Article:
Corrias, MV, Parodi, S, Tchirkov, A et al. (14 more authors) (2018) Event-free survival of infants and toddlers enrolled in the HR-NBL-1/SIOPEN trial is associated with the level of neuroblastoma mRNAs at diagnosis. Pediatric Blood and Cancer, 65 (7). e27052. ISSN 1545-5009

https://doi.org/10.1002/pbc.27052

Event-free survival of infants and toddlers enrolled in the HR-NBL-1/SIOPEN trial is associated with the level of neuroblastoma mRNAs at diagnosis

Maria V. Corrias[1#], Stefano Parodi[1], Andrei Tchirkov[2#], Tim Lammens[3#], Ales Vicha[4#], Claudia Pasqualini[5#], Catarina Träger[6#], Yania Yáñez[7#], Sandro Dallorso[1#], Luigi Varesio[1#,&], Roberto Luksch[8], Genevieve Laureys[3], Dominique Valteau-Couanet[5], Adela Canete[7], Ulrike Pöetschger[9], Ruth Ladenstein[9] and Susan A. Burchill[10#].

[1]Istituto Giannina Gaslini, Genoa, Italy; [2]CHU Clermont-Ferrand, Service de Cytogénétique Médicale and Université Clermont Auvergne, Clermont-Ferrand, France; [3]Ghent University Hospital, Ghent, Belgium; [4]2nd Medical Faculty Charles University and Faculty Hospital Motol, Prague, Czech Republic; [5]Institut Gustave Roussy, Villejuif, France; [6]Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden; [7]Oncología Pediátrica, Hospital Universitari i Politècnic La Fe, Valencia, Spain; [8]Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy; [9]*CCRI/St. Anna Children's Hospital, Vienna, Austria;* [10]Leeds Institute of Cancer and Pathology, Leeds, United Kingdom.

[#] Members of the SIOPEN Molecular Monitoring Group

[&] Dr Luigi Varesio prematurely passed away last December. The colleagues that had the privilege to collaborate with him dedicate this manuscript to his memory.

*** Correspondence:**
Maria Valeria Corrias
Gaslini Institute
Largo Gaslini, 5
16148 Genoa, Italy
Phone: +39 010 5636 3531, Fax: +39 010 3779820
e-mail: mariavaleriacorrias@gaslini.org

Word count for

a) Abstract = 250
b) Main Text = 2781

Tables = 3
Figures = 3

Supplemental files = Supplemental TABLE S1 and supplemental Figures S1, S2, S3, S4 and S5

Running title: infants' survival associates with NB mRNA levels

Keywords: neuroblastoma, infants, toddlers, RTqPCR, outcome.

Abbreviations key table

BM	Bone marrow
DCX	Doublecortin
DFS	Disease-free survival
EFS	Event-free survival
HR	Hazard ratio
HR-NBL-1/SIOPEN	European High-Risk Neuroblastoma trial
MYCNA	MYCN amplification
NB	Neuroblastoma
PB	Peripheral blood
PHOX2B	Paired-like homeobox 2b
ROC	Receiver Operating Characteristic
RTqPCR	Reverse transcription quantitative polymerase chain reaction
TH	Tyrosine hydroxylase

Funding

This research was funded by Cancer Research UK (C5651/A9663; SAB), Neuroblastoma UK and the University of Leeds (SAB), Fondazione Italiana per la Lotta al Neuroblastoma (MVC), French National Hospital research project and Association Les Bagouz'à Manon (AT), MH CZ – DRO, University Hospital Motol, Prague, Czech Republic 00064203 (AV), vzw Kinderkankerfonds (TL), Onderzoeksproject gerealiseerd met de steun van Kom op tegen Kanker (GL) and Asociacion Pablo Ugarte (AC).

ABSTRACT

Background: To evaluate whether levels of neuroblastoma mRNAs in bone marrow and peripheral blood from stage M infants (≤12 months of age at diagnosis, MYCN amplified) and toddlers (between 12 and 18 months, any MYCN status) predict event-free survival (EFS).

