original reports

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

Angela Bellini, PhD[1,2,3]; Ulrike Pötschger, PhD[4,5]; Virginie Bernard, PhD[6]; Eve Lapouble, PhD[7]; Sylvain Baulande, PhD[6]; Peter F. Ambros, PhD[5]; Nathalie Auger, PhD[8]; Klaus Beiske, MD, PhD[9]; Marie Bernkopf, PhD[5]; David R. Betts, PhD[10]; Jaydutt Bhalshankar, MSc[1,2,3]; Nick Bown, PhD[11]; Katleen de Preter, PhD[12]; Nathalie Clément, PhD[1,2,3]; Valérie Combaret, PhD[13]; Jaime Font de Mora, PhD[14]; Sally L. George, MD, PhD[15]; Irene Jiménez, MD[1,2,3]; Marta Jeison, PhD[16]; Barbara Marques, PhD[17]; Tommy Martinsson, PhD[18]; Katia Mazzocco, PhD[19]; Martina Morini, PhD[20]; Annick Mühlethaler-Mottet, PhD[21]; Rosa Noguera, MD[22]; Gaelle Pierron, PhD[7]; Maria Rossing, PhD[23]; Sabine Taschner-Mandl, PhD[5]; Nadine Van Roy, PhD[12]; Ales Vicha, PhD[24]; Louis Chesler, MD, PhD[25]; Walentyna Balwierz, MD[26]; Victoria Castel, MD, PhD[27]; Martin Elliott, MD[28]; Per Kogner, MD, PhD[29]; Geneviève Laureys, MD, PhD[30]; Roberto Luksch, MD[31]; Josef Malis, MD[24]; Maja Popovic-Beck, MD[32]; Shifra Ash, MD[33]; Olivier Delattre, MD[2,3,6]; Dominique Valteau-Couanet, MD, PhD[34]; Deborah A. Tweddle, MD, PhD[35]; Ruth Ladenstein, MD, PhD[36,37]; and Gudrun Schleiermacher, MD, PhD[1,2,3]

abstract

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 (*ALK*a) was detected in 4.5% of cases (41 out of 901), all except one with *MYCN* amplification (MNA). *ALK*a was associated with a significantly poorer overall survival (OS) (5-year OS: *ALK*a [n = 41] 28% [95% CI, 15 to 42]; no-*ALK*a [n = 860] 51% [95% CI, 47 to 54], [$P < .001$]), particularly in cases with metastatic disease. *ALK* mutations (*ALK*m) 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 *ALK*m and MNA ($P < .001$). Among 571 cases with known *ALK*a and *ALK*m status, a statistically significant difference in OS was observed between cases with *ALK*a or clonal *ALK*m versus subclonal *ALK*m or no *ALK* alterations (5-year OS: *ALK*a [n = 19], 26% [95% CI, 10 to 47], clonal *ALK*m [n = 65] 33% [95% CI, 21 to 44], subclonal *ALK*m (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; $P < .001$), *ALK*a (HR, 2.38; $P = .004$), and clonal *ALK*m (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.

J Clin Oncol 39:3377-3390. © 2021 by American Society of Clinical Oncology

ASSOCIATED CONTENT

Appendix

Data Sharing Statement

Protocol

Author affiliations and support information (if applicable) appear at the end of this article.

Accepted on April 21, 2021 and published at ascopubs.org/journal/jco on June 11, 2021: DOI https://doi.org/10.1200/JCO.21.00086

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-GD_2 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-GD_2 immunotherapy.

rapid tumor growth.[9] Other copy-number alterations occur over more extensive chromosome regions, with segmental chromosome alterations being associated with a poor outcome.[10] Recurrent mutations have been described in the RAS-MAPK pathway, chromatin remodeling genes (*ATRX* and *ARID1A*), and *TERT* rearrangements.[11-14]

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 (*ALK*m) observed in 6%-12% of sporadic NBs in all risk groups.[15-18]

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).[19-23]

ALK can also be activated by genomic focal amplification, described in 1%-2% of NBs, almost exclusively with MNA,[17,24] or, more rarely, following structural rearrangements.[25] Genetic alterations of *ALK* are associated with poorer survival in the overall NB population.[24,26] However, their prognostic role in HR-NB has been less well studied.[10,17,24] 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.[27-29]

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.[2-5,30,31] Patients with International Neuroblastoma Staging System stages 2, 3, 4, or 4S with 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[31]; R3—comparison of two induction regimens, rapid-COJEC versus modified N7.[32] HDC was evaluated in the R1 random assignment: busulfan or melphalan versus carboplatin or etoposide or melphalan.[3] Anti-GD_2 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.[5,30] 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[33] for additional molecular analysis with available follow-up data (Fig 1).

MYCN status and tumor genomic copy-number profiles were determined in SIOPEN reference laboratories as described previously.[10,33-36] Samples were required to contain at least 20% tumor cells on pathologic examination.

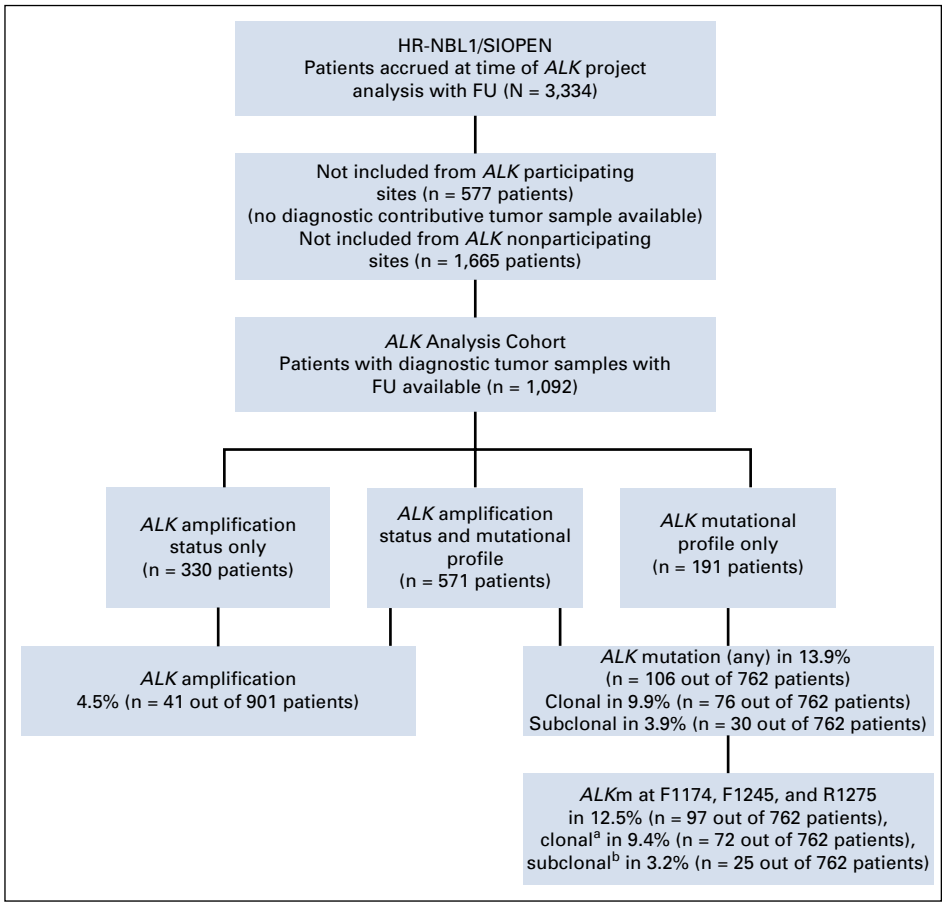


FIG 1. Flow diagram of patient inclusion. A total of 3,334 patients with HR-NB were enrolled in the HR-NBL1 trial from 188 centers. Among these, 2,350 patients were not included in this study, either because no contributive tumor material was available, or because these was no FU data, or both. Thus,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

The *ALK* amplification (*ALK*a) 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 (*ALK*m) 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.

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

	Known *ALK* Amplification Status (N = 901)					Known *ALK* Mutation Status (N = 762)						
	No		Yes			No Mutation		Clonal Mutation		Subclonal Mutation		
Clinical Parameters	**n**	**%**	**n**	**%**	***P***	**n**	**%**	**n**	**%**	**n**	**%**	***P***
Total	860	100	41	100		656	100	76	100	30	100	
Sex												
Female	376	44	16	39	.553	278	42	38	50	11	37	.348
Male	484	56	25	61		378	58	38	50	19	63	
Age, years												
< 1	51	6	7	17	.005	38	6	5	7	0	0	.348
1-1.5	101	12	9	22		79	12	15	20	3	10	
1.5-5	572	67	20	49		428	65	47	62	21	70	
> 5	136	16	5	12		111	17	9	12	6	20	
Stage												
Loc, MNA+	83	10	13	32	< .001	63	10	9	12	4	13	.890
Stage 4	768	89	26	63		586	89	66	87	26	87	
Stage 4s, MNA+	9	1	2	5		7	1	1	1	0	0	
MYCN status												
MNA−	466	54	1	2	< .001	365	56	26	34	9	30	< .001
MNA+	394	46	40	98		291	44	50	66	21	70	
Primary tumor site												
Unknown	20		1		.362	21		1		1		.278
Abdominal adrenal±	606	72	25	63		452	71	47	63	22	76	
Abdominal other±	169	20	10	25		124	20	22	29	6	21	
Other only	65	8	5	13		59	9	6	8	1	3	
Stage 4: MYCN status												
MNA−	466	61	1	4	< .001	365	62	26	39	9	35	< .001
MNA+	302	39	25	96		221	38	40	61	17	65	
Stage 4: MC												
1 MC	91	12	1	4	.091	70	13	11	17	4	17	.788
2 MC	231	32	12	52		177	32	19	29	9	38	
> 2 MC	411	56	10	43		302	55	35	54	11	46	
Overall response: end of induction												
Evaluable	804		39			607		72		28		
CR or VGPR or PR	628	78	31	79	.839	472	78	53	74	24	86	.421
MR or SD or PD	176	22	8	21		135	22	19	26	4	14	

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.

