currently stratifying patients in different protocol arms depending on these features, (see http://www.siopen-net.org). These analyses, however, require a primary tumor biopsy and are costly and time-consuming.

Thus, we decided to retrieve all the results obtained at the Italian reference laboratory where all the analyses for the various markers have been centralized since 1994, and check whether any of these markers was an independent predictor of survival.

Patients and methods
The study cohort includes all patients diagnosed in Italy with NB, according to the International Neuroblastoma Staging System (INSS) criteria, between June 1994 and November 2010, for whom at least one diagnostic analysis was performed at the Italian reference laboratory located at the Gaslini Institute. None of the patients included were diagnosed by mass screening. Moreover, no modifications of the INSS criteria have occurred during the study period. The demographic, clinical and follow-up data (at January 2011) of the 505 study patients (109 stage 1, 39 stage 2, 92 stage 3, 217 stage 4, and 48 stage 4S) were retrieved from the Italian NB Registry.

Laboratory analysis
Urinary HVA and VMA concentrations were determined on samples derived from 24-hour urine collections. Urinary and plasma HVA and VMA were performed by gas chromatography coupled to mass spectrometry (GC-MS) as described by Henneman. Since 2006 urinary HVA and VMA were analyzed by high-pressure liquid chromatography coupled with electrochemical detection (HPLC-EC), using a commercially available method (HVA-VMA by HPLC; Bio Rad Laboratories, Milan, Italy). Urinary HVA and VMA concentrations were expressed as a ratio to creatinine concentration, previously determined using the Jaffé method. LDH, NSE, and ferritin were determined on heparinized plasma, using routine clinical chemistry laboratory methods. Sensitivity and specificity of urinary HVA and VMA are reported in Monsaingeon et al; sensitivity of plasma HVA, VMA, NSE, and LDH in Simon et al; specificity of NSE in Viallard et al; whereas ferritin and LDH are nonspecific markers.

Treatment
After diagnosis the study patients were treated according to the Italian Neuroblastoma protocols, until the commencement of the various International Society of Pediatric Oncology European Neuroblastoma Group (SIOPEN) protocols. All protocols were approved by the local Institutional Review Boards. Before being enrolled, and treated on their respective trials, patients or their guardians signed a consent form allowing the use of their clinical and genetic data for clinical research. No other specific informed consent was required for the purposes of this study.

Statistical analysis
For each marker, statistical analysis was first performed on the entire cohort of the study patients. Then patients were grouped according to the following variables: stage (localized, metastatic NB), age (<18 months, >18 months), and MYCN status (not amplified, amplified). Differences were assessed by Mann–Whitney U test or Fisher’s exact test. Correlations were estimated by the Pearson’s coefficient. Cumulative survival was calculated by the Kaplan–Meier procedure using the first quartile of the marker distribution as cut-off value to stratify the patients. For event-free survival plots, the occurrence of relapse, progression, or death were considered as an event. Statistical significance was estimated by log rank test. Statistical significance was estimated by log rank test. Multivariate analysis was performed by the Cox regression method by including only the significant variables.

Results
In the study period, a total of 1717 children were diagnosed with NB in Italy. Of these, 505 patients had at least one urinary or plasma marker analyzed at diagnosis at the Italian reference laboratory. As shown in Table 1, where the demographic, biological and clinical features of the study patients are summarized, no significant differences were found between the study patients and the entire population of NB patients diagnosed in Italy during the study period. Moreover, the event-free and overall survival (EFS and OS) of the study patients, stratified by stage, age, MYCN status, and treatment protocol was similar to that of the entire population.
The results obtained for each marker in the entire study cohort are summarized in Table 2. Of the 411 patients that had urinary HVA and VMA determined, 174 had also plasma catecholamine determined. Correlation between urinary and plasma values was high (HVA: Pearson’s correlation coefficient = 0.19, P < 0.05; VMA: Pearson’s correlation coefficient = 0.25, P < 0.01), confirming previous findings. As reported previously, the median levels of VMA and HVA increased by stage and the higher levels were found in stage 4S infants (251 and 215 µg/mg creatinine, respectively, with a median ratio of 1.41).

Values for ferritin, NSE, and LDH were available for half of the study patients, but overlapping with urinary HVA and VMA was relatively low (N = 162).