Methods: Bone marrow aspirates and peripheral blood samples from 97 infant-toddlers enrolled in the HR-NBL-1/SIOPEN trial were collected at diagnosis in PAXgene™ blood RNA tubes. Samples were analyzed by RTqPCR according to standardized procedures.

Results: Bone marrow TH or PHOX2B levels in the highest tertile associated with worse EFS; hazard ratios, adjusted for age and MYCN status, were 1.5 and 1.8 respectively. Expression of both TH and PHOX2B in the highest tertile predicted for worse outcome (p=0.015), identifying 20 (23%) infant-toddlers with 5-year EFS of 20% (95%CI: 4%–44%). Prognostic significance was maintained after adjusting for over-fitting bias (p=0.038), age and MYCN status. In peripheral blood, PHOX2B levels in the highest tertile predicted a two-fold increased risk of an event (p=0.032), identifying 23 (34%) infant-toddlers with 5-year EFS of 29% (95%CI: 12%–48%). Time-dependent ROC analysis confirmed the prognostic value of combined TH and PHOX2B in bone marrow and of PHOX2B in peripheral blood during the first year of follow-up.

Conclusions: High levels of bone marrow TH and PHOX2B and of peripheral blood PHOX2B at diagnosis allow early identification of a group of high-risk infant and toddlers with neuroblastoma who may be candidates for alternative treatments. Integration with additional biomarkers, as well as validation in additional international trials is warranted.

INTRODUCTION

Neuroblastoma (NB) clinical presentation is variable, ranging from asymptomatic localized masses to metastatic disease. The main prognostic factors defined by the International Neuroblastoma Risk Group-Stratification System (INRG-SS) are stage, MYCN oncogene status and age at diagnosis [1]. Indeed, younger patients have a better event-free survival (EFS) than older children [2, 3] and MYCN amplification (MYCNA) of the tumor is predictive of a worse EFS in patients with localized disease of any age [4-6] and in young patients with metastatic disease [7]. Metastatic spread is present at diagnosis in 50% of cases and mainly involves bone and bone marrow (BM). Patients presenting with metastatic disease are assigned to stage M and have a worse EFS than patients with localized tumor [1]. A particular case of metastatic NB (MS) may occur in infants with metastases limited to liver and/or skin, and/or limited bone marrow infiltration [8]. In these infants prognosis is good [9-11], unless presenting with MYCNA [7, 12].

Although age is a continuous variable, an age cut-off is used in the clinic at diagnosis to stratify patients for risk and consequently the type of therapeutic intervention. Initially the cut-off was set at 12 months [3], then it has been moved to 18 months [12]. Nonetheless, the cut-off of 12 months is still applied to patients with metastatic disease in some trials, including the European High-Risk (HR-NBL-1/SIOPEN) trial [5, 13].

Recently, a large prospective multicenter study performed on stage M patients enrolled in the HR-NBL-1/SIOPEN trial demonstrated that the levels of Tyrosine hydroxylase (TH), Paired-like homeobox 2b (PHOX2B) and Doublecortin (DCX) mRNAs in BM and peripheral blood (PB) samples, collected at diagnosis and at the end of induction therapy, were predictive of EFS [14]. Given the different survival rate of infants and toddlers as compared with children [1, 5, 7, 13] and because a single country pilot study on infants with stage M and MS, not enrolled in the HR-NBL-1/SIOPEN trial, had suggested that TH, PHOX2B and DCX mRNA levels were significantly lower in infants than in children [15], we sought to evaluate the prognostic value of NB mRNA levels in

the subset of subjects below 18 months of age enrolled in the HR-NBL-1/SIOPEN trial since it was not certain that the predictive power of RT-qPCR demonstrated for the entire high-risk population was truly effective also in this subset of patients. The infant-toddlers included in the previous study represented in fact only 10% of the entire cohort [14], therefore potential differences between children and infant-toddlers could have been lost. Since the frequency of each subset, i.e. infants with MYCNA, toddlers with MYCNA and toddlers with single-copy MYCN is low (around 3%), we preferred considering them as a whole.

