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TH and *DCX* mRNAs in peripheral blood and bone marrow predict outcome in metastatic neuroblastoma patients

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Abstract

Purpose In metastatic neuroblastoma (NB) patients, accurate risk stratification and disease monitoring would reduce relapse probabilities. This study aims to evaluate the independent prognostic significance of detecting tyrosine hydroxylase (*TH*) and doublecortin (*DCX*) mRNAs by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) in peripheral blood (PB) and bone marrow (BM) samples from metastatic NB patients.

Procedures RT-qPCR was performed on PB and BM samples from metastatic NB patients at diagnosis, post-induction therapy and at the end of treatment for *TH* and *DCX* mRNAs detection.

Results High levels of *TH* and *DCX* mRNAs when detected in PB and BM at diagnosis independently predicted worse outcome in a cohort of 162 metastatic NB. In the subgroup of high-risk metastatic NB, *TH* mRNA detected in PB remained as independent predictor of EFS and OS at diagnosis. After the induction therapy, high levels of *TH* mRNA in PB and *DCX* mRNA in BM independently

predicted poor EFS and OS. Furthermore *TH* mRNA when detected in BM predicted worse EFS. *TH* mRNA in PB samples at the end of treatment is an independent predictor of worse outcome.

Conclusion *TH* and *DCX* mRNAs levels in PB and BM assessed by RT-qPCR should be considered in new pre-treatment risk stratification strategies to reliably estimate outcome differences in metastatic NB patients. In those high-risk metastatic NB, *TH* and *DCX* mRNA quantification could be used for the assessment of response to treatment and for early detection of progressive disease or relapses.

Keywords Neuroblastoma · Minimal residual disease · Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) · *TH* · *DCX*

Introduction

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood, and it is characterized by a heterogeneous clinical presentation and outcome. Age (London et al. 2005), stage (Monclair et al. 2009), pathology (Shimada et al. 1999) and some biological tumor characteristics (Brodeur et al. 1984; Spiltz et al. 2006; Lampert et al. 1988; Look et al. 1991) are the most important prognostic factors. Nearly 50 % of all NB cases present metastatic disease and the majority of those children have dissemination in bone marrow (BM). Following the International Neuroblastoma Risk Group Staging System (INRGSS) guidelines, metastatic tumors are defined as stage M (defined as distant metastatic disease), except for stage MS, in which metastases are confined to the skin, liver, and/or bone marrow in children younger than

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18 months of age (Monclair et al. 2009). In Spain, metastatic NB patients (either M or MS with *MYCN* amplification (*MYCNA*) and/or older than 1 year at diagnosis) are currently treated according to the SIOPEL High-Risk Protocol. The treatment schedule includes induction chemotherapy, surgery, myeloablative chemotherapy followed by autologous stem cell reinfusion, radiotherapy and biological-based therapy for minimal residual disease with 13-cis-retinoic acid and anti-GD2 immunotherapy with or without cytokines. Nevertheless, almost 50 % of these patients still have very poor outcome.

Treatment for intermediate-risk metastatic NBs (stage M, younger than 12 months and without *MYCNA*) consists in standard chemotherapy and surgery. Low-risk metastatic patients (MS without *MYCNA*) are treated with standard chemotherapy with or without surgery and in some cases are only observed since spontaneous regression is a hallmark of this subgroup. Despite high survival rates being described for both subgroups, some of these patients die of disease reinforcing thus the importance of accurate pre-treatment risk stratification strategies.

Prognostic and early response biomarkers are of particular relevance in disease assessment and clinical management of NB patients. In the last decades, many efforts have been made in order to best identify the presence of NB cells in peripheral blood (PB) and BM samples. Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is a sensitive and widespread used technique to detect tumor-specific mRNAs as markers of circulating tumor cells (Burchill et al. 2001; Corrias et al. 2004; Viprey et al. 2014). Several groups have studied the specificity and predictive value of large number of mRNAs (Stutterheim et al. 2008; Cheung et al. 2008; Träger et al. 2008). Since catecholamines are produced by NB cells, *tyrosine hydroxylase* (*TH*), the first enzyme in the catecholamines synthesis pathway, is one of the most rigorously evaluated targets for minimal residual disease (MRD) detection in NB (Burchill et al. 2001). *Doublecortin* (*DCX*) gene is specifically expressed in migrating neurons from the central and peripheral nervous system and has been also identified as a sensitive and specific MRD biomarker (Oltra et al. 2005; Viprey et al. 2014).