Table 1 Demographic, biological and clinical features of the study patients stratified by stage, and comparison with the whole NB population diagnosed during the study period

| Study patients | Stage | NB patients | P |
|----------------|-------|-------------|---|
|                | 1     | 2           | 3 | 4 | 4S | Total |                | Total |   |
| N              |       |             |   |   |    |       |                |       |   |
| Sex            |       |             |   |   |    |       |                |       |   |
| Female         | 42    | 21          | 49 | 87 | 21 | 220 (43.6%) | 764 (44.5%) | NS   |
| Male           | 67    | 18          | 43 | 130 | 27 | 285 (56.4%) | 953 (55.5%) |       |
| Age            |       |             |   |   |    |       |                |       |   |
| ≤18 months     | 57    | 25          | 48 | 56 | 48 | 234 (46.3%) | 855 (49.8%) | NS   |
| MYCN status    |       |             |   |   |    |       |                |       |   |
| Normal         | 48    | 23          | 33 | 28 | 37 | 169 (72.2%) | 606 (70.9%) | NS   |
| Amplified      | 2     | 1           | 12 | 18 | 5  | 38 (16.2%)  | 90 (10.5%) | NS   |
| Not known      | 7     | 1           | 3  | 10 | 6  | 27 (11.6%)  | 159 (18.6%) | <0.01 |
| 5 years EFS    | 96.7% | 100%        | 88.5% | 45.9% | 75.0% | 76.9% | 77.8% | NS   |
| 5 years OS     | 98.2% | 100%        | 92.5% | 56.1% | 81.3% | 82.9% | 85.2% | NS   |
| >18 months     | 52    | 14          | 44 | 161 | –  | 271 (53.7%) | 862 (50.2%) | NS   |

Abbreviations: NB, neuroblastoma; NS, not significant; EFS, event-free survival; OS, overall survival.

The results obtained for each marker in the entire study cohort are summarized in Table 2. Of the 411 patients that had urinary HVA and VMA determined, 174 had also plasma catecholamine determined. Correlation between urinary and plasma values was high (HVA: Pearson’s correlation coefficient = 0.19, P < 0.05; VMA: Pearson’s correlation coefficient = 0.25, P < 0.01), confirming previous findings. Indeed, plasma VMA/HVA ratio was shown to be predictive in localized patients. In our cohort, univariate analysis demonstrated that neither VMA nor HVA had any prognostic value, even when patients with localized and metastatic disease were considered separately. However, in patients with localized NB, both urinary and plasma VMA/HVA ratio positively associated with a better outcome (Table 3). Patients with urinary VMA/HVA ratio < 0.50 had 3.44 times greater chance of relapse than patients with a ratio ≥ 0.50 (95% confidence interval [CI]: 1.26–9.39; P < 0.05).

Most importantly, multivariate analysis demonstrated that in patients with localized disease without MYCN amplification, urinary VMA/HVA ratio < 0.5 and plasma VMA/HVA ratio < 0.15 were independent predictors of poor prognosis (Figure 1A and B, respectively; P = 0.017 for both).

Prognostic value of ferritin
Ferritin was evaluated in 272 patients (Table 2). In the whole cohort of patients, univariate analysis indicated a significant positive association between ferritin values higher than the first quartile value and a worse EFS (Figure 1C; P < 0.01). However, multivariate analysis demonstrated that ferritin levels positively associated with stage, thus eliminating its predictive power (Table 3).

Prognostic value of NSE
Neuron-specific enolase was evaluated in 233 patients (Table 2). When the entire cohort of patients was considered, NSE did not associate with EFS or OS. However, in stage 4 patients NSE levels < 200 ng/mL associated with a better EFS (Table 3). Multivariate analysis confirmed that the NSE levels were an independent predictor of prognosis in stage 4 patients without MYCN amplification (Table 3 and Figure 1D; P < 0.01).
Prognostic value of LDH

Lactate dehydrogenase was evaluated in a total of 279 patients. Both in patients with localized and metastatic disease, high LDH values positively associated with a worse prognosis ($P < 0.01$). In particular, patients with localized disease with LDH values $>1300$ IU/mL had 12.9 times greater chance of relapse (95% CI: 3.34–49.78; $P < 0.001$). However, multivariate analysis confirmed LDH predictive power only in patients without MYCN amplification (Table 3 and Figure 1E; $P = 0.0001$). In stage 4 patients, the LDH cut-off value discriminating patients with good and bad prognosis was 2500 IU/mL (Table 3). Also, in stage 4 patients LDH was an independent prognostic factor only in patients without MYCN amplification (Figure 1F; $P = 0.0001$).