METHODS

Clinical samples, children and trial

Eighty-eight BM aspirates (2 x 0.5ml from the right and left site, not pooled) and 74 PB (1 x 2ml) samples were taken at diagnosis from 97 patients entered into the HR-NBL-1/SIOPEN trial (NCT01704716) between April 2002 and June 2015 (www.SIOPEN-R-NET.org [5]). Eligibility criteria for the study were as follows: 1) stage M infants (≤12 months of age at diagnosis) presenting with MYCNA; 2) stage M toddlers (12 to 18 months) with any MYCN status; 3) no death for treatment-related toxicity. Detailed information on the study cohort and the HR-NBL-1/SIOPEN infant-toddlers' cohort is given in Table 1.

Written informed consent was obtained from the legal guardians, and the trial was ethically approved in each participating country according to national practice. This observational, blind

biological study did not impact on clinical management of children in the HR-NBL-1/SIOPEN trial.

Sample processing and analysis

All samples were collected into PAXgene^{TM} blood RNA tubes, stored and analysed using optimised standard operating procedures [16]. Due to limited amounts of RNA isolated from some samples, 88 BM aspirates were analysed for TH, 84 for PHOX2B and 76 for DCX mRNAs, whereas 74 PB samples were analysed for TH, 68 for PHOX2B and 60 for DCX. Quality assurance was maintained across the reference laboratories by biannual quality control [16]. The triplicate result for the target mRNAs and the house keeping gene β2 microglobulin (β2M) were recorded blind to clinical information.

Statistical analysis

Results of RTqPCR were expressed as Log RQ [14] and linked to clinical information. The frequency of data was compared using the Chi Square test or the Fisher exact test, as appropriate. The Mann–Whitney U test was used to compare median values. Association between continuous variables was assessed by the Pearson r correlation coefficient.

EFS included the time from diagnosis to an event (recurrence, progression or death) or the date of the last assessment without event. EFS was analysed by the Kaplan-Meier method and survival curves were compared by the log rank test.

Impact of potential confounders (age at diagnosis and MYCN status) was assessed by the Cox regression model. Over fitting bias, related to the combination of predictive markers at a posteriori

selected cut-offs, was estimated by the method of Harrell et al, using 2,000 bootstrapped samples [17].

The prognostic effect of mRNA levels was also investigated using time-dependent ROC curves [18], adjusted for the potential confounding effect of age at diagnosis and MYCN status [19]. The potential confounding effect of MYCN status was also evaluated by excluding from analysis the toddlers with single copy MYCN.

ROC analysis was performed using the statistical package "survival ROC" implemented in R language [20]. All other analyses were carried out using Stata for Windows statistical package (release 12.1, Stata Corporation, College Station, TX).

RESULTS

Expression of TH, PHOX2B and DCX mRNA in the study patients

Neuroblastoma mRNAs were detected in most samples; 1.2%, 2.4 % and 2.7% of BM and 6.8%, 4.5% and 3.4% of PB were negative (Log = - 4) for TH, PHOX2B and DCX mRNAs respectively (Supplemental Figure S1). This precluded a binary analysis (positive/negative) of neuroblastoma mRNA predictive value. Two patients with BM levels outside the normal distribution

(Supplemental Figure S1) were excluded from the analysis to avoid unnecessary transformation of the data; these children had no unusual clinical features.

The study cohort therefore included 95 infant-toddlers (Table 1). The age distribution, the MYCNA frequency in the toddler subset, as well as the 3-year EFS of the study cohort was representative of the entire infant-toddler population enrolled into the HR-NBL-1/SIOPEN trial (Table 1).