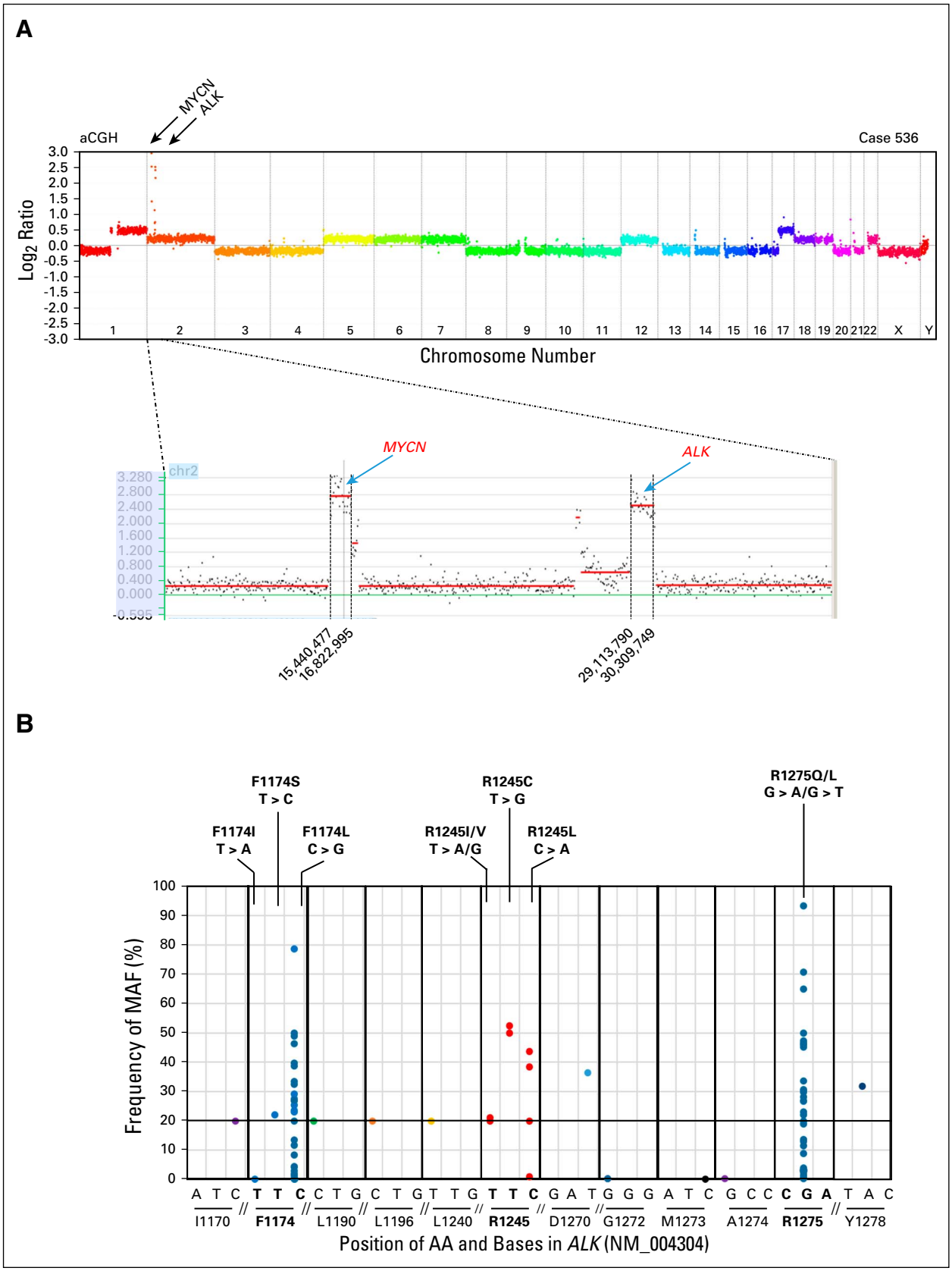


FIG 2. Genetic alterations of *ALK* in patients with HR-NB. (A) Copy-number profile of case 536. Genomic coamplification of *MYCN* and *ALK* is observed on chromosome 2, encompassing the regions between position 15,440,477 and 16,822,999 and between 29,113,790 and 30,309,749 bp (human genome assembly hg19; UCSC Genome Browser Home[38]). (B) Frequency distribution (continued on following page)

FIG 2. (Continued). of mutated *ALK* alleles at the studied chromosome regions, encompassing the AA positions F1174, L1190, L1196, R1245, D1270, G1272, M1273, A1274, R1275, and Y1278 detected, in 762 samples. *ALK* mutations involved the common mutational hotspots (F1174, F1245, and R1275) in 12.5% (97 out of 772) of cases, at a clonal level (MAF 20%-93%) in 72 cases, and at a subclonal level (MAF < 20%) in 25 cases. At the F1174 hotspot (chr2: 29,443,695-29,443,697), alterations were observed in 44 cases: 42 cases harbored a mutation leading to the AA change F1174L, one case with F1174I, and one case with F1174S, with MAFs ranging from 0.12% to 78%. At the R1275 hotspot (chr2: 29,432,849-29,430,139), mutations were detected in 43 cases: 38 cases harbored a mutation leading to the AA change R1275Q and five cases with R1275L, with the MAFs ranging from 0.2% to 93%. Ten cases showed *ALK* mutations at the F1245 hotspot (chr2: 29,436,858-29,436,860) within exon 24. Three samples showed the F1245L mutation, three cases carried the F1245C mutation, three showed the F1245I mutation, and one showed mutation F1245V mutation (Fig 1 and Appendix Table A1). Other *ALK* mutations were detected at residues I1170, L1190 (two cases), L1196, D1270, G1272, M1273, A1274, and Y1278 within the explored regions, leading to a nonsynonymous AA change with a predicted functional impact. All these mutations were clonal (MAF > 20%) except for M1273I (MAF 0.2%) and I1170 (MAF 2.8%). AA, amino acid; aCGH, array comparative genomic hybridization; bp, base pair; HR-NB, high-risk neuroblastoma; MAF, mutated allele fraction; UCSC, University of California, Santa Cruz.

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).[42] The median follow-up period was 6.8 years (0.1-17.4 years).

ALK Alterations

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

ALK alterations were detected in 146 out of 1,092 patients with *ALK*a occurring in 4.5% (41 out of 901 cases) and *ALK*m in 13.9% (106 out of 762 cases). Only one case showed *ALK*a 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*. *ALK*a 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 *ALK*a and response at the end of induction treatment was observed.

A statistically significant poorer 5-year OS was observed in patients whose tumors harbored *ALK*a (5-year OS: *ALK*a 28% [95% CI, 15 to 42] *v* non-*ALK*a 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 = 13), or by TDS (n = 650, including 64 by TDS and Sanger). The biologic data for 52 cases have been reported previously.[22]

Among these, 13.9% (106 out of 762) showed at least one *ALK*m 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).

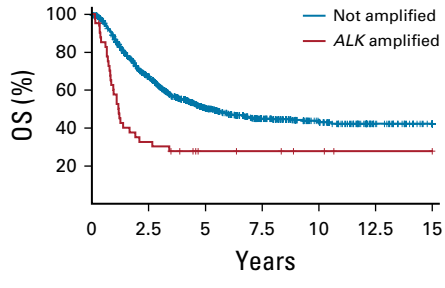
Concordance between results analyzed by two different techniques was observed in 64 cases with clonal *ALK*m (TDS and Sanger). Subclonal *ALK*m were validated by a second independent TDS experiment, with an excellent correlation of MAF between the two experiments ($R2 = 0.9924$; $P < .0001$) (Appendix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

*ALK*m 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 *ALK*m (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).

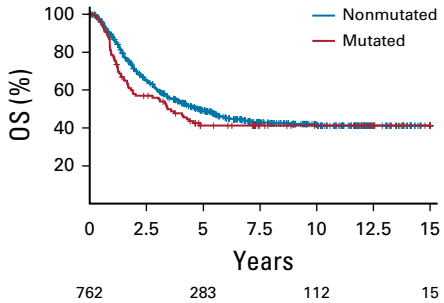
There were no statistically significant correlations between *ALK*m and stage, age at diagnosis, or localization of the primary tumor (adrenal, abdominal, or other) (Table 1). However, a significant correlation was observed between the presence of an *ALK*m and MNA ($P < .001$), with an enrichment of *ALK*m F1174 in MNA tumors ($P = .0005$).