Several studies have previously demonstrated the negative effect on survival of MRD detection in NB patients. However, few studies have performed multivariable analyses to confirm the independent significance of the mRNAs detection in PB and BM (Viprey et al. 2014; Cheung et al. 2015). The present study aims to evaluate the prognostic and predictive significance of *TH* and *DCX* mRNAs in PB and BM from patients with metastatic NB.

Materials and methods

Patients and samples

The present prospective study consists of 162 metastatic NB patients from the cooperating Spanish hospitals that treat children with NB. Staging and risk stratification was established according to INRGSS (Monclair et al. 2009). Samples of fresh or frozen tumor were referred to the Spanish reference center for pathology and molecular biology NB studies. Samples were centrally reviewed and classified according to the International Neuroblastoma Pathology Committee (INPC) system (Shimada et al. 1999; Burgues et al. 2006). Biological studies included status of *MYCN* and 1p, both studied by FISH according to ENQUA guidelines (Noguera et al. 2003; Ambros et al. 2003). Patients were included in national and European studies (INES, LINES, NAR99 and HR-NBL1). Two BM aspirations and two biopsies were performed on routine at diagnosis in all NB cases. All BM were previously analyzed by cytological and histological screening according to standard procedures. The MRD studies were centrally performed at the Spanish NB MRD reference laboratory and for these purpose 2 ml of PB and 0.5 ml of BM were collected in EDTA Vacutainer tubes (BD, UK). All patients had at least one sample, and most of them had both PB and BM. 35 PB and 7 BM samples from healthy donors were assessed as negative controls. Informed consent for samples and data management was obtained in all cases from patients' parents.

Total RNA extraction

Ficoll gradient centrifugation was used to isolate mononuclear cells from BM and PB specimens (Lymphoprep AXIS-SHIELD PoC AS). Once purified, mononuclear cells were lysed in a buffer containing guanidinium thiocyanate and immediately stored at -80°C . Total RNA was isolated using the RNeasy kit (Qiagen) and following the manufacturer's recommendations.

Retro-transcription (RT)

cDNA was synthesized reverse transcribing 1.2 μg RNA in 60 μL total reaction volume using random hexamers-primers and reagents contained in the TaqMan Gold RT-PCR kit (PE Applied Biosystems, Foster City, CA USA). RT was carried out for 30 min at 48°C , followed by RT inactivation for 5 min at 95°C .

Real-time quantitative PCR

RT qPCR for gene expression was performed using specific primers and TaqMan MGB probes for *TH* and *DCX* mRNAs. *Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)* gene served as the endogenous control. Three replicates of each sample were amplified. The PCR mixtures were run in Applied Biosystems 7300 Real-Time PCR System instrument using universal thermal cycling parameters. The normalization was performed by obtaining the Δ CT values in the following way: the mean CT for the marker gene (*TH* or *DCX*) was subtracted by the mean CT of *GAPDH* (Δ CT = CT [Marker]-CT [*GAPDH*]). Protocol details as well as the assays references are previously described (Yáñez et al. 2011). Samples were considered positive if at least two of the three CT values for each marker were lower than 40.

Statistical analyses

Data were summarized using the median for continuous variables and relative frequencies for categorical variables. Correlations between *TH* and *DCX* mRNAs expression values were assessed using Spearman correlation. *TH* and *DCX* values were standardized for univariable and multivariable analyses. Univariable overall survival (OS) and event-free survival (EFS) analyses at diagnosis, after induction therapy and at the end of treatment were performed for each marker in both PB and BM using Cox proportional hazards regression. To assess the independent association of markers with survival, Cox proportional hazard regression models were adjusted including *MYCN* status, stage and age at diagnosis. Since the levels of *TH* and *DCX* mRNA were highly correlated, they could not be included in the same model due multicollinearity. Thus, four models were adjusted for each survival analysis performed. Akaike information criterion (AIC) (Sakamoto et al. 1986) was used to assess which model was the best in each case (lower AIC values meaning better models). Differences in AIC values among models were expressed as Δ AIC (AIC Model 1—AIC Model 2). p values <0.05 were considered statistically significant. All analyses and graphs were performed using R (version 3.1.2). For EFS, time to event was defined as the time from diagnosis until the time of first occurrence of relapse, progression or death. For OS, time to event was defined as time until death or until last contact if the patient was alive.