Discussion

The aim of this work was to investigate the independent prognostic value of different markers commonly evaluated at diagnosis in NB patients as a potential tool to stratify patients in different risk groups. The availability of a routine test, performed at diagnosis in peripheral blood or urine samples, may reduce the time and cost of prognostic analyses with respect to those performed on primary tumors.$^{8,9}$

In our study, LDH values above 1300 IU/mL in patients with localized disease and above 2500 IU/mL in stage 4 patients were positively associated with a worse outcome, independent of age and stage. LDH levels, however, were positively associated with MYCN amplification. Thus, in prospective trials for patients with localized disease without MYCN amplification, LDH levels at diagnosis should be evaluated for their predictive power as compared to the genetic tests performed on the primary tumors. An association between LDH levels and outcome of patients with localized disease was also observed in the Localised Neuroblastoma European Study, but no multivariate analysis was performed.$^{19}$ Similarly, the Task Force of the International Neuroblastoma Risk Group$^{29}$ found that a single LDH cut-off level of 587 IU/mL could dichotomize NB patients into good and poor prognosis. However, LDH was not included in the new INRG Staging System because of its unspecific nature.

Our study demonstrated that in patients with localized disease without MYCN amplification, urinary and plasmatic VMA/HVA ratio were also positively associated with a better event-free survival. In 1992, Berthold et al$^d$ demonstrated that plasma VMA/HVA ratio was an independent predictor of EFS for patients with localized disease. Although Gahr and Hunneman$^{23}$ had shown a good agreement between urinary and plasma catecholamine levels, urinary levels were globally measured. Thus, the report did not receive proper attention.
Table 3 Prognostic value of each marker in the different groups of neuroblastoma patients

| MYCN | Patients with localized NB | | Patients with metastatic NB | Cut-off value |
|------|--------------------------|------------------|--------------------------|--------------|
|      | ≤18 months | >18 months | ≤18 months | >18 months | |
| Urinary VMA/HVA ratio |  |  |  |  | 0.50 |
| N total | 111 | 13 | 41 | 10 | 22 | 14 | 66 | 26 |
| Mean | 1.28 | 0.55 | 0.76 | 0.57 | 1.32 | 0.30 | 1.09 | 0.71 |
| N (%) over cut-off | 14 (12.6) | 8 (61.5) | 14 (34.1) | 6 (60.0) | 5 (22.7) | 11 (78.6) | 15 (22.7) | 17 (65.4) |
| Association with outcome | Y | N | Y | N | N | N | N | N |
| P | 0.017 | NS |  |  |  |  |  |  |
| Plasma VMA/HVA ratio |  |  |  |  | 0.15 |
| N total | 46 | 6 | 25 | 4 | 5 | 7 | 50 | 9 |
| Mean | 0.59 | 0.13 | 0.29 | 0.09 | 0.24 | 0.11 | 0.34 | 0.11 |
| N (%) over cut-off | 5 (10.9) | 5 (83.3) | 6 (24.0) | 4 (100.0) | 3 (60.0) | 6 (85.7) | 19 (38.0) | 8 (88.9) |
| Association with outcome | Y | N | Y | – | – | N | N | N |
| P | 0.017 | NS |  |  |  |  |  |  |
| Ferritin (ng/mL) |  |  |  |  | 119.5 |
| N total | 73 | 14 | 31 | 11 | 11 | 13 | 56 | 15 |
| Mean | 151.4 | 182.4 | 64.0 | 394.7 | 179.1 | 351.3 | 372.2 | 802.2 |
| N (%) over cut-off | 4 (6.1) | 3 (27.3) | 4 (12.9) | 8 (72.7) | 5 (45.5) | 10 (76.9) | 37 (66.1) | 14 (93.3) |
| Association with outcome | N | N | N | N | – | N | N | N |
| P | NS | NS |  |  |  |  |  |  |
| NSE (ng/mL) |  |  |  |  | 200.0 |
| N total | 66 | 11 | 28 | 7 | 10 | 12 | 51 | 12 |
| Mean | 69.9 | 158.1 | 65.8 | 152.7 | 99.0 | 366.2 | 165.2 | 243.2 |
| N (%) over cut-off | 4 (6.1) | 3 (27.3) | 2 (7.1) | 2 (28.6) | 0 (0.0) | 6 (50.0) | 10 (19.6) | 5 (41.7) |
| Association with outcome | N | N | N | N | – | N | Y | N |
| P | NS | NS |  |  | 0.01 |  |  |  |
| LDH (IU/L) |  |  |  |  | 1300/2500 |
| N total | 75 | 14 | 32 | 11 | 12 | 14 | 56 | 16 |
| Mean | 782.4 | 2495.2 | 882.4 | 3449.1 | 1020.8 | 5121.3 | 13670.0 | 4059.2 |
| N (%) over cut-off | 7 (9.3) | 11 (78.6) | 7 (21.9) | 9 (81.8) | 3 (25.0) | 12 (85.7) | 20 (37.5) | 14 (87.5) |
| Association with outcome | Y | N | Y | N | – | N | Y | N |
| P | 0.0001 | 0.0001 |  |  |  |  |  |  |