Since RTqPCR was performed on the right and left (not pooled) BM aspirates, analyses were initially made using either the average value or the highest value. The correlation r values between the left and right aspirates were 0.810, 0.660 and 0.736 for TH, PHOX2B and DCX, respectively (Supplemental Figure S2A). The association between the level of expression of each mRNA in BM aspirate and EFS was slightly better when the highest value was considered (Supplemental Table S1). Therefore, all subsequent analyses on BM status have been performed using the highest value of NB mRNAs measured in either one of the two BM aspirates.

Next, we tested whether the results obtained for each individual NB mRNA correlated with the results obtained with the other two mRNAs. In BM samples, correlation was good between TH and PHOX2B or TH and DCX (Supplemental Figure S2B, $r = 0.80$ and $r = 0.83$, respectively), and excellent between PHOX2B and DCX ($r = 0.90$). In PB there was a good correlation between PHOX2B and DCX mRNAs (Supplemental Figure S2B, $r = 0.88$). However, there was no correlation between NB mRNAs measured in paired BM and PB from the same patient (Supplemental Figure S2C, $r < 0.60$).

Predictive power of neuroblastoma mRNA expression in infants and toddlers

To evaluate the prognostic significance of NB mRNAs, we used the highest tertile of each mRNA level distribution to split patients into two groups with either high or low/intermediate mRNA expression. The comparison of survival in these groups showed that patients with BM levels of TH, PHOX2B and DCX mRNAs in the highest tertile had worse EFS (Figure 1A, 1B and 1C,

188 respectively). Patients with PB levels of PHOX2B and DCX mRNAs in the highest tertile showed
189 shorter EFS (p = 0.024 for PHOX2B, Figure 1E and 1F, respectively), whereas TH mRNA levels
190 in PB were not associated with EFS (Figure 1D). Similar results were observed when toddlers with
191 single copy MYCN were excluded from the analysis.
192 The risk of events as a function of NB mRNA levels was then evaluated using univariate and
193 multivariate Cox regression analysis. The HR for high levels of TH, PHOX2B and DCX
194 expression in BM were 1.8, 1.7 and 1.7, respectively (Table 2). In PB, high levels of PHOX2B
195 mRNA were significantly associated with more than a two-fold increased risk of event (HR = 2.2,
196 p = 0.030, Table 2). The prognostic value of NB mRNAs was confirmed by multivariate analysis
197 including age and MYCN status (Table 2), and after excluding toddlers with normal MYCN status.
198
199 **Predictive power of combined results**
200 Since previous studies have shown that high levels of TH or PHOX2B mRNA predicts for poor
201 outcome [14], survival analysis was performed dichotomising patients by the highest tertile of both
202 TH and PHOX2B mRNAs. In BM, the combination of TH and PHOX2B mRNAs had greater
203 prognostic power (p=0.015, Figure 2A) than either mRNA alone (p=0.063 and p=0.088,
204 respectively, Figure 1A and 1B); this was retained after adjusting for over fitting bias (p = 0.038).
205 Among 29 patients with TH mRNA above the highest tertile and 28 patients with PHOX2B mRNA
206 above the highest tertile, 20 (23%) of infant-toddlers had high levels of both mRNAs (Figure 2B,
207 upper right field). These 20 patients had particularly poor 5-year EFS (20%, 95%CI: 4% – 44%).
208 Patients with higher values of combined TH and PHOX2B mRNAs showed poorer EFS also after