Results

Patients' characteristics

One hundred and sixty two children with metastatic NB tumors were studied, 146 stage M and 16 stage MS. The

median follow-up time was of 42.9 months. Most of the patients presented abdominal or adrenal primary tumors and 64 % had BM metastasis at diagnosis. 73 % of patients were older than 12 months at the time of diagnosis. *MYCN* status was obtained from 95 % of patients, of these, 34 % had *MYCN* amplified tumors. Clinical and biological data are summarized in Table 1.

For the analysis of *TH* and *DCX* mRNA expression at diagnosis, we included not only high-risk metastatic NB patients but also low and intermediate metastatic NB patients (MS patients without *MYCN* amplification and M patients younger than 12 months and without *MYCN* amplification). BM and PB samples were collected from 116 and 101 patients respectively at diagnosis, 81 and 57 after induction therapy, and 36 BM and 33 PB at the end of treatment.

TH and *DCX* mRNAs in PB and BM at diagnosis

The frequency of detection of *TH* and *DCX* mRNAs in PB and BM at diagnosis is detailed in Table 2. A high correlation between both markers was found; in PB the correlation was $\rho = 0.72$ (CI = 0.58–0.83) and in BM $\rho = 0.92$ (CI = 0.86–0.94). The level of *TH* and *DCX* mRNAs in PB and BM and the median expression is represented in Fig. 1a. The median level of expression was higher in BM samples than in PB samples. Univariable analyses revealed that high levels of *TH* and *DCX* mRNAs in PB significantly associated with OS and EFS ($p = 0.006$ and $p = 0.006$ for *TH*; $p = 0.017$ and 0.021 , respectively, for *DCX*). Additionally, high levels of *DCX* mRNA but not *TH* mRNA in BM samples were significantly associated with OS and EFS ($p = 0.005$ and $p = 0.004$, respectively). Further multivariable analysis taking into account age at diagnosis, *MYCN* status and stage, demonstrated that, at the time of diagnosis, high levels of *TH* and *DCX* mRNAs in PB are significant independent predictors of EFS and OS (Table 3a; Fig. 2). In BM, high levels of *DCX* mRNA but not *TH* independently predicted worse OS and EFS (Table 3a). Noteworthy, *TH* mRNA had greater predictive capacity than *DCX* mRNA when applied the AKAIKE criteria in the multivariable models (Δ AIC = 1.35). Univariable and multivariable analyses were also performed in the group of high-risk metastatic NB patient. Only *TH* mRNA in PB remained as independent predictor of poor OS and EFS (Table 3b).

TH and *DCX* mRNAs in PB and BM post induction therapy

MRD monitoring after induction therapy was performed for high-risk metastatic NB patients. Frequencies of detection of *TH* and *DCX* mRNAs as well as the

Table 1 Overview of the 162 metastatic neuroblastoma patients included in the study

Characteristics	INRG staging system		
	M	MS	Total
Number of patients	146	16	162
Pre-treatment risk group			
Low	0	14	14
Intermediate	20	0	20
High	126	2	128
Age at diagnosis in months			
Median	36.7	5.3	33.8
Range	2.0–267	0.2–13.6	0.2–267
Sex			
Female	57	9	66
Male	89	7	96
Primary site			
Adrenal	57	11	68
Abdominal	62	4	66
Cervical	2	0	2
Thoracic	9	0	9
Cervical-thoracic	2	0	2
Thoracic-abdominal	4	1	5
Cervical-thoracic-abdominal	1	0	1
Abdominal-pelvic	2	0	2
Pelvic	3	0	3
Intracranial	1	0	1
No primary tumor	2	0	2
Unknown	1	0	1
BM metastasis at diagnosis	103	2	105
MYCN status			
Amplified (%)	50 (34.2 %)	2 (12.5 %)	52 (32.1 %)
Not amplified (%)	89 (61 %)	13 (81.25 %)	102 (63 %)
Not informed	7 (4.8 %)	1 (6.25 %)	8 (4.9 %)
Patients with relapse (%)	86 (58.9%)	3 (18.75 %)	89 (54.9 %)
Dead (%)	79 (54.1 %)	1 (6.25 %)	80 (49.4 %)
Median follow-up time (months)	41.3	62.8	42.9