A



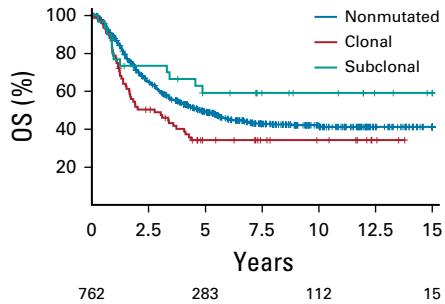
No. at risk:	Patients	Events	5-Year OS, % (95% CI)	HR (95% CI)	*P*
	901	276	94	15	
Not amplified	860	418	51 (47 to 54)	Ref	< .001
ALK amplified	41	29	28 (15 to 42)	2.3 (1.6 to 3.4)	

B



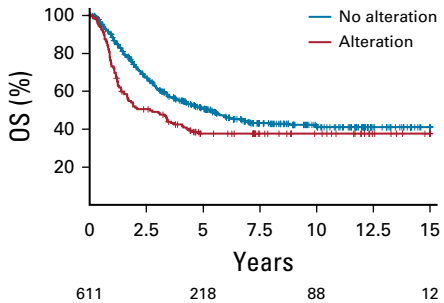
No. at risk:	Patients	Events	5-Year OS, % (95% CI)	HR (95% CI)	*P*
	762	283	112	15	
Nonmutated	656	347	49 (45 to 53)	Ref	.232
Mutated	106	61	41 (31 to 51)	1.2 (0.9 to 1.6)	

C



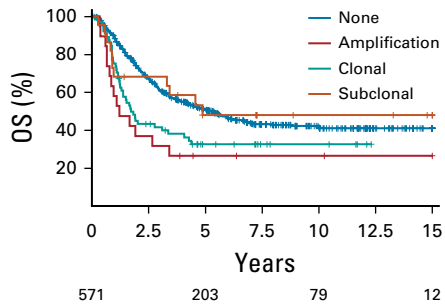
No. at risk:	Patients	Events	5-Year OS, % (95% CI)	HR (95% CI)	*P*
	762	283	112	15	
Nonmutated	656	347	49 (45 to 53)	Ref	.018
Clonal	76	48	34 (23 to 45)	1.4 (1.1 to 2.0)	
Subclonal	30	13	59 (39 to 74)	0.7 (0.4 to 1.2)	

D



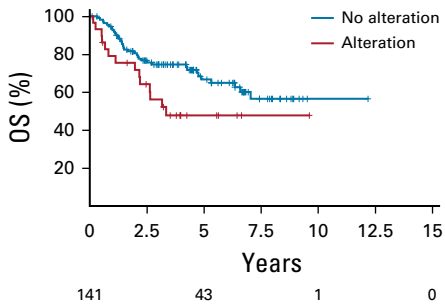
No. at risk:	Patients	Events	5-Year OS, % (95% CI)	HR (95% CI)	*P*
	611	218	88	12	
No alteration	465	241	51 (46 to 55)	Ref	.005
Alteration	146	89	37 (29 to 45)	1.4 (1.1 to 1.8)	

E



No. at risk:	Patients	Events	5-Year OS, % (95% CI)	HR (95% CI)	*P*
	571	203	79	12	
None	465	241	51 (46 to 55)	Ref	.001
Amplification	19	14	26 (10 to 47)	2.2 (1.3 to 3.8)	
Clonal	65	42	33 (21 to 44)	1.7 (1.2 to 2.3)	
Subclonal	22	12	48 (26 to 67)	1.0 (0.5 to 1.8)	

F



No. at risk:	Patients	Events	5-Year OS, % (95% CI)	HR (95% CI)	*P*
	141	43	1	0	
No alteration	112	36	67 (56 to 75)	Ref	.034
Alteration	29	14	48 (28 to 65)	1.9 (1.0 to 3.6)	

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); $P < .0001$. (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)

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 *ALK*m and response at the end of induction treatment was observed.

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

Patients with metastatic disease (stage 4 or 4S MNA) and a clonal *ALK*m showed a trend toward poorer OS. However, in patients with localized disease, the presence of *ALK*m 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 *ALK*a and *ALK*m 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). *ALK*a or clonal *ALK*m were associated with a poorer outcome (5-year OS, *ALK*a 26% [95% CI, 10 to 47], clonal *ALK*m 33% [95% CI, 21 to 44], subclonal *ALK*m 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/SIOPEN trial, the introduction of busulfan and melphalan as standard for HDC, and anti-GD_2 maintenance therapy as a new standard since 2010, has led to significantly improved survival (Appendix Fig A5F, online only).[3-5] Importantly, when considering patients treated according to the SIOPEN standard with busulfan and melphalan HDC and maintenance immunotherapy, the presence of an *ALK* alteration (*ALK*a or clonal *ALK*m) 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 *ALK*m (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 *ALK*a (OS: HR, 2.38 [95% CI, 1.32 to 4.27]; $P = .004$) and clonal *ALK*m (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 *ALK*a or clonal *ALK*m resulted in significantly worse outcome.

Given the oncogenic driver role of *ALK* activation, and the prognostic impact of *ALK*a or clonal *ALK*m, 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),[3-5,7] 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

TABLE 2. EFS and OS According to *ALK* Alterations

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	< .001	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)	
*ALK*m										
Nonmutated	656	347	49 (45 to 53)	Ref	.018	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	< .001	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	.068	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	
Yes	1	1	NA	NA		1	1	NA	NA	
*ALK*m										
Nonmutated	365	202	49 (43 to 54)	Ref	.202	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		1	1	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)

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+										
*ALK*a										
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)	
*ALK*m										
Nonmutated	228	126	44 (38 to 51)	Ref	.453	228	137	40 (33 to 46)	Ref	.666
*ALK*m 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)	
*ALK*m 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
*ALK*a	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)	
*ALK*m 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)	
*ALK*m 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+										
*ALK*a										
No	83	24	71 (59 to 80)	Ref	.059	83	25	69 (57 to 78)	Ref	.015
Yes	13	7	46 (19 to 70)	2.2 (0.9 to 5.1)		13	8	38 (14 to 63)	2.6 (1.2 to 5.8)	
*ALK*m										
Nonmutated	63	19	70 (57 to 80)	Ref	.114	63	20	67 (54 to 77)	Ref	.098
*ALK*m clonal	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)	
*ALK*m subclonal	4	0	NA	NA		4	0	NA		
Known *ALK* alteration status										
Nonmutated	46	13	73 (57 to 83)	Ref	.440	46	14	68 (52 to 80)	Ref	.410
*ALK*a	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)	
*ALK*m 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)	
*ALK*m subclonal	1	0	NA	NA		1	0	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 (*ALK*a, n = 901 patients), *ALK* mutational status (*ALK*m, n = 762 patients), or in patients for whom both the *ALK*a status and *ALK*m 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.

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*a 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.[17] In this extensive cohort of patients, one case harboring both *ALK*a and an R1275 *ALK*m was identified. This indicates that these alterations are not fully mutually exclusive, although co-occurrence is extremely rare.

*ALK*m were found in 13.9% of cases at the studied exonic regions harboring known *ALK* mutational hotspots.[17,24] This is higher than previously reported frequencies of *ALK*m in HR-NB of approximately 10%, most likely as previous reports using Sanger sequencing or standard-resolution NGS approaches.[24,26] Sanger sensitivity is limited to the detection of MAF > 15%-20%, but in NB, *ALK* mutations with lower MAFs have been reported.[14,19-21]

Ultradeep sequencing used in this analysis has a sensitivity limit of MAF of 0.1%.[19,20] 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 3. Multivariate Analysis in 571 Patients With a Known *ALK* Amplification and *ALK* Mutation Status

	OS			EFS		
Clinical Parameters	***P***	**HR**	**95% CL**	***P***	**HR**	**95% CL**
Age, years						
< 1		1.00			1.00	
1-1.5	.269	0.72	0.40 to 1.30	.636	0.87	0.49 to 1.56
1.5-5	.265	0.75	0.45 to 1.24	.830	0.95	0.57 to 1.56
> 5	.662	0.88	0.50 to 1.55	.935	1.02	0.59 to 1.78
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.

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 SNPa 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 *ALK*a 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 *ALK*a patients is concordant with this observation. However, survival of patients whose tumors harbored *ALK*m and MNA was not different from those without MNA, suggesting that *ALK*m cases constitute a more heterogeneous group with regards to the mechanistic tumor classification.[55]

*ALK*a 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 (*ALK*a and *ALK*m) needs to be considered.