209 excluding toddlers with single copy MYCN (Figure 2C). In addition, the predictive power was
210 maintained when the infants and the toddlers were separately analysed (Supplemental Figure S3).
211 Conversely, the combination of TH and PHOX2B mRNAs in PB was not of additional value
212 (p=0.521, Supplemental Figure S4); the prognostic power of PHOX2B mRNA (p=0.024, Figure
213 1E) was lost when combined with TH mRNA (p= 0.910, Figure 1D).
214
215 **Time-dependent prognostic value of molecular analysis in the infant-toddler subset**
216 To estimate the prognostic value of high levels of NB mRNAs in relation to time of events, time-
217 dependent ROC curves [18] were plotted for BM and PB TH, PHOX2B and DCX mRNA levels
218 (Figure 3), and for combined BM TH and PHOX2B levels. As shown in Table 3, the area under
219 the curve (AUC) was statistically significant for the combined BM TH and PHOX2B mRNAs and
220 for PB PHOX2B at 1 year, validating previous findings. However, the average predictive accuracy
221 was moderate (AUC < 70% in each analysis) and limited to the first year after diagnosis, since
222 AUC values at 3 and 5 years were not significant for any NB mRNA (Table 3).
223
224 **Predictive power of previously defined cut-points**
225 The large prospective multicenter study performed on the entire stage M population enrolled into
226 HR-NBL-1/SIOPEN trial defined predictive cut-points for each NB mRNAs [14]. We thus tested
227 whether those cut-points were also predictive for survival within the infant-toddler subset. In BM,
228 the TH cut-point of 2.36 [14] was associated with survival (p=0.060, Supplemental Figure S5A). In
229 contrast the PHOX2B and DCX cut-points (-2.63 and -0.45, respectively) were not predictive of
230 survival (Supplemental Figure S5B and S5C, respectively). This may reflect the distribution of
231 patients since only 2 out of 82 (2.5%) and 13 out of 74 (18%) infant-toddlers, respectively, had
232 mRNA levels below the cut-points. Indeed, median TH mRNA did not discriminate the study
233 patients (Supplemental Figure S5D), in contrast to the median level of BM PHOX2B and DCX

mRNAs (Supplemental Figure S5E and S5F, respectively). In PB, neither the previously defined cut-points [14] nor the median value of the distributions were predictive of EFS.

DISCUSSION

Outcome for children with metastatic NB is variable reflecting the positive effect of young age and the negative effect of MYCNA [1]. Given the difference in outcome of infants and toddlers as compared with children, here we investigated whether the level of NB mRNAs in BM and PB from stage M infants and toddlers enrolled into the HR-NBL-1/SIOPEN trial had an independent prognostic significance. In fact, in our previous study [14], infants and toddlers accounted for only 10% of the cohort and potential differences with children could have been lost.

Here we show that levels of TH, PHOX2B and DCX mRNAs in BM above the highest tertile associated with worse EFS, confirming that NB mRNA levels also had predictive power in this subset of patients with NB. Furthermore, the combination of high TH and PHOX2B mRNAs in BM for 23% of the study cohort was significantly associated with a poor outcome (5-year EFS = 20%, 95%CI: 4% – 44%). Multivariate analysis, after considering over fitting bias, together with the results obtained after exclusion of toddlers with single copy MYCN confirmed that the predictive power was independent of age and MYCN status. In fact, toddlers with single copy MYCN are considered as intermediate risk patients in other trials, but in the HR-NBL-1/SIOPEN trial the age limit for inclusion was 12 months and these patients were thus included. Time-dependent ROC analysis indicated that the predictive power was greater in the first year after

diagnosis, suggesting that high levels of NB mRNAs may characterize treatment refractory or early relapsing children.

Although the expression of the three NB mRNAs correlated to each other, the combination of TH and PHOX2B mRNAs in BM increased the prognostic performance of either alone. This may be due to the limited number of study subjects or may reflect differences in the expression of TH and PHOX2B mRNAs in the neoplastic cells [21, 22], which will require further studies in additional cohorts. In PB, PHOX2B mRNA significantly dichotomized the prognosis of the study cohort, identifying 23 (34%) patients with poor outcome (5-year EFS = 29%, 95%CI: 12% – 48%). It is interesting to note that only half of the patients with poor EFS were identified by high levels of NB mRNAs in both BM and PB analysis, whereas the remainder of the infant-toddlers were identified by NB mRNAs in either BM or PB. This suggests that the analysis of both PB and BM for NB mRNAs may predict outcome most comprehensively. We also demonstrate that the highest level of NB mRNA in one of the two BM aspirates is a stronger predictor of outcome than the average level, supporting recent recommendations for analysis of BM aspirates [23], and advocating separate investigations of right and left BM aspirates [16, 24].