Table 2 Frequencies of detection of *TH* and *DCX* mRNAs in peripheral blood and bone marrow at diagnosis, after induction therapy and at the end of treatment

	Diagnosis		End of induction		End of treatment	
	PB	BM	PB	BM	PB	BM
Frequency of detection (%)						
<i>TH</i> mRNA	70.3	84.5	10.5	44.4	21.2	30.6
<i>DCX</i> mRNA	68.3	87.9	8.8	39.5	12.1	19.4

median expression levels of both markers are shown in Table 2 and Fig. 1b correspondingly. Univariable analyses showed that *TH* mRNA but not *DCX* when detected in PB predicted worse OS and EFS ($p = 0.003$ and $p = 0.04$ respectively). In BM only *DCX* mRNA but not *TH* mRNA was significantly associated with EFS ($p = 0.008$). The

multivariable model included *MYCN* status and age at diagnosis in addition to the *TH* and *DCX* mRNA presence in PB and BM. *TH* mRNA in PB and *DCX* mRNA in BM independently predicted worse OS and EFS. Furthermore, *TH* mRNA when detected in BM predicted poor EFS (Table 3c).

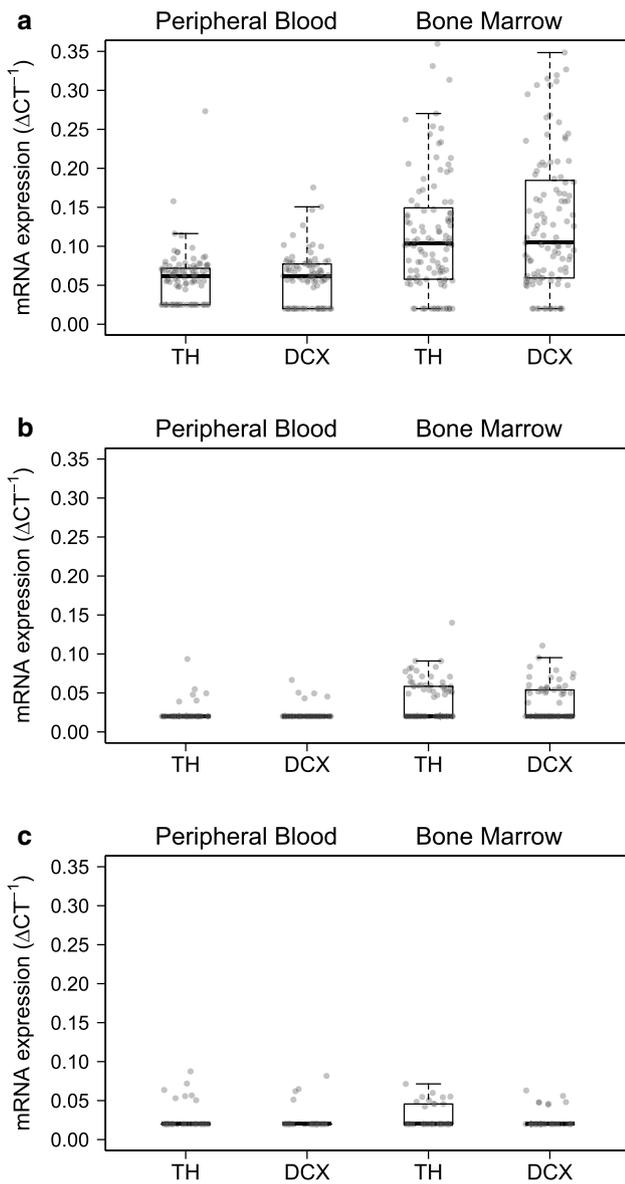


Fig. 1 Levels of *TH* and *DCX* mRNAs in peripheral blood and bone marrow aspirates of metastatic neuroblastoma children: **a** at diagnosis, **b** after induction therapy and **c** at the end of treatment