Note: *For localized/metastatic group.

Abbreviations: A, amplified; HVA, homovanillic acid; LDH, lactate dehydrogenase; N, no, as determined by multivariate analysis; NA, not amplified; NB, neuroblastoma; NS, not significant; NSE, neuron-specific enolase; VMA, vanillylmandelic acid; Y, yes, as determined by multivariate analysis.

As first reported by Laug et al.\textsuperscript{7,24,25} a higher VMA/HVA ratio was found in stage 4S infants. VMA and HVA are the byproducts of two distinct catecholamine metabolic pathways starting from norepinephrine and dopa, respectively. Gitlow et al.\textsuperscript{30} first suggested that high HVA levels were produced by undifferentiated, unfavorable prognosis tumors. Kontras\textsuperscript{31} showed that high VMA levels correlated to a more differentiated phenotype with better prognosis. In our study, information on the differentiation of the primary tumors was not available, thus precluding any study of association. However, our multivariate analysis confirmed the association between low VMA levels and MYCN amplification,\textsuperscript{25–28} and added weight to the association between low VMA levels and poor prognosis previously found in smaller cohorts of NB patients.\textsuperscript{2,4,24} Our multivariate analysis, performed separately in patients with metastatic and localized disease, also explained the contrasting results found in these two subsets of patients by Aydin et al.\textsuperscript{7} with regard to the association of VMA levels with outcome. In fact, the frequency of MYCN amplification in stage 4 patients is higher than in localized patients, whose VMA levels increase from stage 1 to stage 3. In view of this effect, the use of VMA/HVA ratio had greater advantage with respect to the use of VMA alone.

As for the other two markers evaluated in our study, ferritin association with outcome was dependent on the stage of disease, whereas NSE levels > 200 ng/mL positively associated with a worse outcome only in stage 4 patients without MYCN amplification, thus limiting their utility in patient risk stratification.

It is important to note that in our study about 30% of the NB patients had unknown MYCN status, thus the association of each marker with MYCN status must be taken with caution. Further validation of our findings in a future prospective study is needed.
Conclusion
Urinary and plasma VMA/HVA ratio > 0.5 and 0.15, respectively, and LDH values < 1300 IU/mL were found to be independent predictors of good prognosis in patients with localized NB without MYCN amplification. Thus, these data, commonly determined at diagnosis in all NB patients, should be collected in future protocols for this subset of patients, and their prognostic value compared to that of structural and numerical chromosomal aberrations present in the primary tumors.

Acknowledgments
This study was supported in part by Fondazione Italiana per la Lotta al Neuroblastoma. The authors thank Angelo Maffia...
and Daniela Bugnone, Genoa, for keeping the plasma and urinary repositories, and all the nurses and physicians that centralized the samples. SB is recipient of a Fondazione Italiana Neuroblastoma fellowship.

Disclosure

The authors report no conflicts of interest in this work.

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