AFFILIATIONS

[1]Equipe SiRIC RTOP Recherche Translationelle en Oncologie Pédiatrique, Institut Curie, Paris, France
[2]INSERM U830, Laboratoire de Génétique et Biologie des Cancers, Institut Curie, Paris, France
[3]SIREDO: Care, Innovation and Research for Children, Adolescents and Young Adults with Cancer, Institut Curie, Paris, France
[4]Department for Studies and Statistics and Integrated Research, Vienna, Austria
[5]St Anna Children's Cancer Research Institute, Vienna, Austria
[6]Institut Curie Genomics of Excellence (ICGex) Platform, Research Center, Institut Curie, Paris, France
[7]Unité de Génétique Somatique, Service de Génétique, Hospital Group, Institut Curie, Paris, France
[8]Service de Génétique des tumeurs; Institut Gustave Roussy, Villejuif, France
[9]Department of Pathology, Oslo University Hospital, and Medical Faculty, University of Oslo, Oslo, Norway
[10]Department of Clinical Genetics, Children's Health Ireland at Crumlin, Dublin, Ireland
[11]Northern Genetics Service, The Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, United Kingdom
[12]Ghent University, Ghent, Belgium
[13]Translational Research Laboratory, Centre Léon Bérard, Lyon, France
[14]Instituto de Investigación Sanitaria La Fe, Valencia, Spain
[15]Division of Clinical Studies, The Institute of Cancer Research, London, United Kingdom
[16]Schneider Children's Medical Center of Israel, Tel Aviv University, Tel Aviv, Israel
[17]Departamento de Genética Humana, Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisbon, Portugal
[18]Sahlgrenska University Hospital, Göteborg, Sweden
[19]Department of Pathology, IRCCS Istituto Giannina Gaslini, Genova, Italy
[20]Laboratory of Molecular Biology, IRCCS Istituto Giannina Gaslini, Genova, Italy
[21]Pediatric Hematology-Oncology Research Laboratory, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland
[22]Department of Pathology, Medical School, University of Valencia-Incliva Health Research Institute/CIBERONC, Madrid, Spain
[23]Center for Genomic Medicine, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark
[24]Department of Paediatric Haematology and Oncology, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic
[25]Paediatric Tumour Biology, Division of Clinical Studies, The Institute of Cancer Research, Sutton, United Kingdom
[26]Department of Pediatric Oncology and Hematology, Institute of Pediatrics, Jagiellonian University Medical College, Krakow, Poland
[27]Clinical and Translational Oncology Research Group, Health Research Institute La Fe, Valencia, Spain
[28]Leeds Children's Hospital, Leeds General Infirmary, Leeds, United Kingdom
[29]Karolinska University Hospital, Stockholm, Sweden
[30]Department of Paediatric Haematology and Oncology, Princess Elisabeth Children's Hospital, Ghent University Hospital, Ghent, Belgium
[31]Paediatric Oncology, Fondazione IRCCS, Istituto Nazionale dei Tumori, Milan, Italy
[32]Pediatric Hematology-Oncology Unit, Lausanne University Hospital and University of Lausanne, Lausanne, Switzerland
[33]Ruth Rappaport Children's Hospital, Rambam Health Care Campus, Haifa, Israel
[34]Département d'Oncologie Pédiatrique, Gustave Roussy, Villejuif, France
[35]Wolfson Childhood Cancer Research Centre, Newcastle Centre for Cancer, Translational & Clinical Research Institute, Newcastle University, Newcastle upon Tyne, United Kingdom
[36]Department for Studies and Statistics and Integrated Research, St Anna Children's Hospital, St Anna Children's Cancer Research Institute, Vienna, Austria
[37]Department of Paediatrics, Medical University of Vienna, Vienna, Austria

CORRESPONDING AUTHOR

Gudrun Schleiermacher, MD, PhD, SIREDO Pediatric Oncology Center, Laboratory of Translational Research in Pediatric Oncology—INSERMU830 Institut Curie, 26 rue d'Ulm, 75248 Paris Cedex 05, France; e-mail: gudrun.schleiermacher@curie.fr.

EQUAL CONTRIBUTION

A.B. and U.P. contributed equally to this work and are to be considered as joint first authors. D.A.T., R.L., and G.S. contributed equally to this work and are to be considered joint senior authors.

SUPPORT

Supported by the Annenberg Foundation and the Association Hubert Gouin Enfance et Cancer, France. This study was also funded by the Fédération Enfants Cancers Santé, Les Bagouz à Manon, Les amis de Claire. Funding was also obtained from SiRIC/INCa (Grant INCa-DGOS-4654) and PHRC IC2007-09 grant.
High-throughput sequencing was performed by the ICGex NGS platform of the Institut Curie supported by the grants ANR-10-EQPX-03 (Equipex) and ANR-10-INBS-09-08 (France Génomique Consortium) from the Agence Nationale de la Recherche (Investissements d'Avenir program), by the Canceropole Ile-de-France, and by the SiRIC-Curie program - SiRIC Grant INCa-DGOS-4654.
In the United Kingdom, this work was supported by Neuroblastoma UK, Cancer Research UK, Department of Health, Families against Neuroblastoma, Solving Kids' Cancer, and Action Medical Research/Great Ormond Street Hospital Charity. The CCLG Tissue Bank is funded by Cancer Research UK and CCLG.
The funding of the European Commission 5th Frame Work Grant (SIOPEN-R-NET EC Grant No. QLRI-CT-2002-01768, www.siopen-r-net.org) supporting the HR-NBl1/SIOPEN trial is disclosed as funding source in the author statement. Pierre Fabre Médicament providing Busilvex (Paris, France), APEIRON (Vienna, Austria) providing dinutuximab beta (ch14.18/CHO) and the St Anna Kinderkrebsforschung GmbH (Vienna, Austria). The St Anna Kinderkrebsforschung was the academic sponsor of the HR-NBL1/SIOPEN trial providing resources for the remote trial data base and central trial management.
Recloning and production of the ch14.18 monoclonal antibody was done at Polymun, Vienna, Austria, and was enabled by a SIOPEN fundraising effort in 2001. APEIRON provided additional product at a later stage. The authors express their gratitude and appreciation to SIOPEN investigators, treating physicians, clinical research and care teams, and most

importantly to patients and families facing high-risk neuroblastoma for their committed participation in the trial. The European Commission, Pierre Fabre Médicament, and Apeiron had no involvement in the conduct of the research and preparation of the article.
In addition, this work was supported as follows: Belgium: vzw Kinderkankerfonds and Kom op tegen Kanker. Czech Republic: MH CZ—DRO, University Hospital Motol, Prague, Czech Republic; Israel: Hayim Association—for Children with Cancer in Israel, Ramat Gan. Italy: Fondazione Italiana per la Lotta al Neuroblastoma O.N.L.U.S. c/o Istituto G. Gaslini, Genova, Associazione Bianca Garavaglia O.N.L.U.S., Busto Arsizio. Spain: Grant FIS EC10/303, Asociación Pablo Ugarte, Cancercare Xavia, Sumemos Muchas Manos, Heath Institute Carlos III (ISCIII) and FEDER (European Regional Development Fund): Grants PI17/01558 and CIBERONC-CB16/12/00484. NEN Association (Nico contra el cáncer infantil 2017-PVR00157). Switzerland: Oncosuisse, Bern; Swiss Cancer League, Bern; Fond'action contre le Cancer, Lausanne; FORCE Fondation Recherche sur le Cancer de l'Enfant, Ecublens.

CLINICAL TRIAL INFORMATION

NCT01704716 (HR-NBL1/SIOPEN)

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at DOI https://doi.org/10.1200/JCO.21.00086.

AUTHOR CONTRIBUTIONS

Conception and design: Angela Bellini, Ulrike Pötschger, Tommy Martinsson, Louis Chesler, Dominique Valteau-Couanet, Deborah A. Tweddle, Ruth Ladenstein, Gudrun Schleiermacher
Financial support: Deborah A. Tweddle, Gudrun Schleiermacher
Administrative support: Louis Chesler, Olivier Delattre, Gudrun Schleiermacher
Provision of study materials or patients: Angela Bellini, Peter F. Ambros, Nathalie Auger, Klaus Beiske, David R. Betts, Katleen de Preter, Nathalie Clément, Valérie Combaret, Jaime Font de Mora, Irene Jiménez, Marta Jeison, Tommy Martinsson, Katia Mazzocco, Martina Morini, Annick Mühlethaler-Mottet, Rosa Noguera, Gaelle Pierron, Sabine Taschner-Mandl, Nadine Van Roy, Louis Chesler, Victoria Castel, Martin Elliott, Per Kogner, Geneviève Laureys, Josef Malis, Maja Popovic-Beck, Shifra Ash, Olivier Delattre, Dominique Valteau-Couanet, Deborah A. Tweddle, Ruth Ladenstein, Gudrun Schleiermacher
Collection and assembly of data: Angela Bellini, Ulrike Pötschger, Eve Lapouble, Sylvain Baulande, Nathalie Auger, Klaus Beiske, Marie Bernkopf, Jaydutt Bhalshankar, Nick Bown, Katleen de Preter, Nathalie Clément, Valérie Combaret, Jaime Font de Mora, Sally L. George, Irene Jiménez, Marta Jeison, Tommy Martinsson, Katia Mazzocco, Martina Morini, Annick Mühlethaler-Mottet, Rosa Noguera, Gaelle Pierron, Maria Rossing, Sabine Taschner-Mandl, Nadine Van Roy, Ales Vicha, Louis Chesler, Walentyna Balwierz, Victoria Castel, Martin Elliott, Per Kogner, Geneviève Laureys, Josef Malis, Maja Popovic-Beck, Shifra Ash, Olivier Delattre, Dominique Valteau-Couanet, Deborah A. Tweddle, Ruth Ladenstein, Gudrun Schleiermacher
Data analysis and interpretation: Angela Bellini, Ulrike Pötschger, Virginie Bernard, Peter F. Ambros, Nathalie Auger, Marie Bernkopf, David R. Betts, Jaime Font de Mora, Barbara Marques, Tommy Martinsson, Sabine Taschner-Mandl, Nadine Van Roy, Per Kogner, Geneviève Laureys, Roberto Luksch, Deborah A. Tweddle, Ruth Ladenstein, Gudrun Schleiermacher
Manuscript writing: All authors
Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

ACKNOWLEDGMENT

The authors would like to thank the following Biobanks for providing samples: In Italy, the BIT-Gaslini Biobank, IRCCS Istituto Giannina Gaslini, Via G. Gaslini 5, Genova. In Spain, the Clinic Hospital INCLIVA-Valencia NB Tissue Bank (ISCIII, Reference: B0000339). They also thank the Children's Cancer & Leukemia Group (CCLG) Tissue Bank for access to DNA samples (CCLG 2015 BS 04), and contributing CCLG Centers, including members of the Experimental Cancer Medicine Centers Pediatric network.