***TH* and *DCX* mRNAs in PB and BM at the end of treatment**

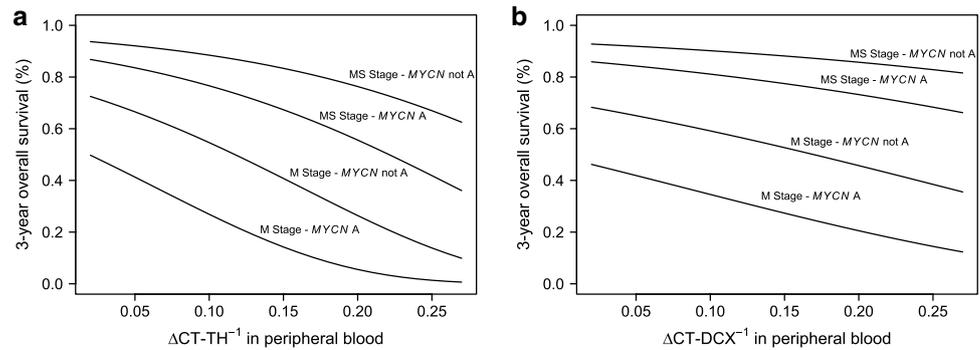
Frequencies of detection and the median expression levels for both markers in PB and BM samples at the end of treatment are detailed in Table 2 and Fig. 1c. Despite the reduced number of samples studied, univariable analyses revealed that *TH* mRNA when detected in PB and *DCX* mRNA when detected in BM associated with worse EFS and OS ($p = 0.011$ and $p = 0.004$ for *TH*, $p = 0.033$ and $p = 0.04$ for *DCX*, respectively). *DCX* mRNA in PB was also predictive of EFS ($p = 0.003$). Multivariable models

Table 3 Event-free survival and overall survival predicted by the levels of *TH* and *DCX* mRNAs in PB and BM at diagnosis, after induction therapy and at the end of treatment

Variable	HR	95 % CI	<i>p</i> value
(a) Multivariable model adjusted for age, MYCN and stage in the cohort of metastatic NB at diagnosis			
Event-free survival			
<i>TH</i> mRNA in PB	1.3	[1.1, 1.5]	0.003
<i>DCX</i> mRNA in PB	1.2	[1.0, 1.3]	0.025
<i>TH</i> mRNA in BM	1.1	[1.0, 1.3]	0.06
<i>DCX</i> mRNA in BM	1.2	[1.0, 1.5]	0.04
Overall survival			
<i>TH</i> mRNA in PB	1.3	[1.1, 1.5]	0.004
<i>DCX</i> mRNA in PB	1.2	[1.0, 1.4]	0.01
<i>TH</i> mRNA in BM	1.1	[1.0, 1.3]	0.054
<i>DCX</i> mRNA in BM	1.3	[1.0, 1.5]	0.025
(b) Multivariable model adjusted for age and MYCN in high-risk NB at diagnosis			
Event-free survival			
<i>TH</i> mRNA in PB	1.2	[1.0, 1.5]	0.015
<i>DCX</i> mRNA in PB	1.1	[1.0, 1.3]	0.11
<i>TH</i> mRNA in BM	1.1	[0.9, 1.2]	0.31
<i>DCX</i> mRNA in BM	1.1	[0.9, 1.4]	0.17
Overall survival			
<i>TH</i> mRNA in PB	1.3	[1.1, 1.5]	0.01
<i>DCX</i> mRNA in PB	1.2	[1.0, 1.3]	0.053
<i>TH</i> mRNA in BM	1.1	[0.9, 1.2]	0.29
<i>DCX</i> mRNA in BM	1.2	[1.0, 1.5]	0.12
(c) Multivariable model adjusted for age and MYCN in high-risk NB after induction therapy			
Event-free survival			
<i>TH</i> mRNA in PB	2.7	[1.0, 7.3]	0.047
<i>DCX</i> mRNA in PB	2.4	[0.5, 11.6]	0.27
<i>TH</i> mRNA in BM	3.9	[1.2, 13.1]	0.026
<i>DCX</i> mRNA in BM	5.2	[1.6, 17.3]	0.007
Overall survival			
<i>TH</i> mRNA in PB	3.5	[1.3, 9.4]	0.013
<i>DCX</i> mRNA in PB	2.4	[0.5, 11.6]	0.27
<i>TH</i> mRNA in BM	3.6	[1.0, 13.3]	0.052
<i>DCX</i> mRNA in BM	3.6	[1.1, 4.2]	0.035
(d) Multivariable model adjusted for age and MYCN in high-risk NB at the end of treatment			
Event-free survival			
<i>TH</i> mRNA in PB	2.4	[1.0, 5.7]	0.056
<i>DCX</i> mRNA in PB	3.4	[1.0, 11.6]	0.055
<i>TH</i> mRNA in BM	0.9	[0.02, 41.5]	0.98
<i>DCX</i> mRNA in BM	1.7	[0.03, 82.9]	0.79
Overall survival			
<i>TH</i> mRNA in PB	3.2	[1.2, 8.8]	0.026
<i>DCX</i> mRNA in PB	2.2	[0.5, 9.9]	0.31
<i>TH</i> mRNA in BM	0.06	[0, 6.9]	0.25
<i>DCX</i> mRNA in BM	2.8	[0.06, 132.1]	0.60

