Frequency and Prognostic Impact of ALK Amplifications and Mutations in the European Neuroblastoma Study Group (SIOPEN) High-Risk Neuroblastoma Trial (HR-NBL1)

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PURPOSE In neuroblastoma (NB), the ALK receptor tyrosine kinase can be constitutively activated through activating point mutations or genomic amplification. We studied ALK genetic alterations in high-risk (HR) patients on the HR-NBL1/SIOPEN trial to determine their frequency, correlation with clinical parameters, and prognostic impact.

MATERIALS AND METHODS Diagnostic tumor samples were available from 1,092 HR-NBL1/SIOPEN patients to determine ALK amplification status (n = 330), ALK mutational profile (n = 191), or both (n = 571).

RESULTS Genomic ALK amplification (ALKa) was detected in 4.5% of cases (41 out of 901), all except one with MYCN amplification (MNA). ALKa was associated with a significantly poorer overall survival (OS) (5-year OS: ALKa [n = 41] 28% [95% CI, 15 to 42]; no-ALKa [n = 860] 51% [95% CI, 47 to 54], P < .001), particularly in cases with metastatic disease. ALK mutations (ALKm) were detected at a clonal level (> 20% mutated allele fraction) in 10% of cases (76 out of 762) and at a subclonal level (mutated allele fraction 0.1%-20%) in 3.9% of patients (30 out of 762), with a strong correlation between the presence of ALKm and MNA (P < .001). Among 571 cases with known ALKa and ALKm status, a statistically significant difference in OS was observed between cases with ALKa or clonal ALKm versus subclonal ALKm or no ALK alterations (5-year OS: ALKa [n = 19], 26% [95% CI, 10 to 47], clonal ALKm [n = 65] 33% [95% CI, 21 to 44], subclonal ALKm [n = 22] 48% [95% CI, 26 to 67], and no alteration [n = 465], 51% [95% CI, 46 to 55], respectively; P = .001). Importantly, in a multivariate model, involvement of more than one metastatic compartment (hazard ratio [HR], 2.87; P < .001), ALKa (HR, 2.38; P = .004), and clonal ALKm (HR, 1.77; P = .001) were independent predictors of poor outcome.

CONCLUSION Genetic alterations of ALK (clonal mutations and amplifications) in HR-NB are independent predictors of poorer survival. These data provide a rationale for integration of ALK inhibitors in upfront treatment of HR-NB with ALK alterations.

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INTRODUCTION Neuroblastoma (NB), the most frequent solid, extracranial malignancy in children, exhibits wide clinical and genetic heterogeneity. High-risk neuroblastoma (HR-NB), defined as metastatic disease over the age of 12 months or MYCN-amplified (MNA) disease at any age, remains associated with long-term survival rates of only 50%.1 Current treatment approaches consist of intensive induction chemotherapy, surgical resection of the primary tumor, consolidation with high-dose chemotherapy (HDC), and autologous stem-cell rescue, and for minimal residual disease, isotretinoin in combination with human or mouse chimeric anti-GD2 antibody, ch14.18.2-8

In NB, several recurrent genetic alterations have been described. MNA is a strong biomarker associated with...
**CONTEXT**

**Key Objective**
High risk neuroblastoma (HR-NB) is one of the most difficult childhood cancers to cure. This study examined whether the presence of an ALK alteration (amplification or mutation) was associated with a poor prognosis in a large patient series treated on the prospective European high-risk neuroblastoma trial (HR-NBL1).

**Knowledge Generated**
We found that ALK amplification or clonal mutation was associated with inferior prognosis in patients with HR-NB and both are independent prognostic variables on multivariate analysis. To our knowledge, this is the first study to report the highly prognostic significance of ALK amplification in HR-NB.

**Relevance**
As ALK can be targeted therapeutically, this study convincingly argues for the introduction of ALK inhibitors for upfront management of patients with HR-NB with ALK aberrations. Importantly, the prognostic significance of ALK alterations included a subgroup of trial patients treated with the current standard of care for HR-NB including anti-GD2 immunotherapy.

rapid tumor growth. Other copy-number alterations occur over more extensive chromosome regions, with segmental chromosome alterations being associated with a poor outcome. Recurrent mutations have been described in the RAS-MAPK pathway, chromatin remodeling genes (ARX and ARID1A), and TERT rearrangements. Activating anaplastic lymphoma kinase (ALK) mutations are the most frequent mutations in NB, occurring in both familial and sporadic cases, with somatically acquired ALK mutations (ALKm) observed in 6%-12% of sporadic NBs in all risk groups.

These ALK activating mutations are localized most frequently within the kinase domain at hotspots identified at the F1174, R1275, and F1245 positions, with mutations occurring both at clonal (> 20% mutated allele fraction [MAF]) or subclonal levels (< 20% MAF).

ALK can also be activated by genomic focal amplification, described in 1%-2% of NBs, almost exclusively with MNA, or, more rarely, following structural rearrangements. Genetic alterations of ALK are associated with poorer survival in the overall NB population. However, their prognostic role in HR-NB has been less well studied. Altogether, ALK alterations are an important molecular target, given the role of ALK as a driver oncogene in NB and its actionability with small molecule therapies.

To determine the frequency of ALK alterations (mutations and amplifications), their correlation with clinical characteristics, and their prognostic impact in HR-NB, we analyzed a large series of 1,092 diagnostic NB samples from patients on the HR-NBL1/SIOPEN trial.

**MATERIALS AND METHODS**

**Patients and Samples**
Patients were treated within the HR-NBL1/SIOPEN Protocol (ClinicalTrials.gov: NCT01704716, EudraCT: 2006-001489-17; Protocol [online only]), an international, randomized, multiarm, open-label, phase III trial. Patients with International Neuroblastoma Staging System stage 4 without MNA or International Neuroblastoma Staging System stage 4 without MNA ≥ 12 months of age at diagnosis were eligible for the trial up to 20 years of age. Within the trial, several randomized treatment arms were conducted over different periods (Appendix Fig A1, online only). Induction random assignments included the following: R0—random assignment of prophylactic granulocyte colony-stimulating factor during rapid COJEC induction; R3—comparison of two induction regimens, rapid-COJEC versus modified N7. HDC was evaluated in the R1 random assignment: busulfan or melphalan versus carboplatin or etoposide or melphalan. Anti-GD2 immunotherapy random assignments during maintenance phase were explored in R2 (2009-2013) and R4 (2014-2017), both comparing dinutuximab beta with oral isotretinoin to dinutuximab beta and subcutaneous interleukin-2 with oral isotretinoin, but with altered schedules. In the interim, dinutuximab beta with oral isotretinoin was the recommended standard.

Patients were enrolled on the HR-NBL1/SIOPEN trial after approval by national regulatory authorities and by national, and institutional, ethical committees or review boards in participating countries. Parents or guardians and patients according to age provided written informed consent for treatment, data collection, and analysis.

The ALK analysis cohort consisted of patients for whom a contributive tumor sample obtained at diagnosis was available in a SIOPEN reference laboratory. Samples were required to contain at least 20% tumor cells on pathologic examination.
The ALK amplification (ALKa) status was evaluated using either fluorescence in situ hybridization and/or multiplex ligation polymerase chain reaction–dependent amplification, array comparative genomic hybridization (aCGH), and/or array single-nucleotide polymorphism according to established guidelines.\(^{10,33,34,37}\) ALK gene amplification was defined as more than fourfold increase of ALK signals in relation to numbers of chromosome 2 by fluorescence in situ hybridization, or as more than 10 copies of the gene estimated by multiplex ligation–dependent amplification, aCGH, or array single-nucleotide polymorphism.

The ALK mutational (ALKm) status was determined by Sanger sequencing, next-generation sequencing (NGS) techniques (coverage > 80×), targeted deep sequencing (TDS), or a combination of the latter techniques, covering the ALK regions of interest (exon 23: chr2:29443647-29443776; exon 24: chr2:29436830-29436935; exon 25: chr2:29432603-29432704; UCSC Genome Browser Home,\(^{38}\) hg19) containing the ALK mutational hotspots F1174 (exon 23), F1245 (exon 24), and R1275 (exon 25).\(^{20,22}\)

MAF ≥ 20% were defined as clonal events and MAF < 20% as subclonal events, as reported previously.\(^{20,22}\) No correction for tumor cell content was undertaken when reporting MAF. Mutations identified by Sanger sequencing were considered clonal. All detected mutations were validated by a second independent experiment: for clonal events, TDS data were validated by Sanger sequencing, and for subclonal events, NGS or TDS was validated in an independent second experiment.

Standard bioinformatics were used to detect mutations in NGS experiments as previously reported. Mutations in TDS experiments were determined as described previously.\(^{20,22}\) In brief, to highlight mutations, in each NB sample, the frequencies of each base at each position of the analyzed regions were compared with those observed in all other samples and controls. This approach enabled the identification of mutations with a statistically significant increase in percentage of a variant base, compared with background noise.