REFERENCES

1. Matthay KK, Maris JM, Schleiermacher G, et al: Neuroblastoma. Nat Rev Dis Primers 2:16078, 2016
2. Holmes K, Potschger U, Pearson ADJ, et al: Influence of surgical excision on the survival of patients with stage 4 high-risk neuroblastoma: A report from the HR-NBL1/SIOPEN study. J Clin Oncol 38:2902-2915, 2020
3. 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 18:500-514, 2017
4. Ladenstein R, Potschger U, Valteau-Couanet D, et al: Investigation of the role of dinutuximab beta-based immunotherapy in the SIOPEN high-risk neuroblastoma 1 trial (HR-NBL1). Cancers (Basel) 12:309, 2020
5. Ladenstein R, Potschger U, Valteau-Couanet D, et al: Interleukin 2 with anti-GD2 antibody ch14.18/CHO (dinutuximab beta) in patients with high-risk neuroblastoma (HR-NBL1/SIOPEN): A multicentre, randomised, phase 3 trial. Lancet Oncol 19:1617-1629, 2018
6. Ozkaynak MF, Gilman AL, London WB, et al: A comprehensive safety trial of chimeric antibody 14.18 with GM-CSF, IL-2, and isotretinoin in high-risk neuroblastoma patients following myeloablative therapy: Children's Oncology Group study ANBL0931. Front Immunol 9:1355, 2018
7. Park JR, Kreissman SG, London WB, et al: Effect of tandem autologous stem cell transplant vs single transplant on event-free survival in patients with high-risk neuroblastoma: A randomized clinical trial. JAMA 322:746-755, 2019
8. Pinto N, Naranjo A, Hibbitts E, et al: Predictors of differential response to induction therapy in high-risk neuroblastoma: A report from the Children's Oncology Group (COG). Eur J Cancer 112:66-79, 2019
9. Seeger RC, Brodeur GM, Sather H, et al: Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med 313: 1111-1116, 1985
10. Janoueix-Lerosey I, Schleiermacher G, Michels E, et al: Overall genomic pattern is a predictor of outcome in neuroblastoma. J Clin Oncol 27:1026-1033, 2009
11. Peifer M, Hertwig F, Roels F, et al: Telomerase activation by genomic rearrangements in high-risk neuroblastoma. Nature 526:700-704, 2015
12. Molenaar JJ, Koster J, Zwijnenburg DA, et al: Sequencing of neuroblastoma identifies chromothripsis and defects in neuritogenesis genes. Nature 483: 589-593, 2012
13. Pugh TJ, Morozova O, Attiyeh EF, et al: The genetic landscape of high-risk neuroblastoma. Nat Genet 45:279-284, 2013
14. Sausen M, Leary RJ, Jones S, et al: Integrated genomic analyses identify ARID1A and ARID1B alterations in the childhood cancer neuroblastoma. Nat Genet 45:12-17, 2012
15. Chen Y, Takita J, Choi YL, et al: Oncogenic mutations of ALK kinase in neuroblastoma. Nature 455:971-974, 2008
16. George RE, Sanda T, Hanna M, et al: Activating mutations in ALK provide a therapeutic target in neuroblastoma. Nature 455:975-978, 2008

17. Janoueix-Lerosey I, Lequin D, Brugieres L, et al: Somatic and germline activating mutations of the ALK kinase receptor in neuroblastoma. Nature 455:967-970, 2008
18. Mosse YP, Laudenslager M, Longo L, et al: Identification of ALK as a major familial neuroblastoma predisposition gene. Nature 455:930-935, 2008
19. Bellini A, Bessoltane-Bentahar N, Bhalshankar J, et al: Study of chromatin remodeling genes implicates SMARCA4 as a putative player in oncogenesis in neuroblastoma. Int J Cancer 145:2781-2791, 2019
20. Javanmardi N, Fransson S, Djos A, et al: Low frequency ALK hotspots mutations in neuroblastoma tumours detected by ultra-deep sequencing: Implications for ALK inhibitor treatment. Sci Rep 9:2199, 2019
21. Combaret V, Iacono I, Bellini A, et al: Detection of tumor ALK status in neuroblastoma patients using peripheral blood. Cancer Med 4:540-550, 2015
22. Bellini A, Bernard V, Leroy Q, et al: Deep sequencing reveals occurrence of subclonal ALK mutations in neuroblastoma at diagnosis. Clin Cancer Res 21: 4913-4921, 2015
23. Eleveld TF, Oldridge DA, Bernard V, et al: Relapsed neuroblastomas show frequent RAS-MAPK pathway mutations. Nat Genet 47:864-871, 2015
24. Bresler SC, Weiser DA, Huwe PJ, et al: ALK mutations confer differential oncogenic activation and sensitivity to ALK inhibition therapy in neuroblastoma. Cancer Cell 26:682-694, 2014
25. Fransson S, Hansson M, Ruuth K, et al: Intragenic anaplastic lymphoma kinase (ALK) rearrangements: Translocations as a novel mechanism of ALK activation in neuroblastoma tumors. Genes Chromosomes Cancer 54:99-109, 2015
26. De Brouwer S, De Preter K, Kumps C, et al: Meta-analysis of neuroblastomas reveals a skewed ALK mutation spectrum in tumors with MYCN amplification. Clin Cancer Res 16:4353-4362, 2010
27. Friboulet L, Li N, Katayama R, et al: The ALK inhibitor ceritinib overcomes crizotinib resistance in non-small cell lung cancer. Cancer Discov 4:662-673, 2014
28. Guan J, Tucker ER, Wan H, et al: The ALK inhibitor PF-06463922 is effective as a single agent in neuroblastoma driven by expression of ALK and MYCN. Dis Model Mech 9:941-952, 2016
29. Solomon BJ, Besse B, Bauer TM, et al: Lorlatinib in patients with ALK-positive non-small-cell lung cancer: Results from a global phase 2 study. Lancet Oncol 19: 1654-1667, 2018
30. Ladenstein R, Potschger U, Siabalis D, et al: Dose finding study for the use of subcutaneous recombinant interleukin-2 to augment natural killer cell numbers in an outpatient setting for stage 4 neuroblastoma after megatherapy and autologous stem-cell reinfusion. J Clin Oncol 29:441-448, 2010
31. 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 28:3516-3524, 2010
32. Garaventa A, Poetschger U, Valteau-Couanet D, et al: Randomized trial of two induction therapy regimens for high-risk neuroblastoma: HR-NBL1.5 International Society of Pediatric Oncology European Neuroblastoma Group study. J Clin Oncol 39:2552-2563, 2021
33. Ambros PF, Ambros IM, Brodeur GM, et al: International consensus for neuroblastoma molecular diagnostics: Report from the International Neuroblastoma Risk Group (INRG) Biology Committee. Br J Cancer 100:1471-1482, 2009
34. Ambros IM, Brunner B, Aigner G, et al: A multilocus technique for risk evaluation of patients with neuroblastoma. Clin Cancer Res 17:792-804, 2011
35. Schleiermacher G, Michon J, Ribeiro A, et al: Segmental chromosomal alterations lead to a higher risk of relapse in infants with MYCN-non-amplified localised unresectable/disseminated neuroblastoma (a SIOPEN collaborative study). Br J Cancer 105:1940-1948, 2011
36. Schleiermacher G, Mosseri V, London WB, et al: Segmental chromosomal alterations have prognostic impact in neuroblastoma: A report from the INRG project. Br J Cancer 107:1418-1422, 2012
37. Ambros IM, Brunner C, Abbasi R, et al: Ultra-high density SNParray in neuroblastoma molecular diagnostics. Front Oncol 4:202, 2014
38. UCSC Genome Browser Home: https://genome.ucsc.edu/index.html
39. Kaplan E, Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53:457-481, 1958
40. Peto R, Pike MC, Armitage P, et al: Design and analysis of randomized clinical trials requiring prolonged observation of each patient. II. Analysis and examples. Br J Cancer 35:1-39, 1977
41. Andersen PK, Perme MP: Pseudo-observations in survival analysis. Stat Methods Med Res 19:71-99, 2011
42. Morgenstern DA, Potschger U, Moreno L, et al: Risk stratification of high-risk metastatic neuroblastoma: A report from the HR-NBL-1/SIOPEN study. Pediatr Blood Cancer 65:e27363, 2018
43. Depuydt P, Boeva V, Hocking TD, et al: Genomic amplifications and distal 6q loss: Novel markers for poor survival in high-risk neuroblastoma patients. J Natl Cancer Inst 110:1084-1093, 2018
44. Mosse YP, Lim MS, Voss SD, et al: Safety and activity of crizotinib for paediatric patients with refractory solid tumours or anaplastic large-cell lymphoma: A Children's Oncology Group phase 1 consortium study. Lancet Oncol 14:472-480, 2013
45. Guan J, Fransson S, Siaw JT, et al: Clinical response of the novel activating ALK-I1171T mutation in neuroblastoma to the ALK inhibitor ceritinib. Cold Spring Harb Mol Case Stud 4:a002550, 2018
46. Krytska K, Ryles HT, Sano R, et al: Crizotinib synergizes with chemotherapy in preclinical models of neuroblastoma. Clin Cancer Res 22:948-960, 2016
47. Bogen D, Brunner C, Walder D, et al: The genetic tumor background is an important determinant for heterogeneous MYCN-amplified neuroblastoma. Int J Cancer 139:153-163, 2016
48. Marrano P, Irwin MS, Thorner PS: Heterogeneity of MYCN amplification in neuroblastoma at diagnosis, treatment, relapse, and metastasis. Genes Chromosomes Cancer 56:28-41, 2017
49. Berbegall AP, Bogen D, Potschger U, et al: Heterogeneous MYCN amplification in neuroblastoma: A SIOP Europe Neuroblastoma study. Br J Cancer 118: 1502-1512, 2018
50. Turajlic S, Sottoriva A, Graham T, et al: Resolving genetic heterogeneity in cancer. Nat Rev Genet 20:404-416, 2019
51. Williams JB, Li S, Higgs EF, et al: Tumor heterogeneity and clonal cooperation influence the immune selection of IFN-gamma-signaling mutant cancer cells. Nat Commun 11:602, 2020
52. Padovan-Merhar OM, Raman P, Ostrovnaya I, et al: Enrichment of targetable mutations in the relapsed neuroblastoma genome. PLoS Genet 12:e1006501, 2017
53. Schleiermacher G, Javanmardi N, Bernard V, et al: Emergence of new ALK mutations at relapse of neuroblastoma. J Clin Oncol 32:2727-2734, 2014
54. Chicard M, Colmet-Daage L, Clement N, et al: Whole-exome sequencing of cell-free DNA reveals temporo-spatial heterogeneity and identifies treatment-resistant clones in neuroblastoma. Clin Cancer Res 24:939-949, 2018
55. Ackermann S, Cartolano M, Hero B, et al: A mechanistic classification of clinical phenotypes in neuroblastoma. Science 362:1165-1170, 2018
56. Koneru B, Lopez G, Farooqi A, et al: Telomere maintenance mechanisms define clinical outcome in high-risk neuroblastoma. Cancer Res 80:2663-2675, 2020