Statistically significant *p*-values are indicated in bold
PB peripheral blood, *BM* bone marrow, *HR* hazard ratio

Fig. 2 Three-year overall survival graphs representing the prognostic impact of *TH* mRNA **a** and *DCX* mRNA detection **b** in peripheral blood from metastatic NB patients considering stage and *MYCN* status



including the previously mentioned risk factors demonstrated that only *TH* mRNA when detected in PB remained as an independent predictor of adverse outcome (Table 3d).

Discussion

RT- qPCR has shown tremendous potential for detecting MRD in PB and BM from NB patients (Viprey et al. 2014; Stutterheim et al. 2011; Cheung et al. 2003). However, the proof of the clinical utility of the mRNAs detection has been limited by several factors. An important one has been the small number of samples investigated at the scheduled time points for disease evaluation. Another significant factor has been the lack of multivariable analysis to assess the independent significance of MRD detection. The majority of previous studies have focused on high-risk metastatic NB patients. This approach has helped identify children with ultrahigh-risk disease who may benefit from new treatment strategies (Viprey et al. 2014). However, this approach does not take into consideration low and intermediate metastatic NB patients at the time of diagnosis. Since in the group of low and intermediate metastatic NB there are a percentage of patients who die of disease, it is also important to identify such group to treat them more accurately. In our study we analyzed a cohort of metastatic NB patients without previously stratifying by risk factors. The levels of *TH* and *DCX* mRNAs when detected in PB and BM independently predicted worse EFS and OS. Given the heterogeneity of the metastatic NB group, considering the mRNA levels in new pre-treatment risk stratification strategies would help predict outcome differences and identify children for whom current treatment is failing or insufficient. In low and intermediate metastatic NB, the study of MRD at different time points of evolution/treatment could be another interesting challenge.

TH and *DCX* mRNAs are not 100 % specific biomarkers for NB cell detection since low levels of expression have been detected in hematopoietic cells and in PB from healthy donors (Kuçi et al. 2006; Corrias et al. 2012; Hartomo

et al. 2012). In order to address the issue of the illegitimate expression of the NB mRNAs, previous multicenter international studies have established cut points for data analyses with a threshold beyond which the mRNAs most reliably predict worse EFS and OS (Viprey et al. 2014). Medical researchers often converted continuous variables into categorical variables by grouping values into two or more categories. The simplicity achieved may cause considerable loss of power and residual confounding if categorization is applied to the original prognostic variables (Royston et al. 2006). Under our experimental conditions, we have not detected *TH* neither *DCX* mRNAs in PB and BM samples from healthy donors. In this study the *TH* and *DCX* mRNA levels were considered continuous variables. We assessed the expected continuous relationship between increasing levels of *TH* and *DCX* mRNAs and an increasing risk of events. This also allowed us to address the problem of illegitimate expression: patients with low mRNA levels have a lower risk of events than patients with higher mRNA levels.