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**FIG 1.** Flow diagram of patient inclusion. A total of 3,334 patients with HR-NB were enrolled in the HR-NBL1 trial from 188 centers. Among these, 2,350 patients were not included in this study, either because no contributive tumor material was available, or because there was no FU data, or both. Thus, 1,092 patients from 132 centers were included in this study. *Clonal level: > 20% MAF. Subclonal level: MAF 0.1%-20%. FU, follow-up; HR-NB, high-risk neuroblastoma; MAF, mutated allele fraction.*
Statistical Analysis

Event-free survival (EFS) was calculated from diagnosis to the first relapse, progressive disease, secondary malignancy, or death from any cause, or until last patient contact. Overall survival (OS) was calculated from diagnosis to death from any cause, or until the last patient contact. EFS and OS were estimated using the Kaplan-Meier method and compared using the logrank test, and if indicated with pseudo-value regression for 5-year OS.39-41 EFS and OS are presented as 5-year point estimates together with 95% CIs using log-log transformation.41 To adjust for established risk-factors (age at diagnosis, stage, number of metastatic compartments, and MYCN amplification), a Cox proportional hazards regression model was used.

TABLE 1. Characteristics of Patients According to the ALK Amplification or ALK Mutation Status

| Clinical Parameters | Known ALK Amplification Status (N = 901) | Known ALK Mutation Status (N = 762) |
|---------------------|------------------------------------------|-------------------------------------|
| n                   | %                                       | n                                   | %                                   |
| Total               | 860 100                                  | 656 100                             | 76 100                              | 30 100                             |
| Sex                 |                                          |                                     |                                     |
| Female              | 376 44                                   | 278 42                              | 11 37                               | .348                               |
| Male                | 484 56                                   | 378 58                              | 19 63                               | .348                               |
| Age, years          |                                          |                                     |                                     |
| < 1                 | 51 6                                     | 38 6                                | 0 0                                 | .348                               |
| 1-1.5               | 101 12                                   | 79 12                               | 3 10                                | .348                               |
| 1.5-5               | 572 67                                   | 428 65                              | 21 70                               | .348                               |
| > 5                 | 136 16                                   | 111 17                              | 6 20                                | .348                               |
| Stage               |                                          |                                     |                                     |
| Loc, MNA+           | 83 10                                    | 63 10                               | 4 13                                | .890                               |
| Stage 4             | 768 89                                   | 586 89                              | 87 26                               | .890                               |
| Stage 4s, MNA+      | 9 1                                      | 7 1                                 | 1 0                                 | 0                                  |
| MYCN status         |                                          |                                     |                                     |
| MNA−                | 466 54                                   | 365 56                              | 9 30                                | <.001                              |
| MNA+                | 394 46                                   | 291 44                              | 21 70                               | .278                               |
| Primary tumor site  |                                          |                                     |                                     |
| Unknown             | 20 1                                      | 21 1                                | 1 1                                 | .278                               |
| Abdominal adrenal+  | 606 72                                   | 452 71                              | 63 22                               | 76                                 |
| Abdominal other+    | 169 20                                   | 124 20                              | 29 6                                | 21                                 |
| Other only          | 65 8                                      | 59 9                                | 8 1                                 | 3                                  |
| Stage 4: MYCN status|                                          |                                     |                                     |
| MNA−                | 466 61                                   | 365 62                              | 9 35                                | <.001                              |
| MNA+                | 302 39                                   | 221 38                              | 17 65                               | .788                               |
| Stage 4: MC         |                                          |                                     |                                     |
| 1 MC                | 91 12                                    | 70 13                               | 17 4                                 | 17                                 |
| 2 MC                | 231 32                                   | 177 32                              | 29 9                                 | 38                                 |
| > 2 MC              | 411 56                                   | 302 55                              | 54 11                                | 46                                 |
| Overall response: end of induction |                     |                                     |                                     |
| Evaluable           | 804 39                                   | 607 72                              | 28                                  | .421                               |
| CR or VGPR or PR    | 628 78                                   | 472 78                              | 24 86                                | .389                               |
| MR or SD or PD      | 176 22                                   | 135 22                              | 4 14                                | .278                               |

NOTE. Patients studied for ALK amplifications (n = 901) and ALK mutations (n = 762). Abbreviations: CR, complete response; MC, metastatic compartments; MNA, MYCN amplification; MR, minor response; PD, progressive disease; PR, partial response; SD, stable disease; VGPR, very good partial response.
FIG 2. Genetic alterations of ALK in patients with HR-NB. (A) Copy-number profile of case 536. Genomic coamplification of MYCN and ALK is observed on chromosome 2, encompassing the regions between position 15,440,477 and 16,822,999 and between 29,113,790 and 30,309,749 bp (human genome assembly hg19; UCSC Genome Browser Home38). (B) Frequency distribution (continued on following page).
Correlations between patient and disease characteristics and ALK genetic alterations were explored using chi-square tests.

To allow for sufficient follow-up time, only patients enrolled until December 31, 2019, were considered. The data cutoff for the final analysis was October 3, 2020. We calculated median follow-up using the inverse Kaplan-Meier estimate.

Statistical analysis was performed using SAS (version 9.4).

RESULTS

Of 3,334 patients enrolled on the HR-NBL1/SIOPEN trial between November 24, 2002, and December 31, 2019, 1,092 patients were included in the ALK analysis cohort (Fig 1; Appendix Table A1, online only). Patients were accrued from 132 SIOPEN member institutions or hospitals in 19 countries (Appendix Table A2, online only). Among these 1,092 patients, 81% (889 out of 1,092) were > 18 months of age at diagnosis, 47% (521 out of 1,092) showed MNA, and 88% (966 out of 1,092) had stage 4 disease, with no statistically significant difference in EFS or OS between the ALK analysis cohort and the overall HR-NBL1 cohort (Appendix Fig A2, online only). The median follow-up period was 6.8 years (0.1-17.4 years).

ALK Alterations

Within the ALK cohort, the ALKm status was analyzed in 762 patients, the ALKa status in 901 cases, with both ALKm and ALKa studied in 571 patients (Fig 1, Table 1).

ALK alterations were detected in 146 out of 1,092 patients with ALKa occurring in 4.5% (41 out of 901 cases) and ALKm in 13.9% (106 out of 762 cases). Only one case showed ALKa and a concomitant ALK R1275Q mutation with an MAF of 93%, suggesting that the mutated allele is contained in the amplicon (Appendix Fig A3, online only).

ALK Amplification and Correlation With Risk Factors

High-level genomic amplification of the ALK gene was found in 4.5% (41 out of 901) of cases (Fig 2A, Table 1). All but one also had MNA. ALKa significantly correlated with MNA (P < .001), non-stage 4 disease (P < .001), and age at diagnosis < 18 months (P = .005). No correlation between the presence of ALKa and response at the end of induction treatment was observed.

A statistically significant poorer 5-year OS was observed in patients whose tumors harbored ALKa (5-year OS: ALKa 28% [95% CI, 15 to 42%] vs non-ALKa 51% [95% CI, 47 to 54%]; P < .0001; Fig 3A, Table 2) with a stronger prognostic effect in patients with stage 4 or 4S MNA.

ALK Mutation and Correlation With Risk Factors

ALK mutational status was studied in 762 cases by Sanger sequencing (n = 163), by NGS techniques (n = 15), or by TDS (n = 650, including 64 by TDS and Sanger). The biologic data for 52 cases have been reported previously.

Among these, 13.9% (106 out of 762) showed at least one ALKm within the explored ALK regions of interest, with 10% (76 out of 762) harboring mutations at a clonal level (MAF > 20%) and 3.9% (30 out of 762) at a subclonal level (MAF ≤ 20%); nine cases—MAF 0.1% to < 1%; 10 cases MAF 1% to < 5%; two cases MAF 5% to < 10%, and nine cases MAF 10% to < 20% (Figs 1 and 2B; Table 1).

Concordance between results analyzed by two different techniques was observed in 64 cases with clonal ALKm (TDS and Sanger). Subclonal ALKm were validated by a second independent TDS experiment, with an excellent correlation of MAF between the two experiments (R2 = 0.9924; P < .0001) (Appendix Fig A4, online only).

ALKm involved the common mutational hotspots (F1174, F1245, and R1275) in 12.5% (97 out of 762) of cases, comprising 91% (97 out of 106) of all detected ALKm (Fig 2B).

Interestingly, three cases harbored two or more distinct mutations. In the first case, both F1174L and F1245L mutations were observed (MAF 2% and 0.8%, respectively). The second case showed three subclonal mutations F1174L, R1275Q, and R1275L (MAF 2.9%, 8.9%, and 2.9%, respectively). A third case harbored a mutation at the F1174 and R1275 hotspots (MAF 27% and 1.3%, respectively).