The predictive power of high levels of NB mRNAs in BM from the infant-toddlers is consistent with previous observations [14]. The level of TH mRNAs that dichotomized the study patients was close to the previously defined cut-point (1.89 vs. 2.36, respectively). Conversely, PHOX2B and DCX levels were higher in the infants/toddlers than in the whole population of children with NB (2.6 vs. -2.6 and 2.65 vs. -0.45, respectively, [14]). This may reflect high levels of PHOX2B and DCX mRNAs during neuronal development [25, 26]. In PB, the levels of PHOX2B and DCX mRNA that dichotomize infants was close to the previously defined cut-points (0.07 vs. 0.28, and 0.16 vs. 0.41, respectively [14]), whereas TH mRNA levels in blood were not predictive of outcome. This may be related to either expression of TH mRNA in more differentiated, sympathetic-committed neuronal cells [21, 22, 24] or to its reported illegitimate transcription [24, 27-32]. Taken together, the

differences observed in NB mRNAs in infant-toddlers, as compared to children [14] may be linked to their better outcome. However, we cannot exclude the possibility that the differences observed here in the infant-toddler population were due to the smaller sample size, confirming the need for validation in additional cohorts.

The results described here do not confirm that stage M infants have lower levels of NB mRNAs than older stage M children [15]. The distribution of TH, PHOX2B and DCX mRNA expression in BM aspirates and PB samples from the infant-toddlers cohort was similar to that observed when patients of all ages were considered [14]. Discrepancy may be due to the small size and very low frequency of high-risk infants included in the pilot, single-country study [15].

In conclusion, high TH and PHOX2B mRNA levels in BM, and high PHOX2B mRNA levels in PB identify 23% and 34% respectively of infant-toddlers that have poor 5-year EFS. Given the low number of infants and toddlers with high-risk NB, integration with additional biomarkers, such as ploidy [33], and validation in additional international trials are necessary. However, early identification of those infant-toddlers for whom current treatment appears to provide no substantial survival benefit may lead to improved outcome, if they can be offered alternative, more effective, treatment.

CONFLICT OF INTEREST STATEMENT

TL received two travel grants from Jazz Pharmaceutical. All the other Authors have no conflict of interest to declare.

ACKNOWLEDGMENTS

The Authors wish to thank all the physicians and pathologists that collected and centralized the samples, and the parents and legal guardians that allowed the use of their children's samples for research studies.

REFERENCES

1. Cohn SL, Pearson AD, London WB, et al. The International Neuroblastoma Risk Group (INRG) classification system: an INRG Task Force report. J Clin Oncol. 2009;27(2):289-97.
2. London WB, Boni L, Simon T, et al. The role of age in neuroblastoma risk stratification: the German, Italian, and children's oncology group perspectives. Cancer Lett. 2005;228(1-2):257-266.
3. Brodeur GM, Pritchard J, Berthold F, et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol. 1993;11(8):1466-1477.
4. De Bernardi B, Gerrard M, Boni L, et al. Excellent outcome with reduced treatment for infants with disseminated neuroblastoma without MYCN gene amplification. J Clin Oncol. 2009;27(7):1034-1040.
5. Ladenstein R, Valteau-Couanet D, Brock P, et al. Randomized Trial of prophylactic granulocyte colony-stimulating factor during rapid COJEC induction in pediatric patients with

high-risk neuroblastoma: the European HR-NBL1/SIOPEN study. J Clin Oncol. 2010;28(21):3516-3524.