TH is the most rigorously evaluated and most used target for the detection of disease in PB and BM from children with NB. There are some important factors to take into consideration when analyzing the *TH* expression. One relevant factor is that hematopoietic cells can also produce, metabolize and take up catecholamines. This means that discriminating hematopoietic cells from NB cells by detecting *TH* mRNA is not as precise as desired, since low expression levels of *TH* have been detected in hematopoietic cells. In addition, an inverse correlation of noradrenaline transporter and *TH* expression has been described (Lode et al. 1995). Metaiodobenzylguanidine (MIBG) is accumulated in the NB cell by the noradrenaline transporter. Thus, it is possible that NBs with high MIBG uptake may express very low levels of *TH*, perhaps even undetectable by RT-qPCR. Testing multiple targets for MRD detection may increase sensitivity and specificity and also overcome the heterogeneity of the NB cells.

In the subgroup of high-risk metastatic NB patients, we found that high levels of *TH* mRNA in PB independently

predicted worse outcome when detected at diagnosis, post-induction therapy and at the end of treatment. The use of PB and not BM samples for the study is an additional advantage taking into consideration that patients will not be exposed to an invasive technique. Our results at diagnosis agree with some previously published reports (Viprey et al. 2014; Burchill et al. 2001; Träger et al. 2008).

After induction therapy, *TH* and *DCX* mRNAs when detected in BM independently predicted worse outcome in high-risk metastatic NB patients. Additionally, *TH* mRNA when detected in PB is an independent predictor of poor EFS and OS. Viprey et al. have previously described that high levels of these mRNAs predicted worse EFS and OS in BM but not in PB. The methodological differences between the two studies could be the principal reason for such discrepancy. Sensitivity limitations of cytological screening for accurate detection of disease in BM reinforce the importance of the mRNAs assessment by RT-qPCR to evaluate the effectiveness of the induction chemotherapy. Additional alternative treatment should be considered for children with insufficient molecular response to treatment at this time point.

The study of the MRD at the end of treatment was carried out with fewer samples as compared to the one used during diagnostics. The decrease in the number of samples is in part a result of the evolution of the disease, that is, the patients die before reaching the end of treatment. Prospective multicenter collaborative studies with larger cohorts of patients are needed to determine the clinical relevance of the mRNAs detection at the end of treatment. To the best of our knowledge, we described here for the first time that *TH* mRNA when detected in PB at the end of treatment is an independent predictor of worse outcome. Circulating tumor cells detected at the end of treatment could generate metastases or die by apoptotic mechanisms induced by the maintenance therapy. Considering the low survival rates of metastatic NB who relapse or progress, new maintenance treatment strategies should be contemplated for this patient population based on the true remission status. Accurate detection of minimal disease, undetectable by conventional methods, would identify patients with worse outcome before the appearance of overt clinical symptoms.

RT-qPCR is a sensitive, easy and widely applicable method to objectively detect MRD in NB patients. There are very few published studies focused on MRD detection in PB and BM samples with an evolutionary focus on the different evaluation points in the disease. Sequential MRD monitoring throughout the disease course by RT-qPCR should be included as a standard method for disease evaluation using standard operating procedures for analysis and reporting.

In conclusion, *TH* and *DCX* mRNA levels in PB and BM samples assessed by RT-qPCR at diagnosis could be used to

better assign metastatic NB patients into risk groups and tailor the intensity of treatment. MRD detection in PB and BM after induction therapy has important prognostic implication for high-risk metastatic NB. When MRD is monitored at scheduled times as per protocol, it would give relevant insights into the effectiveness of treatment. Also, monitoring MRD would aid clinicians to recognize critical elements in practice such as how and when to modify treatment.

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Compliance with ethical standards

Conflict of interest The authors indicated no potential conflicts of interest.

Ethical approval The study was approved by the Hospital La Fe Ethical Committee and was performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent All parents or guardians signed an informed consent statement for sample and data management from all patients included in the study.

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