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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

The following represents disclosure information provided by authors of this manuscript. All relationships are considered compensated unless otherwise noted. Relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information about ASCO's conflict of interest policy, please refer to www.asco.org/rwc or ascopubs.org/jco/authors/author-center.

Open Payments is a public database containing information reported by companies about payments made to US-licensed physicians (Open Payments).

Walentyna Balwierz
Honoraria: Shire, Gilead Sciences, Novartis, Amgen
Consulting or Advisory Role: Amgen, Novartis, Roche, Takeda
Travel, Accommodations, Expenses: Jazz Pharmaceuticals, Shire, Roche, Servier

Martin Elliott
Consulting or Advisory Role: Bayer

Dominique Valteau-Couanet
Consulting or Advisory Role: EUSA Pharma
Research Funding: Orphelia Pharma
Patents, Royalties, Other Intellectual Property: Royalties from Apeiron to SIOPEN
Travel, Accommodations, Expenses: EUSA Pharma, Jazz Pharmaceuticals

Deborah A. Tweddle
Honoraria: Eusa Pharma
Travel, Accommodations, Expenses: EUSA Pharma

Ruth Ladenstein
Honoraria: Apeiron Biologics, Boehringer Ingelheim, EUSA Pharma
Consulting or Advisory Role: Apeiron Biologics, Boehringer Ingelheim, EUSA Pharma
Research Funding: Apeiron Biologics, EUSA Pharma
Patents, Royalties, Other Intellectual Property: Apeiron Biologics, EUSA Pharma
Expert Testimony: Apeiron Biologics, EUSA Pharma
Travel, Accommodations, Expenses: Apeiron Biologics, EUSA Pharma

Gudrun Schleiermacher
Honoraria: BMS
Research Funding: Bristol Myers Squibb, Pfizer, MSDavenir, Roche
Travel, Accommodations, Expenses: Roche

No other potential conflicts of interest were reported.

APPENDIX

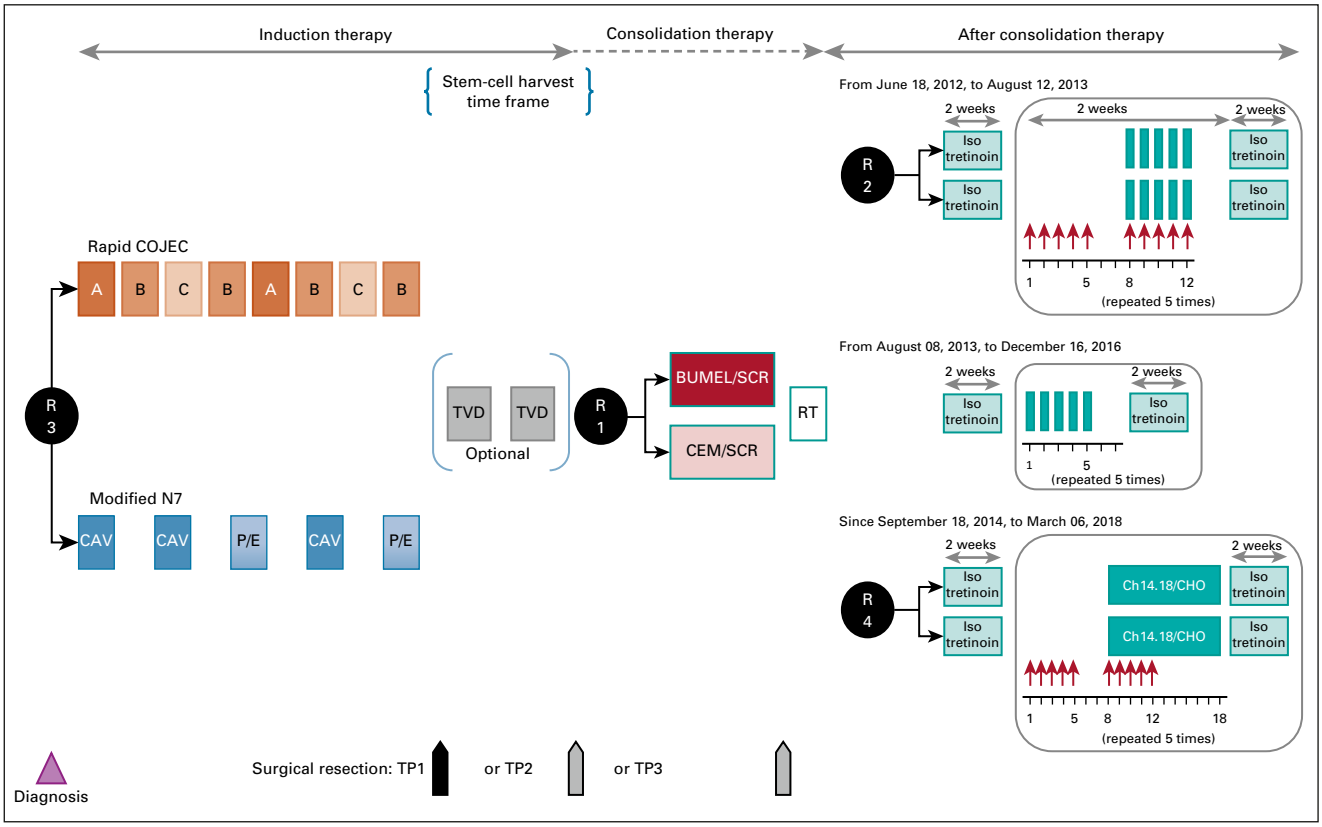


FIG A1. Treatment flowchart of the HR-NBL1 Protocol (ClinicalTrials.gov: NCT01704716, EudraCT: 2006-001489-17) over the whole period. [a]Infants and children with a body weight below 12 kg will be dosed at 0.67 mg/kg/d. In infants weighing ≤ 5 kg, a further 1/3 dose reduction is advised. AUC, area under 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)