There were no statistically significant correlations between ALKm and stage, age at diagnosis, or localization of the primary tumor (adrenal, abdominal, or other) (Table 1). However, a significant correlation was observed between the presence of ALKm and MNA (P < .001), with an enrichment of ALKm F1174 in MNA tumors (P = .0005).
FIG 3. Survival in the ALK analysis cohort. (A) OS according to ALK amplification status in 901 patients: presence of ALK amplification (n = 41), 5-year OS 28% (95% CI, 15 to 42) versus absence of ALK amplification (n = 860), 5-year OS 51% (95% CI, 47 to 54); P < .001. (B) OS according to ALK mutation status in 762 patients: presence of an ALK mutation (n = 106), 5-year OS 41% (95% CI, 31 to 51) versus absence of an ALK mutation (n = 656), 5-year OS 49% (95% CI, 45 to 53); P = NS. (C) OS according to ALK clonal or subclonal (continued on following page)
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FIG 3. (Continued). mutation status in 762 patients: no mutation (n = 656), 5-year OS 49% (95% CI, 45 to 53); clonal mutations (n = 76), 5-year OS 34% (95% CI, 23 to 45); and subclonal mutations (n = 30), 5-year OS 59% (95% CI, 39 to 74), respectively; P = .018. (D) OS according to the presence of any ALK alterations in 611 patients with known ALK amplification and ALK mutation status: presence of an ALK alteration (n = 146), 5-year OS 37% (95% CI, 29 to 45); versus absence of ALK alterations (n = 465), 5-year OS 51% (95% CI, 46 to 55); P = .005. (E) OS according to the type of ALK alteration in the cohort of 571 patients with known ALK amplification and ALK mutation status: no alteration (n = 465), 5-year OS 51% (95% CI, 46 to 55); clonal mutations (n = 65), 5-year OS 33% (95% CI, 21 to 44); subclonal mutations (n = 12), 5-year OS 48% (95% CI, 26 to 67); and ALK amplification (n = 19), 5-year OS 26% (95% CI, 10 to 47), respectively; P = .001. (F) OS according to ALK alterations (ALK amplification or clonal ALK mutation) in patients who received immunotherapy (n = 141): To evaluate the impact of ALK alterations (ALK amplification or clonal ALK mutation) in patients who received dinutuximab beta, OS was calculated from the start of dinutuximab beta treatment and evaluated using the same approaches as described in the Materials and Methods section. ALK alteration (ALK amplification or clonal ALK mutation, n = 29, 5-year OS 48% (95% CI, 28 to 65)) versus no ALK alteration (n = 112) 67% (95% CI, 56 to 75); P = .034. Patient details: Appendix Table A3. HR, hazard ratio; NS, not significant; OS, overall survival; ref, reference.

This was also observed when analyzing only stage 4 tumors. No correlation between ALKm and response at the end of induction treatment was observed.

No statistically significant difference in outcome was observed between patients harboring any ALKm versus none (Fig 3B, Table 2). However, when distinguishing clonal and subclonal mutations, a poorer OS was observed only in patients with clonal ALKm, as opposed to subclonal or no mutations (5-year OS, clonal ALKm 34% [95% CI, 23 to 45], subclonal ALKm 59% [95% CI, 39 to 74], and no ALKm 49% [95% CI, 45 to 53]; P = .018) (Fig 3C, Table 2).

Patients with metastatic disease (stage 4 or 4S MNA) and a clonal ALKm showed a trend toward poorer OS. However, in patients with localized disease, the presence of ALKm did not confer poorer survival (Table 2).

Overall Prognostic Impact of ALK Genetic Alterations

To determine the overall prognostic impact of ALK genetic alterations, we focused on the subgroup of 571 patients with both known ALKα and ALKm status. In this subgroup of patients, a statistically significant poorer OS was observed in patients whose tumors harbored any ALK alteration (5-year OS, any alteration 37% [95% CI, 29 to 45] v no alteration 51% [95% CI, 46 to 55]; P = .005; Fig 3D). ALKα or clonal ALKm were associated with a poorer outcome (5-year OS, ALKα 26% [95% CI, 10 to 47], clonal ALKm 33% [95% CI, 21 to 44], subclonal ALKm 48% [95% CI, 26 to 67], and no ALK alteration 51% [95% CI, 46 to 55]; P = .001; Fig 3E, Table 2). Among the subgroup of patients with known ALK status, we sought to determine the prognostic impact of ALK alterations according to the different treatment arms of HR-NBL1. Indeed, in the HR-NBL01/SIOOPEN trial, the introduction of busulfan and melphalan as standard for HDC, and anti-GD2 maintenance therapy as a new standard since 2010, has led to significantly improved survival (Appendix Fig A5F, online only). Importantly, when considering patients treated according to the SIOOPEN standard with busulfan and melphalan HDC and maintenance immunotherapy, the presence of an ALK alteration (ALKα or clonal ALKm) remained associated with a poorer 5-year OS of 48% (95% CI, 28 to 65), versus no ALK alteration 67% (95% CI, 56 to 75); P = .03 (Fig 3F, Appendix Table A3, online only), with a trend also observed when taking into account all ALKm (clonal and subclonal, P = .059).

Based on univariate risk factor exploration of the whole ALK analysis cohort (Appendix Fig A5), we developed a Cox model for multivariate analysis including clinical and biologic parameters previously shown to be of prognostic impact (n = 571 patients). Involvement of two or more metastatic compartments (OS: hazard ratio [HR], 2.87 [95% CI, 1.73 to 4.78]; P = .001) and the presence of ALKα (OS: HR, 2.38 [95% CI, 1.32 to 4.27]; P = .004) and clonal ALKm (OS: HR, 1.77 [95% CI, 1.25 to 2.49]; P = .001) were of independent prognostic significance, whereas MNA and age were not (Table 3).

DISCUSSION

In HR-NB, the identification of prognostic biomarkers is crucial for the development of new treatment approaches. Recent studies have shown that MNA is not associated with poorer outcome among the overall cohort of patients with HR-NB, but the presence of genomic amplifications other than MYCN might constitute a poor outcome biomarker.43 We now show in this large ALK analysis cohort that the presence of ALKα or clonal ALKm resulted in significantly worse outcome.

Given the oncogenic driver role of ALK activation, and the prognostic impact of ALKα or clonal ALKm, the introduction of frontline ALK-targeted treatment is now strongly supported by the current study. Although early phase clinical trials of first- and second-generation ALK inhibitors showed modest efficacy of the first-generation inhibitor crizotinib in NB with F1174 hotspot mutations being resistant,44 third-generation ALK inhibitors such as lorlatinib exhibit improved efficacy alone and when combined with chemotherapy.28,44-46 Crizotinib is currently being administered with chemotherapy in a phase III upfront trial for patients with HR-NB with ALK alterations (ClinicalTrials.gov: NCT03126916).

