

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

Giuliana Cangemi¹
 Giorgio Reggiardo²
 Sebastiano Barco¹
 Laura Barbagallo¹
 Massimo Conte³
 Paolo D'Angelo^{4,#}
 Maurizio Bianchi^{5,#}
 Claudio Favre^{6,#}
 Barbara Galleni³
 Giovanni Melioli¹
 Riccardo Haupt⁷
 Alberto Garaventa³
 Maria V Corrias⁸

¹Clinical Pathology Laboratory Unit, Giannina Gaslini Institute, Genoa, Italy; ²Data Management and Biostatistics Unit, Medi Service, Genoa, Italy; ³Department of Hematology-Oncology, Giannina Gaslini Institute, Genoa, Italy; ⁴Pediatric Oncology Unit, Azienda Civico, Di Cristina e Benfratelli, Palermo, Italy; ⁵Pediatric Onco-Hematology, Stem Cell Transplantation and Cellular Therapy Division, Regina Margherita Children's Hospital, Turin, Italy; ⁶Department of Hematology-Oncology, Pisana University Hospital, Pisa, Italy; ⁷Epidemiology and Biostatistics Unit, Giannina Gaslini Institute, Genoa, Italy; ⁸Laboratory of Oncology, Giannina Gaslini Institute, Genoa, Italy

[#]On behalf of the Italian Association of Pediatric Hematology and Oncology (AIEOP)

Correspondence: Maria V Corrias
 Head Molecular Oncology, Laboratory of Oncology, Gaslini Institute, Largo Gaslini, 5, 16147, Genoa, Italy
 Tel +39 010 563 6524
 Fax +39 010 377 9820
 Email mariavaleriacorrias@ospedale-gaslini.ge.it

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.¹ Several studies performed over more than 3 decades^{2–7} 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.^{8,9} 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,¹⁰ 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.¹¹

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 Hunneman.¹² 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 Jaffè 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 al¹³; sensitivity of plasma HVA, VMA, NSE, and LDH in Simon et al¹⁴; specificity of NSE in Viillard et al¹⁵; whereas ferritin and LDH are nonspecific markers.

Treatment

After diagnosis the study patients were treated according to the Italian Neuroblastoma protocols,^{11,16–18} until the commencement of the various International Society of Pediatric Oncology European Neuroblastoma Group (SIOPEN) protocols.^{19–22} 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. 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.¹¹

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 Total	P
	1	2	3	4	4S	Total		
N	109	39	92	217	48	505	1717	
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
MYCN status								
Normal	27	10	16	86	–	139 (51.3%)	433 (50.2%)	NS
Amplified	2	2	15	31	–	50 (18.4%)	123 (14.3%)	<0.05
Not known	23	2	13	44	–	82 (30.3%)	306 (35.5%)	NS
5 years EFS	100%	83.3%	63.6%	22.5%	–	38.2%	37.6%	NS
5 years OS	100%	92.9%	75.2%	30.1%	–	50.6%	50.8%	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.^{6,23} As reported previously,^{2,7,24,25} the median levels of VMA and HVA increased by stage and the higher levels were found in stage 4S infants (251 and 215 $\mu\text{g}/\text{mg}$ 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 ($N = 162$).

Prognostic values of urinary and plasmatic VMA/HVA ratio

VMA and HVA are the end product of two different pathways of catecholamine catabolism, thus their relative abundance seems to associate with different phenotypes, each characterized by different prognosis.^{2,26–28} Indeed, plasma VMA/HVA ratio was shown to be predictive in localized patients.⁶ 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; $P < 0.01$).

Table 2 Concentration of each analyte in the entire cohort of study patients (N = 505) and in patients with localized disease (N = 288)

N	VMA/creatinine ($\mu\text{g}/\text{mg}$)	HVA/creatinine ($\mu\text{g}/\text{mg}$)	Plasma VMA (ng/mL)	Plasma HVA (ng/mL)	Ferritin (ng/mL)	NSE (ng/mL)	LDH (IU/mL)
Study patients (N = 505)							
Valid	411	402	174	174	272	233	279
Missing	94	103	331	331	233	272	226
Mean	167.5	195.1	318.9	1265.9	262.7	130.2	1653.3
Median	53.0	73.6	98.0	553.5	119.5	56.6	912.0
Standard deviation	320.5	542.2	792.9	2434.1	407.9	175.5	2104.9
Percentiles							
25	14.4	25.2	39.0	205.0	47.25	23.1	579.0
50	53.0	73.6	98.0	553.5	119.5	56.6	912.0
75	180.6	209.6	295.4	1367.7	280.2	169.2	1727.0
Patients with localized disease (N = 288)							
Valid	227	226	93	93	155	132	159
Missing	61	62	195	195	133	156	129
Mean	144.6	125.9	278.1	795.5	150.4	80.7	1259.8
Median	30.7	41.2	64.0	269.0	69.0	30.2	687.0
Standard deviation	319.0	204.1	793.7	1321.8	214.7	115.3	1637.6
Percentiles							
25	10.4	19.3	33.5	154.7	26.0	17.1	513.0
50	30.7	41.2	64.0	269.0	69.0	30.3	687.0
75	100.7	128.1	172.3	880.8	193.0	93.7	1334.0

Abbreviations: HVA, homovanillic acid; LDH, lactate dehydrogenase; NSE, neuron-specific enolase; VMA, vanillylmandelic acid.