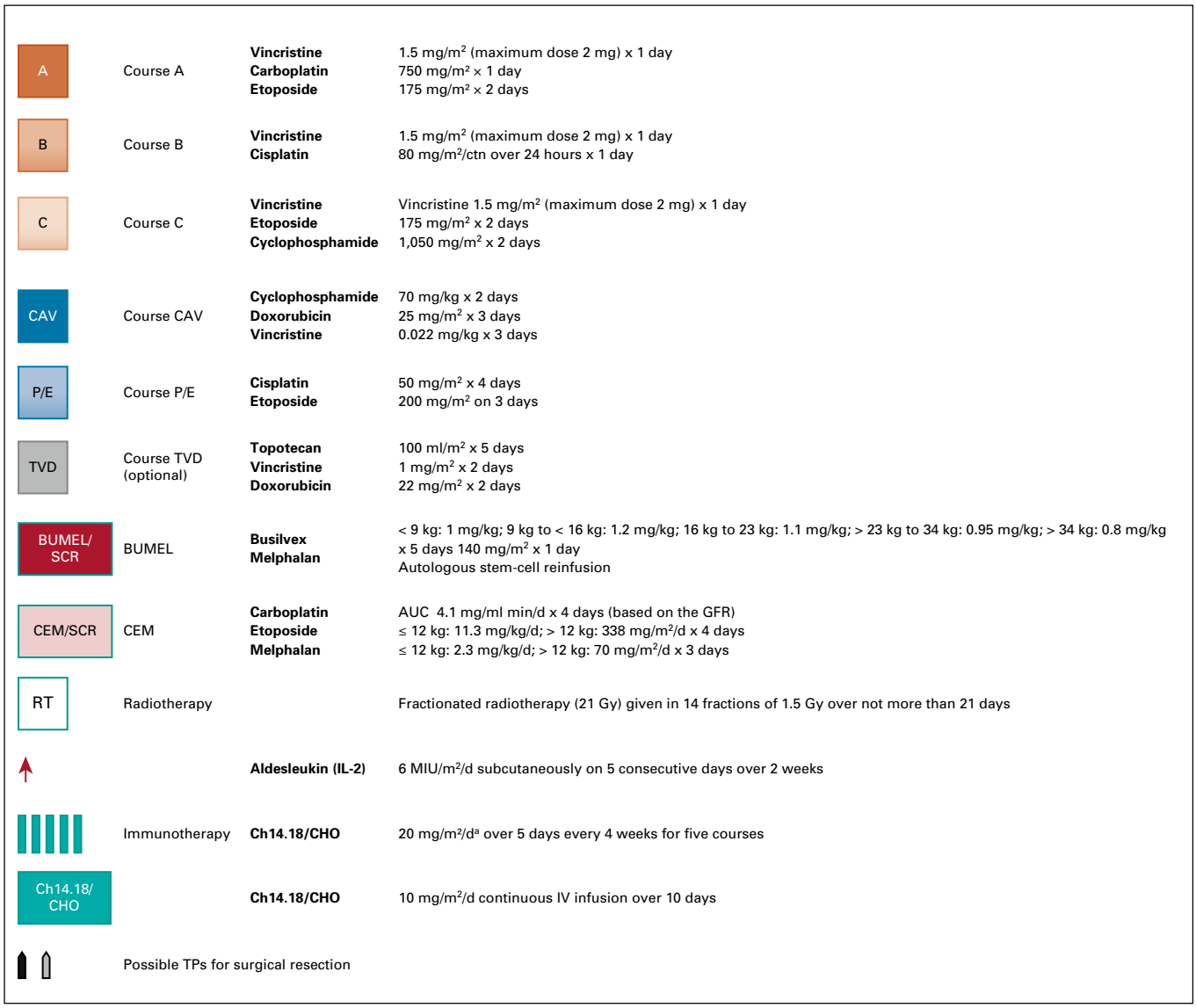
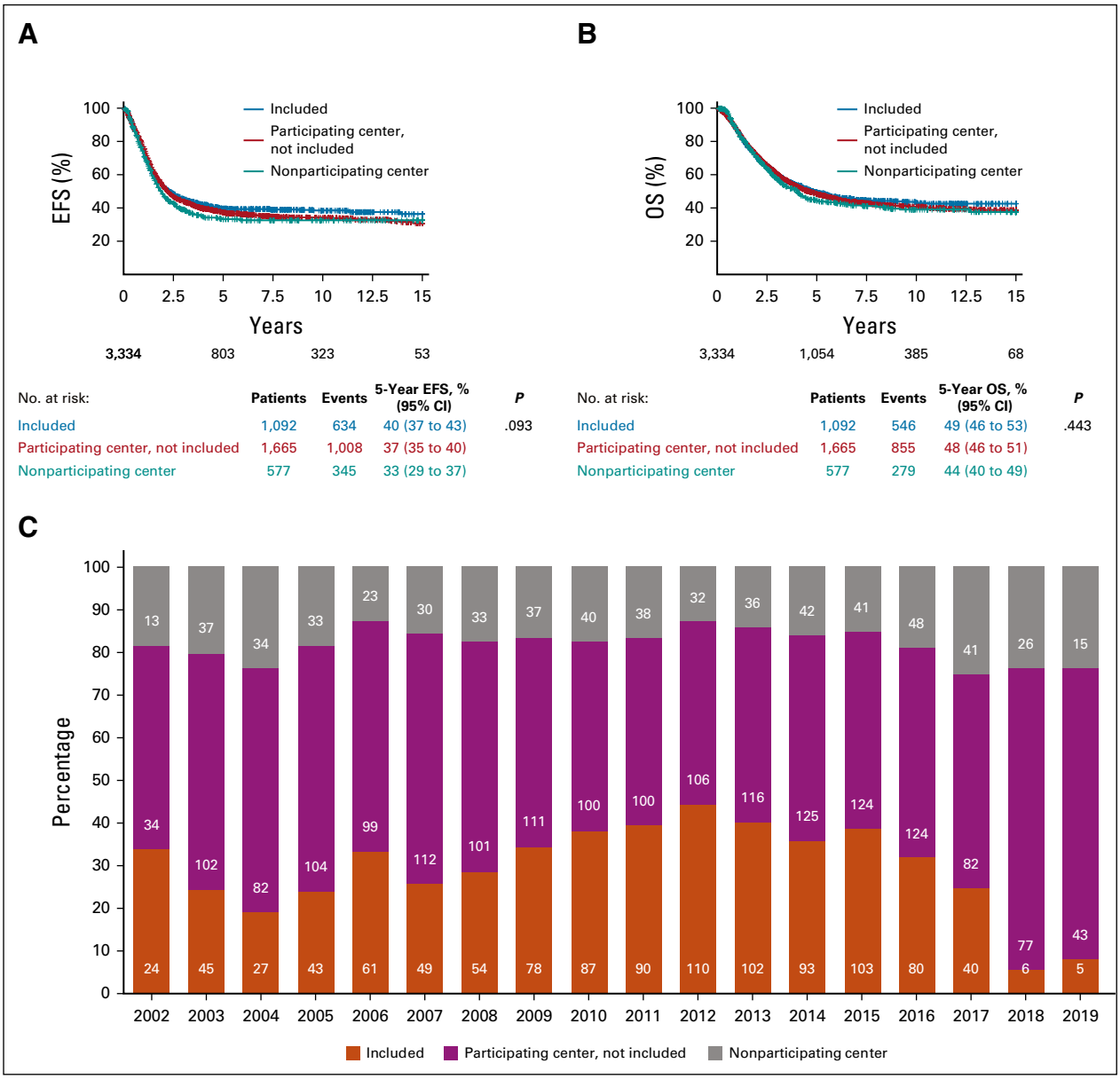


FIG A1. (Continued).



No. at risk:	Patients	Events	5-Year EFS, % (95% CI)	P
Included	1,092	634	40 (37 to 43)	.093
Participating center, not included	1,665	1,008	37 (35 to 40)	
Nonparticipating center	577	345	33 (29 to 37)	

No. at risk:	Patients	Events	5-Year OS, % (95% CI)	P
Included	1,092	546	49 (46 to 53)	.443
Participating center, not included	1,665	855	48 (46 to 51)	
Nonparticipating center	577	279	44 (40 to 49)	

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.