6. Gatta G, Botta L, Rossi S, et al. Childhood cancer survival in Europe 1999-2007: results of EUROCARE-5--a population-based study. Lancet Oncol. 2014;15(1):35-47.
7. Canete A, Gerrard M, Rubie H, et al. Poor survival for infants with MYCN-amplified metastatic neuroblastoma despite intensified treatment: the International Society of Paediatric Oncology European Neuroblastoma Experience. J Clin Oncol. 2009;27(7):1014-1019.
8. D'Angio GJ, Evans AE, Koop CE. Special pattern of widespread neuroblastoma with a favourable prognosis. Lancet. 1971;1(7708):1046-1049.
9. Matthay KK. Stage 4S neuroblastoma: what makes it special? J Clin Oncol. 1998;16(6):2003-2006.
10. Benard J, Raguenez G, Kauffmann A, et al. MYCN-non-amplified metastatic neuroblastoma with good prognosis and spontaneous regression: a molecular portrait of stage 4S. Mol Oncol. 2008;2(3):261-271.
11. Hero B, Simon T, Spitz R, et al. Localized infant neuroblastomas often show spontaneous regression: results of the prospective trials NB95-S and NB97. J Clin Oncol. 2008;26(9):1504-1510.
12. London WB, Castleberry RP, Matthay KK, et al. Evidence for an age cutoff greater than 365 days for neuroblastoma risk group stratification in the Children's Oncology Group. J Clin Oncol. 2005;23(27):6459-6465.
13. Ladenstein R, Potschger U, Pearson ADJ, et al. Busulfan and melphalan versus carboplatin, etoposide, and melphalan as high-dose chemotherapy for high-risk neuroblastoma (HR-

NBL1/SIOPEN): an international, randomised, multi-arm, open-label, phase 3 trial. Lancet Oncol. 2017;18(4):500-514.

14. Viprey VF, Gregory WM, Corrias MV, et al. Neuroblastoma mRNAs predict outcome in children with stage 4 neuroblastoma: a European HR-NBL1/SIOPEN study. J Clin Oncol. 2014;32(10):1074-1083.
15. Parodi S, Erminio G, Carlini B, et al. Infants and children with stage 4 neuroblastoma express significantly different levels of specific molecular markers J Mol Biomark Diagn 2012;S(2):8.
16. Viprey V, Corrias MV, Kagedal B, et al. Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: quality assurance on behalf of SIOPEN-R-NET. Eur J Cancer. 2007;43(2):341-350.
17. Harrell FE, Jr., Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. Stat Med. 1996;15(4):361-387.
18. Heagerty PJ, Zheng Y. Survival model predictive accuracy and ROC curves. Biometrics. 2005;61(1):92-105.
19. Janes H, Longton G, Pepe M. Accommodating Covariates in ROC Analysis. Stata J. 2009;9(1):17-39.
20. Team RC. R: A language and environment for statistical computing. . R Foundation for Statistical Computing, Vienna, Austria 2015.
21. Boeva V, Louis-Brennetot C, Peltier A, et al. Heterogeneity of neuroblastoma cell identity defined by transcriptional circuitries. Nature Gen. 2017;49(9):1408-1413.
22. van Groningen T, Koster J, Valentijn LJ, et al. Neuroblastoma is composed of two super-enhancer-associated differentiation states. Nature Gen. 2017;49(8):1261-1266.
23. Burchill SA, Beiske K, Shimada H, et al. Recommendations for the standardization of bone marrow disease assessment and reporting in children with neuroblastoma; on behalf of the

International Neuroblastoma Response Criteria Bone Marrow Working Group. Cancer. 2017;123(7):1095-1105.

24. Viprey VF, Lastowska MA, Corrias MV, Swerts K, Jackson MS, Burchill SA. Minimal disease monitoring by QRT-PCR: guidelines for identification and systematic validation of

molecular markers prior to evaluation in prospective clinical trials. J Pathol. 2008;216(2):245-252.