Improvements in HR-NB patient survival have been achieved with intensification of HDC and immunotherapy with dinutuximab (ch14.18/Sp02 and ch14.18/CHO),3,5 and our results highlight the potential of ALK inhibition as an attractive upfront precision-medicine strategy in patients with ALK alterations to further improve survival. Importantly, in patients reaching the maintenance treatment phase...
| Parameters | OS | EFS |
|------------|----|-----|
|             | Patients, No. | Events, No. | 5-Year OS, % (95% CI) | HR (95% CI) | P | Patients, No. | Events, No. | 5-Year EFS, % (95% CI) | HR (95% CI) | P |
| **Total**   |               |               |                       |             |   |               |               |                       |             |   |
| ALK  a      | No            | 860           | 418                   | 51 (47 to 54) | Ref | 860           | 492           | 40 (36 to 43)          | Ref | < .001 |
|             | Yes           | 41            | 29                    | 28 (15 to 42) | 2.3 (1.6 to 3.4) | 41            | 31            | 24 (13 to 38)          | 2.0 (1.4 to 2.9) | .001 |
| ALK  m      | Nonmutated   | 656           | 347                   | 49 (45 to 53) | Ref | 656           | 395           | 38 (35 to 42)          | Ref | .081 |
| ALK  m clonal | 76           | 48            | 34 (23 to 45)         | 1.4 (1.1 to 2.0) | .   | 76            | 51            | 31 (21 to 42)          | 1.3 (1.0 to 1.7) | .   |
| ALK  m subclonal | 30        | 13            | 59 (39 to 74)         | 0.7 (0.4 to 1.2) | .   | 30            | 16            | 49 (30 to 65)          | 0.8 (0.5 to 1.3) | .   |
| Known ALK alteration status |               |               |                       |             |   |               |               |                       |             |   |
| Nonmutated | 465           | 241           | 51 (46 to 55)         | Ref | .001 | 465           | 280           | 38 (33 to 43)          | Ref | .057 |
| ALK  a      | 19            | 14            | 26 (10 to 47)         | 2.2 (1.3 to 3.8) | .   | 19            | 14            | 26 (10 to 47)          | 1.7 (1.0 to 2.9) | .   |
| ALK  m clonal | 65           | 42            | 33 (21 to 44)         | 1.7 (1.2 to 2.3) | .   | 65            | 43            | 33 (22 to 44)          | 1.4 (1.0 to 1.9) | .   |
| ALK  m subclonal | 22        | 12            | 48 (26 to 67)         | 1.0 (0.5 to 1.8) | .   | 22            | 14            | 39 (19 to 59)          | 1.0 (0.6 to 1.8) | .   |
| Stage 4, 4s |               |               |                       |             |   |               |               |                       |             |   |
| ALK  a      | No            | 777           | 394                   | 48 (44 to 52) | Ref | 777           | 467           | 37 (33 to 40)          | Ref | < .001 |
|             | Yes           | 28            | 22                    | 19 (7 to 35)  | 2.9 (1.8 to 4.6) | 28            | 23            | 18 (7 to 34)          | 2.9 (1.8 to 4.6) | .   |
| ALK  m      | Nonmutated   | 593           | 328                   | 47 (43 to 51) | Ref | 593           | 375           | 35 (31 to 39)          | Ref | .216 |
| ALK  m clonal | 67           | 43            | 33 (22 to 45)         | 1.4 (1.0 to 1.9) | .   | 67            | 46            | 30 (19 to 41)          | 1.4 (1.0 to 1.9) | .   |
| ALK  m subclonal | 26        | 13            | 52 (31 to 70)         | 0.8 (0.4 to 1.4) | .   | 26            | 16            | 41 (22 to 59)          | 0.8 (0.4 to 1.4) | .   |
| Known ALK alteration status |               |               |                       |             |   |               |               |                       |             |   |
| Nonmutated | 419           | 228           | 48 (43 to 53)         | Ref | .000 | 419           | 266           | 35 (30 to 39)          | Ref | .042 |
| ALK  a      | 15            | 12            | 20 (5 to 42)          | 2.6 (1.3 to 4.7) | .   | 15            | 12            | 20 (5 to 42)          | 1.8 (1.0 to 3.4) | .   |
| ALK  m clonal | 57           | 38            | 30 (18 to 43)         | 1.7 (1.2 to 2.4) | .   | 57            | 39            | 30 (19 to 42)          | 1.4 (1.0 to 1.9) | .   |
| ALK  m subclonal | 21        | 12            | 45 (23 to 65)         | 1.0 (0.5 to 1.8) | .   | 21            | 14            | 36 (16 to 56)          | 1.0 (0.6 to 1.8) | .   |
| Stage 4, MNA |               |               |                       |             |   |               |               |                       |             |   |
| ALK  a      | No            | 466           | 236                   | 49 (44 to 54) | NA | 466           | 292           | 33 (28 to 38)          | NA | NA |
|             | Yes           | 1             | 1                     | NA          | NA | 1             | 1             | NA                    | NA | NA |
| ALK  m      | Nonmutated   | 365           | 202                   | 49 (43 to 54) | Ref | 365           | 238           | 33 (28 to 38)          | Ref | .245 |
| ALK  m clonal | 26           | 18            | 28 (13 to 46)         | 1.5 (0.9 to 2.5) | .   | 26            | 20            | 23 (9 to 40)          | 1.5 (0.9 to 2.3) | .   |
| ALK  m subclonal | 9           | 4             | 53 (18 to 80)         | 0.9 (0.3 to 2.3) | .   | 9             | 5             | 42 (11 to 71)          | 0.9 (0.4 to 2.3) | .   |
| Known ALK alteration status |               |               |                       |             |   |               |               |                       |             |   |
| Nonmutated | 269           | 146           | 50 (43 to 56)         | Ref | .010 | 269           | 174           | 32 (27 to 38)          | Ref | .029 |
| ALK  a      | 1             | 1             | NA                     | NA          | NA | 1             | 1             | NA                    | NA | NA |
| ALK  m clonal | 20           | 15            | 22 (7 to 42)          | 2.1 (1.3 to 3.6) | .   | 20            | 16            | 20 (6 to 39)          | 1.8 (1.1 to 2.9) | .   |
| ALK  m subclonal | 6           | 3             | 44 (7 to 78)          | 1.2 (0.4 to 3.7) | .   | 6             | 4             | 25 (1 to 65)          | 1.4 (0.5 to 3.9) | .   |

(continued on following page)
with dinutuximab beta in the HR-NBL1/SIOPEN trial, the presence of an ALK alteration was still associated with poorer survival, thus strongly suggesting that integration of ALK-targeted therapy is warranted throughout all treatment phases of modern-era HR-NB therapy. ALK was observed in 4% of NB cases, accounting for approximately 1 out of 3 of ALK-activated NB cases. To date, co-occurrence of ALK hotspot mutations and genomic amplification has rarely been reported in NB. In this extensive cohort of patients, one case harboring both ALKa and an R1275 ALKm was identified. This indicates that these alterations are not fully mutually exclusive, although co-occurrence is extremely rare.

ALK were found in 13.9% of cases at the studied exonic regions harboring known ALK mutational hotspots. This is higher than previously reported frequencies of ALKm in HR-NB of approximately 10%, most likely as previous reports using Sanger sequencing or standard-resolution NGS approaches. Sanger sensitivity is limited to the detection of MAF. MAFs with lower MAFs have been reported. Ultradeep sequencing used in this analysis has a sensitivity limit of MAF of 0.1%. This approaches the theoretical limit of detection based on the genomic DNA input of 50 ng for one experiment, equivalent to 5,000 diploid genomes.

### Table 2. EFS and OS According to ALK Alterations (continued)

| Parameters | OS | EFS |
|------------|----|-----|
|            | Patients, No. | Events, No. | 5-Year OS, % (95% CI) | HR (95% CI) | P | Patients, No. | Events, No. | 5-Year EFS, % (95% CI) | HR (95% CI) | P |
| Stage 4, 4s MNA+ | | | | | | | | | |
| ALKa | No | 311 | 158 | 48 (42 to 54) | Ref | < .001 | 311 | 175 | 43 (37 to 48) | Ref | < .001 |
| | Yes | 27 | 21 | 19 (7 to 36) | 2.3 (1.4 to 3.7) | | 27 | 22 | 19 (7 to 35) | 2.0 (1.3 to 3.3) | |
| ALKm | Nonmutated | 228 | 126 | 44 (38 to 51) | Ref | .453 | 228 | 137 | 40 (33 to 46) | Ref | .666 |
| | ALKm clonal | 41 | 25 | 37 (22 to 51) | 1.2 (0.8 to 1.8) | | 41 | 26 | 34 (20 to 49) | 1.2 (0.8 to 1.8) | |
| | ALKm subclonal | 17 | 9 | 52 (27 to 73) | 0.8 (0.4 to 1.5) | | 17 | 11 | 41 (19 to 63) | 0.9 (0.5 to 1.7) | |
| Known ALK alteration status | Nonmutated | 150 | 82 | 46 (37 to 54) | Ref | .085 | 150 | 92 | 39 (31 to 47) | Ref | .372 |
| | ALKa | 14 | 11 | 21 (5 to 45) | 1.9 (1.0 to 3.7) | | 14 | 11 | 21 (5 to 45) | 1.6 (0.8 to 3.0) | |
| | ALKm clonal | 37 | 23 | 35 (20 to 51) | 1.3 (0.8 to 2.1) | | 37 | 23 | 36 (20 to 51) | 1.2 (0.7 to 1.9) | |
| | ALKm subclonal | 15 | 9 | 46 (20 to 68) | 0.9 (0.4 to 1.8) | | 15 | 10 | 40 (16 to 63) | 1.0 (0.5 to 1.9) | |
| Localized, MNA+ | Nonmutated | 63 | 19 | 70 (57 to 80) | Ref | .114 | 63 | 20 | 67 (54 to 77) | Ref | .098 |
| | ALKa | 9 | 5 | 42 (11 to 71) | 2.2 (0.8 to 5.8) | | 9 | 5 | 42 (11 to 71) | 2.2 (0.8 to 5.9) | |
| | ALKm clonal | 4 | 0 | NA | NA | | 4 | 0 | NA | NA | |
| Known ALK alteration status | Nonmutated | 46 | 13 | 73 (57 to 83) | Ref | .440 | 46 | 14 | 68 (52 to 80) | Ref | .410 |
| | ALKa | 4 | 2 | 50 (6 to 84) | 2.0 (0.4 to 8.7) | | 4 | 2 | 50 (6 to 84) | 1.8 (0.4 to 7.9) | |
| | ALKm clonal | 8 | 4 | 50 (15 to 77) | 2.1 (0.7 to 6.5) | | 8 | 4 | 50 (15 to 77) | 2.2 (0.7 to 6.8) | |
| | ALKm subclonal | 1 | 0 | NA | NA | | 1 | 0 | NA | NA | |

**NOTE.** EFS and OS in the ALK analysis cohort, according to different clinical parameters: complete summary of all risk-factor–based 5-year EFS and OS rates in patients according to the ALK amplification status (ALKa, n = 901 patients), ALK mutational status (ALKm, n = 762 patients), or in patients for whom both the ALKa status and ALKm status are known (known ALK alteration status, n = 571).

**Abbreviations:** EFS, event-free survival; MNA, MYCN-amplified; NA, not available; OS, overall survival; ref, reference.