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 Group²⁹ 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 al⁶ demonstrated that plasma VMA/HVA ratio was an independent predictor of EFS for patients with localized disease. Although Gahr and Hunneman²³ 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		
	NA	A	NA	A	NA	A	NA	A	
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				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	1367.0	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 al² and confirmed by several groups,^{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 al³⁰ first suggested that high HVA levels were produced by undifferentiated, unfavorable prognosis tumors. Kontras³¹ 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,^{25–28} and added weight to the association between low VMA levels and poor prognosis previously found in smaller cohorts of NB patients.^{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 al⁷ 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.

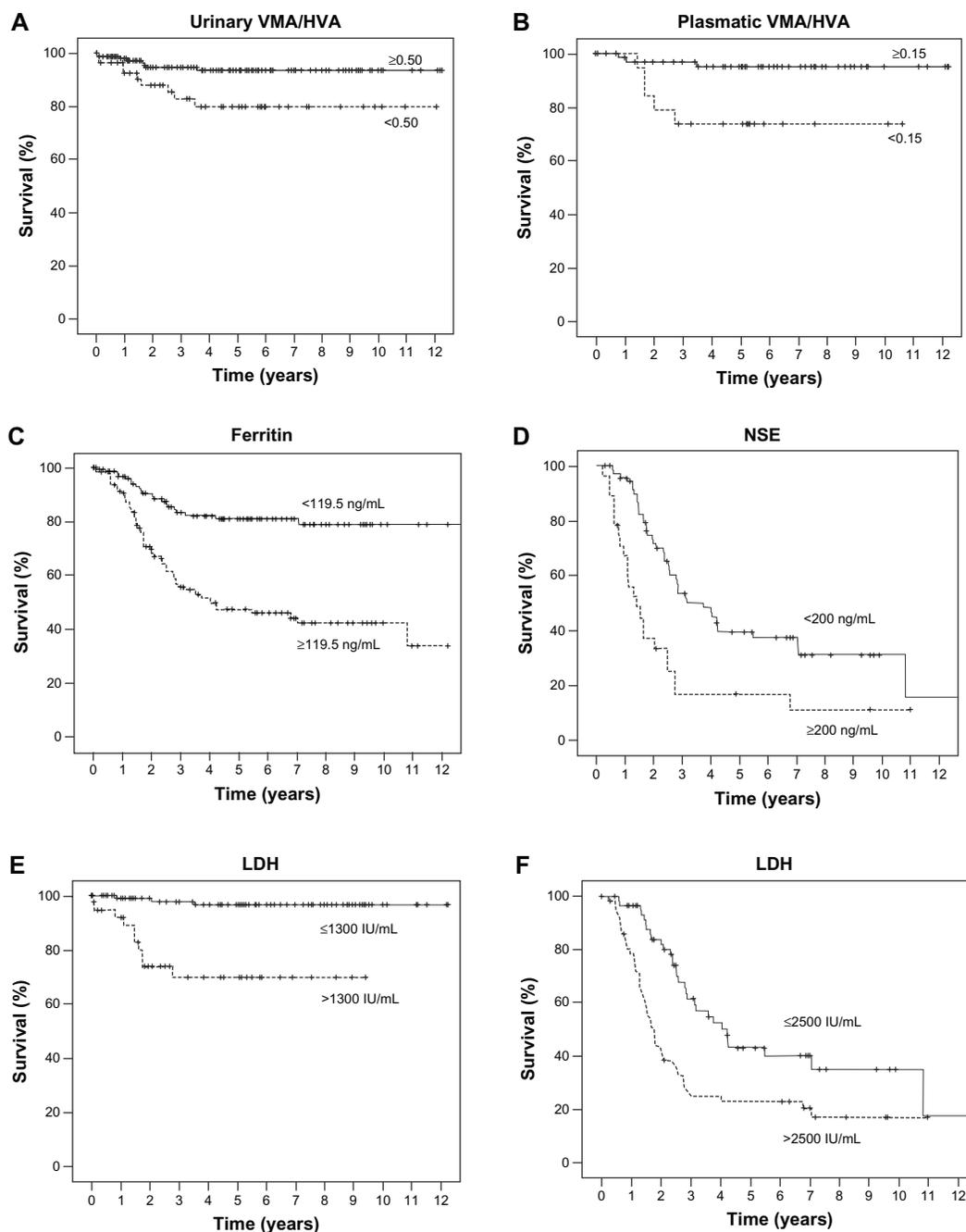


Figure 1 Kaplan-Meier plots of event-free survival of (A) patients with localized NB stratified by urinary VMA/HVA cut-off value; (B) patients with localized disease stratified by plasma VMA/HVA cut-off value; (C) the entire cohort of study patients stratified by ferritin cut-off value; (D) patients with stage 4 disease stratified by NSE cut-off value; (E) patients with localized disease stratified by LDH cut-off value; and (F) patients with stage 4 disease stratified by LDH cut-off value.

Note: For each marker the cut-off value corresponded to the first quartile of the marker distribution in that group of patients.

Abbreviations: HVA, homovanillic acid; LDH, lactate dehydrogenase; NB, neuroblastoma; NSE, neuron-specific enolase; VMA, vanillylmandelic acid.

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.

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Disclosure

The authors report no conflicts of interest in this work.

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