25. van Limpt V, Chan A, Schramm A, Eggert A, Versteeg R. Phox2B mutations and the Delta-Notch pathway in neuroblastoma. Cancer Lett. 2005;228(1-2):59-63.
26. Sharma V, Nag TC, Wadhwa S, Roy TS. Temporal distribution of mRNA expression levels of various genes in the developing human inferior colliculus. Neuroscience letters. 2009;461(3):229-234.
27. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al. Detecting minimal residual disease in neuroblastoma: the superiority of a panel of real-time quantitative PCR markers. Clin Chem. 2009;55(7):1316-1326.
28. Stutterheim J, Zappeij-Kannegieter L, Versteeg R, Caron HN, van der Schoot CE, Tytgat GA. The prognostic value of fast molecular response of marrow disease in patients aged over 1 year with stage 4 neuroblastoma. Eur J Cancer. 2011;47(8):1193-1202.
29. Corrias MV, Haupt R, Carlini B, et al. Multiple target molecular monitoring of bone marrow and peripheral blood samples from patients with localized neuroblastoma and healthy donors. Pediatr Blood Cancer. 2012;58(1):43-49.
30. Stutterheim J, Zappeij-Kannegieter L, Ora I, et al. Stability of PCR targets for monitoring minimal residual disease in neuroblastoma. J Mol Diagn. 2012;14(2):168-175.
31. van Wezel EM, Stutterheim J, Vree F, et al. Minimal residual disease detection in autologous stem cell grafts from patients with high risk neuroblastoma. Pediatr Blood Cancer. 2015;62(8):1368-1373.
32. Stutterheim J, Gerritsen A, Zappeij-Kannegieter L, et al. PHOX2B is a novel and specific marker for minimal residual disease testing in neuroblastoma. J Clin Oncol. 2008;26(33):5443-5449.

33. George RE, London WB, Cohn SL, et al. Hyperdiploidy plus nonamplified MYCN confers a favorable prognosis in children 12 to 18 months old with disseminated neuroblastoma: a Pediatric Oncology Group study. J Clin Oncol. 2005;23(27):6466-6473.

LEGENDS TO FIGURES

Figure 1. Kaplan-Meier event-free survival estimates (EFS) obtained by stratifying the infant-toddler cohort by the highest tertile value in BM (**A, B** and **C**) and in PB (**D, E** and **F**).

Figure 2. A) Kaplan-Meier survival estimates obtained by stratifying the whole infant-toddler cohort by combining TH and PHOX2B RNA levels in BM. **B)** Distribution of BM TH and PHOX2B mRNA levels in the study patients. The infant-toddlers with both TH and PHOX2B in the highest tertile are included in the upper right quadrant (closed circles). **C)** Kaplan-Meier survival estimates obtained by stratifying the subset of infant-toddlers presenting with MYCN amplification by combining TH and PHOX2B RNA levels in BM.

Figure 3. Time-dependent ROC curves obtained by considering levels of TH, PHOX2B, DCX (**A, B,** and **C**, respectively), and levels of TH, PHOX2B and DCX mRNAs in PB (**D**, **E** and **F**, respectively).

Supplemental Figure S1. Expression levels of NB mRNAs in the infant-toddler cohort. Arrows indicate the occurrence of outliers in either left or right BM aspirate (closed symbols) from two patients. For one patient only one BM site was available; for the other patient open symbols indicate the other BM aspirate while arrows indicated his/her PB values (TH was not evaluated).

Supplemental Figure S2. A) Correlation between left and right BM aspirates. B) Correlation between NB mRNAs in BM (upper row) and PB (lower row). C) Correlation between BM and PB mRNAs in the same patient.

Supplemental Figure S3. Kaplan-Meier EFS estimates obtained by stratifying the infants (panel A) and the toddlers (panel B) by both TH and PHOX2B highest tertiles in BM.

Supplemental Figure S4. Kaplan-Meier survival estimates obtained by stratifying the infant-toddlers by TH and PHOX2B highest tertiles in PB.

Supplemental Figure S5. Kaplan-Meier EFS estimates obtained by stratifying the study patients by the published BM cut-points (14) (panels A, B and C) or by the median value of each NB mRNA distribution (panels D, E and F).

Supplemental TABLE S1. Cox regression model for event-free survival analysis in relation to levels of NB RNAs in BM, obtained by considering the average value of the right and left aspirate or the highest value in either one of the two aspirates.