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This study demonstrates that use of higher-resolution techniques enables a higher detection rate of ALK m. The MAF distribution indicated a majority of clonal events (76 out of 106 cases). Importantly, clonal ALK m were associated with poorer outcome and were of independent prognostic significance, but subclonal events were not. Subclonal events, defined in this study by MAF, 20%, comprised 28% (30 out of 106) of all ALK m, with a very low MAF, <5%, observed in 19 cases. However, when considering ALK m, the OS remains poor in all patient subgroups (5-year OS < 62%). Furthermore, although of different prognostic impact in this study, the biomarker (ALK mutation) might not be of distinct predictive impact, and even in patients with subclonal ALK mutations, ALK inhibitor treatment might be effective in the targeted cell population. Thus, future upfront trials should consider ALK-targeted treatment based on clinically applicable reliable detection limits (for instance MAF 5% for NGS techniques) rather than the MAF defining prognostic subgroups.

As tumor samples harbored at least 20% tumor cells by pathologic examination, with additional confirmation provided by a dynamic aCGH or SNPp array profile in the majority of cases, the observed low MAF is likely to correspond to intratumoral heterogeneity. In NB, intratumor heterogeneity has been reported for MNA and segmental chromosome alterations.47-49 The coexistence of ALK nonmutated and mutated cells within a single tumor suggests that these different subclones might coexist in an advantageous equilibrium, which might crucially affect the dynamics of cancer progression.50,51 Correlation with pathologic findings, single-cell RNA or DNA experiments, and in situ approaches will elucidate how ALK-mutated cells are distributed throughout an NB. A higher frequency of ALK m at NB relapse has been demonstrated, suggesting clonal evolution of a minor ALK-mutated subclone to a dominant ALK mutated clone at relapse, but these cases might not represent clinically unfavorable cases initially.23,52,53 Further studies focusing on serial blood samples for ctDNA studies will further elucidate clonal evolution, also under targeted therapy.54

In HR-NB, mutations in the p53 or RAS-MAPK pathways, including ALK, together with telomere maintenance caused by induction of telomerase or ALT (alternative lengthening of telomere) are thought to increase tumor aggressiveness, resulting in even poorer survival among patients with HR-NB.55,56 As MYCN leads to upregulation of TERT expression, MNA associated with any ALK alteration might lead to inferior outcome. Cases with ALKa show both ALK pathway activation and activation of telomere maintenance through MNA, with a suggested additive effect of these genetic events. The very poor survival of ALKa patients is concordant with this observation. However, survival of patients whose tumors harbored ALKm and MNA was not different from those without MNA, suggesting that ALKm cases constitute a more heterogeneous group with regards to the mechanistic tumor classification.55

### Table 3. Multivariate Analysis in 571 Patients With a Known ALK Amplification and ALK Mutation Status

| Clinical Parameters | OS | | | | | | EFS | | |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                      | P   | HR   | 95% CL          | P   | HR   | 95% CL          |
| Age, years          |     |      |                 |     |      |                 |
| < 1                 | .269| .72  | 0.40 to 1.30    | .636| .87  | 0.49 to 1.56    |
| 1-1.5               | .265| .75  | 0.45 to 1.24    | .830| .95  | 0.57 to 1.56    |
| 1.5-5               | .662| .88  | 0.50 to 1.55    | .935| 1.02 | 0.59 to 1.78    |
| > 5                 |     |      |                 |     |      |                 |
| Metastatic compartments |      |      |                 |     |      |                 |
| Localized-none      | 1.00|      |                 | 1.00|      |                 |
| 1 MC                | .122| 1.60 | 0.88 to 2.90    | .096| 1.63 | 0.92 to 2.88    |
| 2 MC                | .001| 2.41 | 1.44 to 4.04    | .001| 2.38 | 1.44 to 3.94    |
| > 2 MC              | < .0001 | 2.87 | 1.73 to 4.78 | < .0001 | 2.88 | 1.76 to 4.72 |
| MYCN amplification  |      |      |                 |     |      |                 |
| MNA+                | .135| 1.23 | 0.94 to 1.62    | .797| 1.03 | 0.80 to 1.34    |
| ALK alteration      |      |      |                 |     |      |                 |
| No alteration       | 1.00|      |                 | 1.00|      |                 |
| ALKa                | .004| 2.38 | 1.32 to 4.27    | .026| 1.94 | 1.08 to 3.47    |
| ALKm clonal         | .001| 1.77 | 1.25 to 2.49    | .017| 1.50 | 1.08 to 2.10    |
| ALKm subclonal      | .696| 0.88 | 0.46 to 1.68    | .934| 1.02 | 0.58 to 1.81    |

Abbreviations: EFS, event-free survival; MC, metastatic compartments; MNA, MYCN-amplified; OS, overall survival.
ALKα and ALK clonal mutation were both independent predictors of poor outcome in our multivariate Cox model. Notably, the end-of-induction response rate was not associated with ALK genetic alterations, suggesting that ALK-altered tumor cells are unlikely to be primarily chemotherapy resistant.

In summary, our data contribute to the rationale for future clinical trials introducing ALK-targeted treatment in the frontline setting together with chemotherapy and immunotherapy, and the distinct prognostic impact of different ALK alterations (ALKα and ALKfm) needs to be considered.

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Frequency and Prognostic Impact of ALK Amplifications and Mutations in the European Neuroblastoma Study Group (SIOPEN) High-Risk Neuroblastoma Trial (HR-NBL1)

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FIG A1. Treatment flowchart of the HR-NBL1 Protocol (ClinicalTrials.gov: NCT01704716, EudraCT: 2006-001489-17) over the whole period. *Infants and children with a body weight below 12 kg will be dosed at 0.67 mg/kg/d. In infants weighing ≤5 kg, a further 1/3 dose reduction is advised. AUC, area under the curve; BUMEL, busulfan and melphalan; CAV, cyclophosphamide plus doxorubicin or vincristine; CEM, carboplatin, etoposide, and melphalan; CH14.18/CHO, human-mouse chimeric monoclonal anti-disialoganglioside GD2 antibody ch14.18 produced in Chinese hamster ovary (CHO) cells; COJEC, chemotherapy schedule COJEC defined below; GFR, glomerular filtration rate; IL-2, interleukin-2; IV, intravenous; P or E, cisplatin or etoposide; R1, randomization 1; R2, randomization 2; R3, randomization 3; R4, randomization 4; RT, radiotherapy; SCR, stringent complete response; TP, time period; TVD, topotecan-vincristine-doxorubicin. (continued on following page)
### Course A
- **Vincristine**: 1.5 mg/m² (maximum dose 2 mg) x 1 day
- **Carboplatin**: 750 mg/m² x 1 day
- **Etoposide**: 175 mg/m² x 2 days

### Course B
- **Vincristine**: 1.5 mg/m² (maximum dose 2 mg) x 1 day
- **Carboplatin**: 80 mg/m²/ctn over 24 hours x 1 day

### Course C
- **Vincristine**: 1.5 mg/m² (maximum dose 2 mg) x 1 day
- **Etoposide**: 175 mg/m² x 2 days
- **Cyclophosphamide**: 1,050 mg/m² x 2 days

### CAV
- **Cyclophosphamide**: 70 mg/kg x 2 days
- **Doxorubicin**: 25 mg/m² x 3 days
- **Vincristine**: 0.022 mg/kg x 3 days

### P/E
- **Cisplatin**: 50 mg/m² x 4 days
- **Etoposide**: 200 mg/m² on 3 days

### TVD
- **Topotecan**: 100 ml/m² x 5 days
- **Vincristine**: 1 mg/m² x 2 days
- **Doxorubicin**: 22 mg/m² x 2 days

### BUMEL
- **Busilvex**: < 9 kg: 1 mg/kg; 9 kg to < 16 kg: 1.2 mg/kg; 16 kg to 23 kg: 1.1 mg/kg; > 23 kg to 34 kg: 0.95 mg/kg; > 34 kg: 0.8 mg/kg
- **Melphalan**: x 5 days 140 mg/m² x 1 day
- **Autologous stem-cell reinfusion**

### CEM/SCR
- **Carboplatin**: AUC 4.1 mg/ml min/d x 4 days (based on the GFRI)
- **Etoposide**: < 12 kg: 11.3 mg/kg/d; > 12 kg: 339 mg/m²/d x 4 days
- **Melphalan**: < 12 kg: 2.3 mg/kg/d; > 12 kg: 70 mg/m²/d x 3 days

### Radiotherapy
- Fractionated radiotherapy (21 Gy) given in 14 fractions of 1.5 Gy over not more than 21 days

### Immunotherapy
- **Ch14.18/CHO**: 20 mg/m²/d over 5 days every 4 weeks for five courses
- **Ch14.18/CHO**: 10 mg/m²/d continuous IV infusion over 10 days

### Possible TPs for surgical resection

![FIG A1. (Continued).](journal_of_clinical_oncology)
FIG A2. Comparison of patients in the ALK analysis cohort and patients not in the ALK analysis cohort. (A and B) EFS and OS of the ALK analysis cohort and patients not in the ALK cohort. (A) No statistically significant difference in EFS and (B) OS was observed between patients included in the ALK analysis cohort (n = 1,092, from 132 centers; red line), patients not included in this study from the same centers (n = 1,665, blue line) and patients not included in this study from centers not participating in this study (n = 577, green line) (5-year EFS: 40% [95% CI, 37 to 43] v 37% [95% CI, 35 to 40] v 33% [95% CI, 29 to 37]; 5-year OS: 49% [95% CI, 46 to 53] v 48% [95% CI, 46 to 51] v 44% [95% CI, 40 to 59]; P = NS). (C) Recruitment, by year (x-axis), in the ALK analysis cohort (% of patients: y-axis; absolute numbers: in the blue bars). The % and number of patients not included in the ALK analysis cohort from centers participating, and from nonparticipating centers, are indicated in orange and gray, respectively. EFS, event-free survival; NS, not significant; OS, overall survival.
FIG A3. Double event of ALK amplification and ALK mutation detected in one case (case 15). The SNP array shows an amplified region in chromosome 2 encompassing the ALK gene. Sanger sequencing profile shows R1275Q mutation (MAF = 93.3%) in the same case. HD, high definition; MAF, mutated allele fraction; SNP, single-nucleotide polymorphism.
**FIG A4.** MAF of subclonal ALK mutations detected by TDS and confirmed by a second independent TDS experiment. Red spots representing the MAF for each ALK mutation are plotted on the x-axis (first TDS experiment) and y-axis (second TDS experiment), with a strong correlation between the two independent experiments ($r^2 = 0.9924$, $P < .0001$). Blue spots represent subclonal ALK mutations with a very low MAF (< 0.1%) not confirmed in an independent experiment and not retained in the analysis ($n = 6$). MAF, mutated allele fraction; TDS, targeted deep sequencing.
### FIG A5.
Survival in the ALK analysis cohort (n = 1,092 patients) according to known prognostic factors. (A) EFS and OS in the ALK analysis cohort population (n = 1,092 patients). Five-year EFS (blue line) 40% (95% CI, 37 to 43); 5-year OS (red line) 49% (95% CI, 46 to 53). (B) OS according to age. Five-year OS in patients ≤ 1 year of age at diagnosis (red line) 50% (95% CI, 37 to 61); in patients 1-1.5 years of age at diagnosis (blue line) 58% (95% CI, 49 to 66); in patients 1.5-5 years of age at diagnosis (green line) 50% (95% CI, 46 to 53); and in patients > 5 years of age at diagnosis (purple line) 43% (95% CI, 35 to 50); P = NS (pseudo-value regression). (continued on following page)
FIG A5. (Continued).  (C) OS according to number of involved MCs. Five-year OS in patients with localized disease (red line) 67% (95% CI, 58 to 75), in patients with involvement of one MC (blue line) 65% (95% CI, 55 to 73), two MCs (green line) 52% (95% CI, 46 to 58), or over two MCs (purple line) 41% (95% CI, 36 to 46);  \( P < .001 \).  (D) OS according to stage. Five-year OS in patients with localized disease (red line) 67% (95% CI, 41 to 51), in patients with stage 4 disease (blue line) 47% (95% CI, 44 to 50), or stage 4s disease (green line) 54% (95% CI, 25 to 76);  \( P < .001 \).  (E) OS according to MYCN amplification in stage 4 disease. Five-year OS in patients with MNA (blue line) 46% (95% CI, 41 to 51), in patients without MNA (red line) 48% (95% CI, 44 to 53), NS (pseudo-value regression).  (F) OS according to treatment period, before (\( \leq \) March 2010) or after (\( > \) March 2010) the definition of HDC by BUMEL and immunotherapy maintenance as standard treatment. A significant improvement survival because of BUMEL and GD2 standard therapy is observed. Five-year OS in patients having been treated before March 2010 (red line) 46% (95% CI, 41 to 51) versus after March 2010 (blue line) 51% (95% CI, 47 to 56);  \( P = .039 \).  BUMEL, busulfan and melphalan; cHR, crude hazard ratio; EFS, event-free survival; HDC, high-dose chemotherapy; HR, hazard ratio; MC, metastatic compartment; MNA, MYCN-amplified; NS, not significant; OS, overall survival; ref, reference.

### TABLE A1. Clinical Characteristics of 1,092 Patients Included in the ALK Analysis Cohort

|                          | Localized MNA+ | Total | MNA− | MNA+ | Stage 4s MNA+ | Total |
|--------------------------|----------------|-------|------|------|--------------|-------|
|                          | 113            | 966   | 571  | 395  | 13           | 1,092 |
| Sex, No. (%)             |                |       |      |      |              |       |
| Female                   | 45 (40)        | 423 (44) | 258 (45) | 165 (42) | 5 (38)  | 473 (43) |
| Male                     | 68 (60)        | 543 (56) | 313 (55) | 230 (58) | 8 (62)  | 619 (57) |
| Age at diagnosis, years  |                |       |      |      |              |       |
| < 1, No. (%)             | 5 (4)          | 50 (5)  | 0 (0) | 49 (12) | 13 (100) | 67 (6)  |
| 1-1.5, No. (%)           | 22 (19)        | 113 (12) | 39 (7)  | 75 (19) | 0 (0)   | 136 (12) |
| 1.5-5, No. (%)           | 79 (70)        | 634 (66) | 392 (69) | 242 (61) | 0 (0)   | 713 (65) |
| 5-10, No. (%)            | 7 (6)          | 169 (17) | 140 (25) | 29 (7)  | 0 (0)   | 176 (16) |
| Median (min-max)         | 2.1 (0.6-8.3)  | 2.9 (0.12-20) | 3.5 (1-20) | 2 (0.12-12) | 0.23 (0-0.65) | 2.8 (0-20) |
| Primary tumor, No. (%)   |                |       |      |      |              |       |
| No data                  | 1              | 31     | 21   | 10   | —            | 32    |
| Cervical                 | 5 (4)          | 54 (6)  | 37 (7) | 17 (4)  | 0 (0)   | 59 (6)  |
| Thoracic                 | 4 (4)          | 157 (17) | 108 (20) | 49 (13) | 0 (0)   | 161 (15) |
| Abdominal adrenal        | 85 (76)        | 655 (70) | 341 (62) | 242 (61) | 0 (0)   | 753 (71) |
| Abdominal other          | 41 (37)        | 329 (35) | 203 (37) | 126 (33) | 3 (23)  | 373 (35) |
| Pelvic                   | 4 (4)          | 59 (6)  | 30 (5) | 29 (8) | 0 (0)   | 63 (6)  |
| Metastatic sites, No. (%)|                |       |      |      |              |       |
| None                     | 113            | —      | —    | —    | 13         | 113    |
| Not specified            | 55             | 26     | 29   | 2    |            |       |
| 1 MC                     | 111 (12)       | 51 (9)  | 60 (16) | 4 (36)  |            |       |
| 2 MC                     | 299 (33)       | 180 (33) | 119 (33) | 3 (27)  |            |       |
| > 2 MC                   | 501 (55)       | 314 (58) | 187 (51) | 4 (36)  |            |       |
| ALK alteration, No. (%)  |                |       |      |      |              |       |
| Yes                      | 25 (22)        | 118 (12) | 36 (6)  | 82 (21) | 3 (23)  | 146 (13) |
| No                       | 88 (78)        | 848 (88) | 535 (94) | 313 (79) | 10 (77) | 946 (87) |
| ALK amplification, No. (%)|                |       |      |      |              |       |
| Yes                      | 13 (12)        | 26 (3)  | 1 (0)  | 25 (6)  | 2 (15)  | 41 (4)  |
| No                       | 83 (73)        | 768 (80) | 466 (82) | 302 (76) | 9 (69)  | 860 (79) |
| Missing data             | 17 (15)        | 172 (18) | 104 (18) | 68 (17) | 2 (15)  | 191 (17) |
| ALK mutations, No. (%)   |                |       |      |      |              |       |
| ALKm clonal              | 9 (8)          | 66 (7)  | 26 (5) | 40 (10) | 1 (8)   | 76 (7)  |
| ALKm subclonal           | 4 (4)          | 26 (3)  | 9 (2)  | 17 (4)  | 0 (0)   | 30 (3)  |
| No                       | 63 (56)        | 586 (61) | 365 (64) | 221 (56) | 7 (54)  | 656 (60) |
| Missing data             | 37 (33)        | 288 (30) | 171 (30) | 117 (30) | 5 (38)  | 330 (30) |

Abbreviations: MC, metastatic compartments; MNA, MYCN-amplified.
| Country | Center | Patients, No. |
|---------|--------|--------------|
| Total   | 1,092  |              |
| FR      | Total  | 344          |
|         | Institut Curie | 65        |
|         | Center Léon Berard | 34        |
|         | Hopitaux de Marseille La Timone | 30        |
|         | Center Oscar Lambret de Lille | 26        |
|         | CHR de Nantes | 23        |
|         | Hopital Hautepierre-CHU Strasbourg | 20        |
|         | Hôpital Trousseau Paris | 18        |
|         | Institut Gustave Roussy | 17        |
|         | Hôpital D’Enfants de Toulouse | 14        |
|         | CHU de Grenoble | 13        |
|         | CHU de Nancy Brabois | 11        |
|         | CHU Montpellier Hôpital Arnaud Villeneuve | 11        |
|         | CHU Rouen | 10        |
|         | Hopital Jean Bernard La Miletrie Poitiers | 8        |
|         | CHR de Caen | 8        |
|         | CHU-Saint Etienne | 6        |
|         | Hôpital de L’Archet Nice | 5        |
|         | CHR Hôpital Sud de rennes | 5        |
|         | Center Hospitalier Angers | 5        |
|         | CHU Morvan de Brest | 4        |
|         | Hotel Dieu de Clermont-ferrand | 4        |
|         | CHRU Nord d’Amiens | 4        |
|         | Hopital d’Enfants Dijon | 2        |
|         | Hopital American de Reims | 1        |
| UK      | Total  | 292          |
|         | Great Ormond Street Hospital | 40        |
|         | Royal Marsden Hospital Surrey | 34        |
|         | Newcastle: Royal Victoria Infirmary | 29        |
|         | Dublin: OLHSC | 13        |
|         | Oxford: John Radcliffe Hospital | 20        |
|         | Bristol Royal Hospital for Children | 19        |
|         | Glasgow Royal Hospital for Sick Children | 19        |
|         | Manchester: Royal Manchester Children’s Hospital | 18        |
|         | Southampton General Hospital | 16        |
|         | Cambridge: Addenbrooke’s NHS Trust | 14        |
|         | Liverpool: Alder Hey Children’s Hospital | 14        |
|         | Birmingham Children’s Hospital | 11        |
|         | Leeds: St. James’s University Hospital | 11        |
|         | Belfast: Royal Belfast Hospital for Sick Children | 9        |
|         | Sheffield Children’s Hospital | 7        |
|         | Cardiff: Llandough Hospital | 5        |
|         | Aberdeen: Royal Aberdeen Children’s Hospital | 4        |
|         | Edinburgh Royal Hospital for Sick Children | 4        |
|         | Leicester Royal Infirmary | 3        |
|         | UCLH University College London Hospital | 2        |

(continued on following page)
| Country | Center                              | Patients, No. |
|---------|-------------------------------------|---------------|
| ES      | Total                               | 152removal (continued) |
|         | H Nino Jesus                        | 15removal (continued) |
|         | Hospital Infantil La Fe             | 13removal (continued) |
|         | Carlos Haya                         | 11removal (continued) |
|         | H Central de Asturias               | 10removal (continued) |
|         | Hospital Infantil La Paz            | 10removal (continued) |
|         | H. Virgen de la Arrixaca            | 8removal (continued) |
|         | Hospital de Cruces                  | 7removal (continued) |
|         | Hospital materno infantil Virgen de las Nieves | 7removal (continued) |
|         | Hospital Vall d’Hebron              | 6removal (continued) |
|         | H. Miguel Servet                    | 6removal (continued) |
|         | Hospital Clinico                    | 5removal (continued) |
|         | H. Virgen del Camino                | 4removal (continued) |
|         | H. Son Dureta                       | 5removal (continued) |
|         | H. General de Galicia               | 4removal (continued) |
|         | Hospital Gregorio Maranon           | 4removal (continued) |
|         | Hospital 12 de Octubre              | 4removal (continued) |
|         | H. de Donostia Ntra. Sra. de Aranzazu, | 4removal (continued) |
|         | Materno Infantil de Badajoz         | 3removal (continued) |
|         | H. General de Alicante              | 3removal (continued) |
|         | Virgen del Rocio                    | 3removal (continued) |
|         | Hospital Germans Triasi Pujol        | 2removal (continued) |
|         | H Sant Pau                          | 2removal (continued) |
|         | Hospital Universitario de Canarias  | 2removal (continued) |
|         | H. Torrecardenas                    | 2removal (continued) |
|         | Hospital Reina Sofia                | 2removal (continued) |
|         | H. C. U. de Salamanca               | 2removal (continued) |
|         | H. Virgen de la Salud               | 1removal (continued) |
|         | H. Materno-Infantil Teresa Herrera  | 1removal (continued) |
|         | H. SanT Joan de Deu                 | 1removal (continued) |
|         | H. Monteprincipe                    | 1removal (continued) |
|         | Complejo Hospitalario de Jaen       | 1removal (continued) |
|         | H. Virgen de la Macarena            | 1removal (continued) |
|         | Hospital Universitario Nuestra Sra de la Candelaria | 1removal (continued) |
|         | Hospital Xeral-Ces                  | 1removal (continued) |
| AT      | Total                               | 57removal (continued) |
|         | St Anna Kinderspital                | 23removal (continued) |
|         | Landes-Kinderklinik Linz            | 12removal (continued) |
|         | Univ.Klinik f. Kinder-u. Jugendheilkunde Innsbruck | 10removal (continued) |
|         | Univ.-Klinik für Kinder- und Jugendheilkunde Graz | 6removal (continued) |
|         | St. Johanns Spital LKH Salzburg     | 6removal (continued) |

(continued on following page)
### TABLE A2. Number of Patients Included in the ALK Analysis Cohort by Country and Center (continued)

| Country | Center | Patients, No. |
|---------|--------|--------------|
| SE      | Total  | 44           |
|         | Stockholm | 14           |
|         | Lund    | 11           |
|         | Uppsala | 8            |
|         | Children’s Hospital Linkoping | 5 |
|         | Queen Silvia’s Children’s Hospital (Gothenburg) | 5 |
|         | Reykjavik | 1           |
| CZ      | Total  | 38           |
|         | University Hospital Motol, Prague | 5 |
| IT      | Total  | 29           |
|         | Ospedale S. Orsola | 7 |
|         | Clinica di Oncoematologia Pediatrica Padova | 5 |
|         | Istituto per l’Infanzia Burlo Garofolo | 3 |
|         | Ospedale Bambino Gesu | 3 |
|         | Policlinico Universitario | 2 |
|         | Istituto Gianna Gaslini | 2 |
|         | Istituto Nazionale Tumori di Milano | 2 |
|         | Policlinico San Matteo | 1 |
|         | Ospedali Riuniti | 1 |
|         | Ospedale dei bambini, Palermo | 1 |
|         | Azienda Ospedaliera Universitaria di Parma-Oncoematologia Pediatrica | 1 |
|         | Policlinico Borgo Roma | 1 |
| CH      | Total  | 25           |
|         | CHUV | 11           |
|         | University Children’s Hospital (Geneva) | 5 |
|         | Inseelspitalk Bern | 3 |
|         | Kantonspital Aarau | 3 |
|         | Ostschweizer Kinderspital | 2 |
|         | Luzerner Kantonsspital - Kinderspital Luzern | 1 |
| PL      | Total  | 23           |
|         | University Children’s Hospital Krakow | 14 |
|         | Wroclaw Medical University | 3 |
|         | Children’s Hospital in Chorzów | 2 |
|         | University of Medical Sciences Poznan | 2 |
|         | Medical University of Bydgoszcz | 1 |
|         | Medical University in Gdansk | 1 |
| BE      | Total  | 21           |
|         | University Hospital Gent | 9 |
|         | UZ Gasthuisberg | 8 |
|         | Clinique de l’Espérance, | 2 |
|         | Cliniques universitaires St-Luc | 1 |
|         | CHR Citadelle | 1 |
| IL      | Total  | 18           |
|         | Schneider Children’s Medical Center of Israel | 17 |
|         | Dana Children’s Hosp., Suraski Tel-Aviv Med. Cent. | 1 |

(continued on following page)
**TABLE A2.** Number of Patients Included in the *ALK* Analysis Cohort by Country and Center (continued)

| Country | Center | Patients, No. |
|---------|--------|---------------|
| PT      | Total  | 14            |
|         | IPOFG-CRL | 14           |
| HK      | Total  | 10            |
|         | University of Hong Kong | 10 |
| NO      | Total  | 10            |
|         | Rikshospitalet | 5           |
|         | Haukeland University Hospital | 4 |
|         | St Olavs Hospital Trondheim | 1 |
| IE      | Total  | 7             |
|         | Dublin: OLHSC | 7           |
| FI      | Total  | 4             |
|         | University of Tampere | 4 |
| DK      | Total  | 2             |
|         | Aarhus Universitetshospital | 1 |
|         | University Hospital of Odense | 1 |
| GR      | Total  | 1             |
|         | Aghia Sophia Children’s Hospital, Athens | 1 |
| SI      | Total  | 1             |
|         | University Children’s Hospital Ljubljana | 1 |

Abbreviations: AT, Austria; BE, Belgium; CH, Switzerland; CZ, Czech Republic; DK, Denmark; ES, Spain; FI, Finland; FR, France; GR, Greece; HK, Hong Kong; IE, Ireland; IL, Israel; IT, Italy; NO, Norway; PL, Poland; PT, Portugal; SE, Sweden; SI, Slovenia; UK, United Kingdom.
| Patient No. | Sex | Age at Diagnosis, years | INSS Stage | Induction Treatment | Status Post Induction | HDC | Relapse | Last Status | MYCN Status | ALK Amplification Status | ALK Mutations | Type of ALK Mutation | MAF, % | Technique Used to Study ALK Mutations |
|------------|-----|-------------------------|------------|--------------------|----------------------|-----|---------|------------|--------------|---------------------------|--------------|--------------------------|-------|--------------------------------------|
| 1          | M   | 2.0                     | 4          | Rapid COJEC        | CR                   | CEM | No      | Alive      | MN-NA        | ALK-NA                    | Yes          | R1275Q                  | 26.911 | TDS and Sanger                   |
| 2          | M   | 2.2                     | 4          | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MNA          | ALK-A                     | No           | NA                      | NA    | TDS                                |
| 3          | F   | 4.9                     | Loc        | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MNA          | ALK-A                     | No           | NA                      | NA    | TDS                                |
| 4          | M   | 1.9                     | Loc        | Rapid COJEC        | PR                   | BUMEL | Yes     | Dead       | MNA          | ALK-A                     | No           | NA                      | NA    | TDS and Sanger                   |
| 5          | F   | 3.5                     | 4          | Rapid COJEC        | VGPR                 | BUMEL | Yes     | Dead       | MNA          | ALK-A                     | No           | NA                      | NA    | TDS                                |
| 6          | M   | 2.3                     | 4          | Rapid COJEC        | MR                   | BUMEL | Yes     | Dead       | MN-NA        | ALK-NA                    | Yes          | R1275Q                  | 30.584 | TDS and Sanger                   |
| 7          | M   | 2.5                     | Loc        | Rapid COJEC        | SD                   | BUMEL | No      | Alive      | MNA          | ALK-NA                    | Yes          | F1174L                  | 50    | TDS and Sanger                   |
| 8          | F   | 1.5                     | 4          | Rapid COJEC        | PR                   | BUMEL | Yes     | Dead       | MN-NA        | ALK-NA                    | Yes          | F1245C                  | 50    | TDS and Sanger                   |
| 9          | F   | 2.0                     | 4          | Rapid COJEC        | VGPR                 | CEM   | Yes     | Dead       | MNA          | ALK-NA                    | Yes          | R1275Q                  | 45.123 | TDS and Sanger                   |
| 10         | M   | 2.6                     | Rapid COJEC| PR                 | BUMEL                | No    | Alive   | MNA        | ALK-NA       | Yes                      | F1174L > 20 | Sanger                  |       |                                     |
| 11         | M   | 2.3                     | 4          | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MNA          | ALK-A                     | No           | NA                      | NA    | TDS                                |
| 12         | F   | 1.2                     | 4          | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MNA          | ALK-A                     | No           | NA                      | NA    | TDS                                |
| 13         | M   | 2.6                     | 4          | Rapid COJEC        | VGPR                 | BUMEL | No      | Alive      | MNA          | ALK-NA                    | Yes          | R1275Q                  | 3.994  | TDS                                |
| 14         | M   | 4.8                     | MOD. N7    | PR                 | BUMEL                | No    | Alive   | MNA        | ALK-NA       | Yes                      | I1170S > 20 | TDS and Sanger           |       |                                     |
| 15         | F   | 1.3                     | 4          | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MNA          | ALK-NA                    | Yes          | F1174L                  | 0.135  | TDS                                |
| 16         | F   | 2.0                     | 4          | MOD. N7            | PR                   | BUMEL | No      | Alive      | MN-NA        | ALK-NA                    | Yes          | R1275Q                  | 45.986 | TDS and Sanger                   |
| 17         | M   | 4.0                     | 4          | Rapid COJEC        | VGPR                 | BUMEL | Yes     | Alive      | MNA          | ALK-NA                    | Yes          | A1274S/G1272V/G1272W | 0.352/0.302/0.275 | TDS        |
| 18         | M   | 1.3                     | MOD. N7    | PR                 | BUMEL                | No    | Alive   | MNA        | ALK-NA       | Yes                      | F1174L       | 32.382                  | TDS and Sanger |
| 19         | F   | 4.3                     | 4          | Rapid COJEC        | PR                   | BUMEL | Yes     | Alive      | MNA          | ALK-NA                    | Yes          | F1174L      > 20         | Sanger            |
| 20         | M   | 1.1                     | 4          | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MN-NA        | ALK-NA                    | Yes          | F1174L      > 20         | Sanger            |
| 21         | M   | 9.7                     | 4          | Rapid COJEC        | PR                   | BUMEL | Yes     | Dead       | MNA          | ALK-NA                    | Yes          | F1174L       4.37         | TDS               |
| 22         | M   | 2.0                     | 4          | Rapid COJEC        | PR                   | BUMEL | Yes     | Dead       | MNA          | ALK-NA                    | Yes          | F1174L      26.982       | TDS and Sanger |
| 23         | F   | 1.6                     | 4          | Rapid COJEC        | VGPR                 | BUMEL | Yes     | Dead       | MNA          | ALK-NA                    | Yes          | R1275Q      0.24         | TDS               |
| 24         | F   | 6.8                     | 4          | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MN-NA        | NA                      | Yes          | I1170N      2.8          | NGS               |
| 25         | F   | 2.1                     | 4          | Rapid COJEC        | PR                   | CEM   | Yes     | Dead       | MNA          | ALK-A                     | No           | NA                      | NA    | TDS                                |
| 26         | M   | 2.7                     | 4          | Rapid COJEC        | PR                   | BUMEL | Yes     | Dead       | MN-NA        | ALK-NA                    | Yes          | F1174L       23.554       | TDS and Sanger |
| 27         | M   | 1.7                     | 4          | Rapid COJEC        | PR                   | BUMEL | Yes     | Dead       | MNA          | ALK-NA                    | No           | NA                      | NA    | TDS                                |
| 28         | M   | 1.7                     | 4          | Rapid COJEC        | VGPR                 | BUMEL | Yes     | Dead       | MNA          | ALK-NA                    | Yes          | F1245L      38.402       | TDS and Sanger |
| 29         | F   | 3.9                     | 4          | Rapid COJEC        | VGPR                 | BUMEL | No      | Alive      | MNA          | ALK-NA                    | Yes          | F1245V > 20           | Sanger            |
| 30         | M   | 2.8                     | 4          | Rapid COJEC        | PR                   | BUMEL | No      | Alive      | MNA          | ALK-NA                    | Yes          | F1174L > 20            | Sanger            |

(continued on following page)
| Patient No. | Sex | Age at Diagnosis, years | INSS Stage | Induction Treatment | Status Post Induction | HDC | Relapse | Last Status | MYCN Status | ALK Amplification Status | ALK Mutations | Type of ALK Mutation | MAF, % | Technique Used to Study ALK Mutations |
|------------|-----|------------------------|------------|---------------------|-----------------------|-----|---------|------------|-------------|------------------------|--------------|------------------------|-------|-------------------------------------|
| 31         | M   | 2.1                    | 4          | Rapid COJEC         | PR                    | BUMEL | No      | Alive      | MNA         | ALK-NA                 | Yes          | L1240V                | > 20   | Sanger                             |
| 32         | F   | 2.2                    | 4          | Rapid COJEC         | VGPR                  | BUMEL | Yes     | Alive      | MN-NA       | ALK-NA                 | Yes          | R1275L                | > 20   | Sanger                             |
| 33         | F   | 2.2                    | 4          | Rapid COJEC         | PR                    | BUMEL | Yes     | Dead       | MNA         | ALK-NA                 | Yes          | F1174L                | > 20   | Sanger                             |
| 34         | M   | 1.9                    | Loc        | Rapid COJEC         | VGPR                  | BUMEL | No      | Alive      | MNA         | ALK-NA                 | Yes          | F1174L                | > 20   | Sanger                             |
| 35         | F   | 2.0                    | 4          | Rapid COJEC         | PR                    | BUMEL | No      | Alive      | MNA         | ALK-NA                 | Yes          | L1190M                | > 20   | Sanger                             |

NOTE. Among these patients, ALK amplifications were detected in eight cases, and clonal ALK mutations were detected in 21 cases. In addition, six cases with subclonal mutations are also listed. Abbreviations: ALK-A, ALK-amplified; ALK-NA, ALK not amplified; BUMEL, busulfan and melphalan; CEM, carboplatin, etoposide, and melphalan; COJEC, chemotherapy regimen, details in Figure A1; CR, complete remission; F, female; HDC, high-dose chemotherapy; INSS, International Neuroblastoma Staging System; M, male; MAF, mutated allele fraction; MNA, MYCN-amplified; MN-NA, MYCN not amplified; MR, minor response; NA, not applicable; NGS, next-generation sequencing; PR, partial remission; SD, stable disease; TDS, targeted deep sequencing; VGPR, very good partial response.