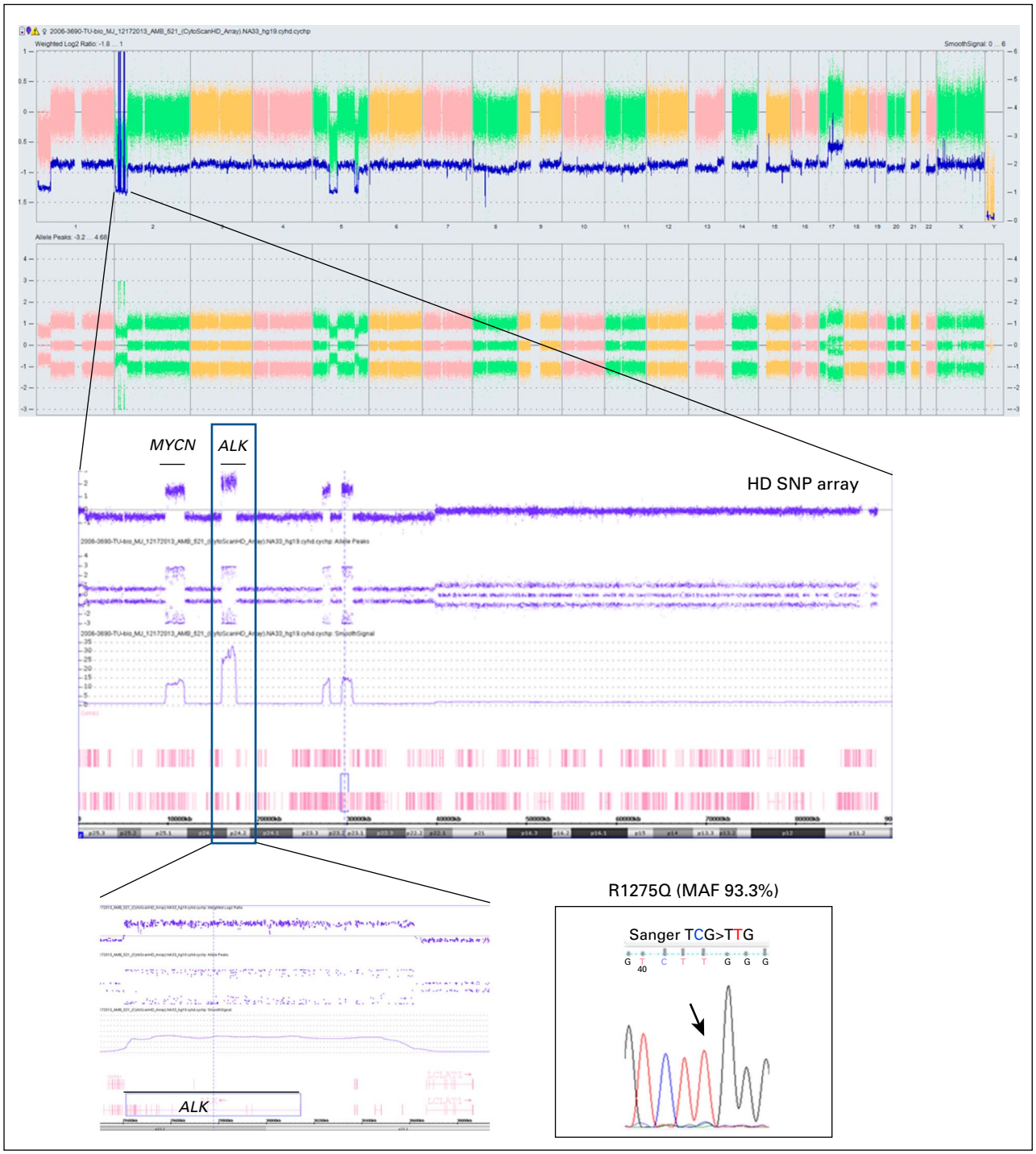


FIG A3. Double event of *ALK* amplification and *ALK* mutation detected in one case (case 15). The SNP array shows an amplified region in chromosome 2 encompassing the *ALK* gene. Sanger sequencing profile shows R1275Q mutation (MAF = 93.3%) in the same case. HD, high definition; MAF, mutated allele fraction; SNP, single-nucleotide polymorphism.

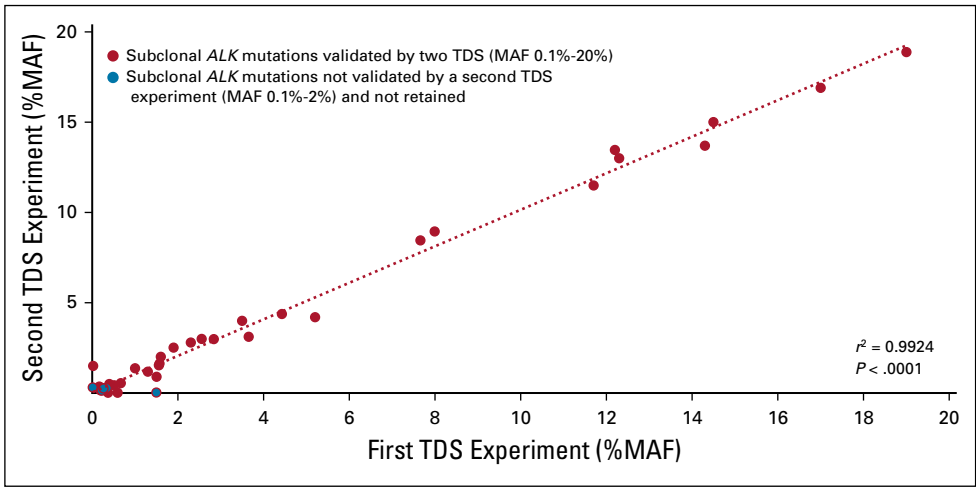


FIG A4. MAF of subclonal *ALK* mutations detected by TDS and confirmed by a second independent TDS experiment. Red spots representing the MAF for each *ALK* mutation are plotted on the *x*-axis (first TDS experiment) and *y*-axis (second TDS experiment), with a strong correlation between the two independent 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.

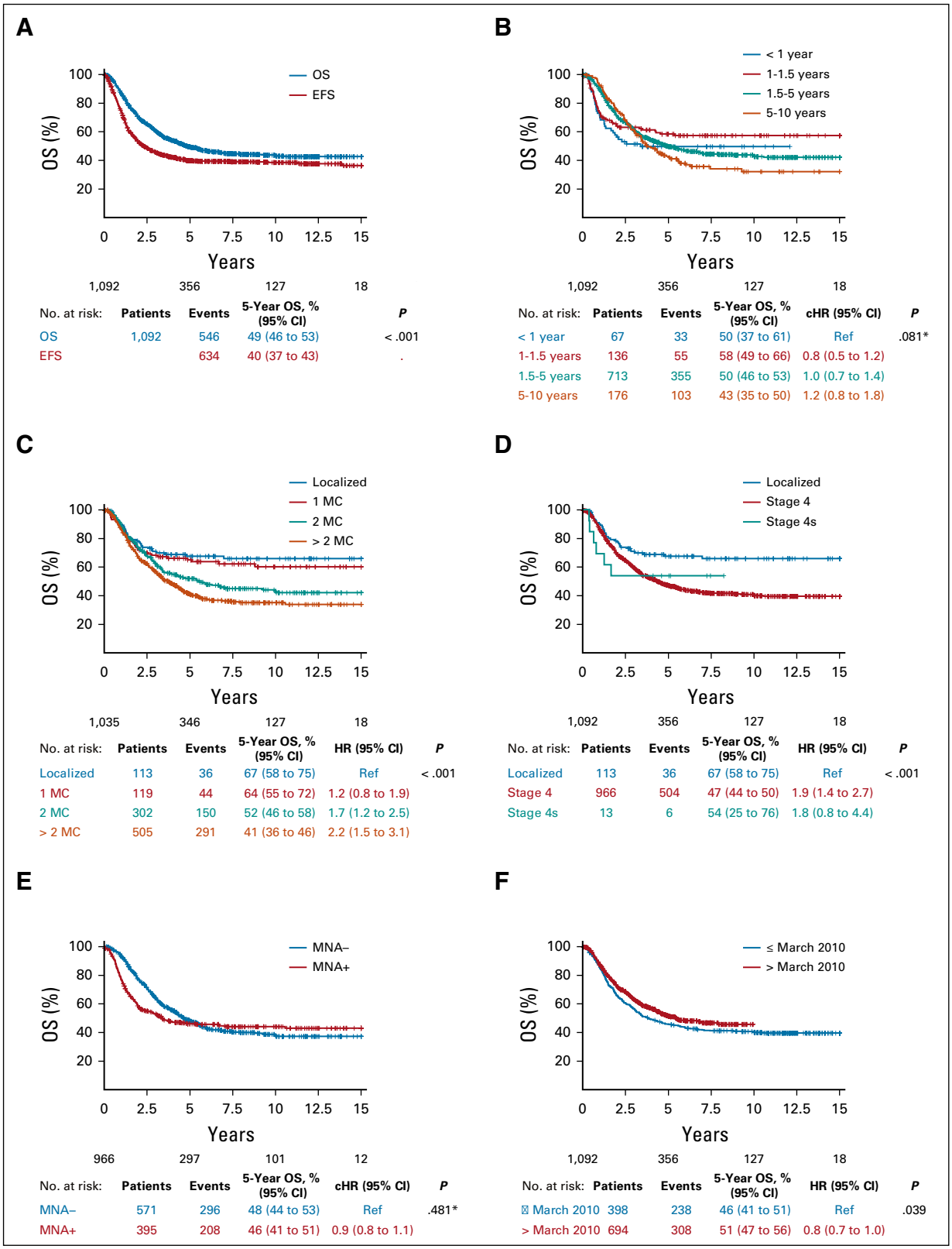


FIG A5. Survival in the *ALK* analysis cohort (n = 1,092 patients) according to known prognostic factors. (A) EFS and OS in the *ALK* analysis cohort population (n = 1,092 patients). Five-year EFS (blue line) 40% (95% CI, 37 to 43); 5-year OS (red line) 49% (95% CI, 46 to 53). (B) OS according to age. 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, 58 to 75), 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 (< 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); $P = .039$.[3-5] 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

		Stage 4				
	Localized MNA+	Total	MNA−	MNA+	Stage 4s MNA+	Total
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)	314 (82)	13 (100)	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. (%)						
*ALK*m clonal	9 (8)	66 (7)	26 (5)	40 (10)	1 (8)	76 (7)
*ALK*m 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.

TABLE A2. Number of Patients Included in the *ALK* Analysis Cohort by Country and Center

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 Americain 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)

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

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

(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	38
IT	All	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 Giannina 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
	Inselspital Bern	3
	Kantonspital Aarau	3
	Ostschweizer Kinderspital	2
	Luzerner Kantonspital - 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.

TABLE A3. Clinical Characteristics of 35 Patients Treated by Immunotherapy Whose Tumors Harbored *ALK* Genetic Alterations

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	4	Rapid COJEC	PR	BUMEL	Yes	Dead	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	Yes	Dead	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	4	MOD. N7	PR	BUMEL	Yes	Dead	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	4	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	Dead	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	No	Alive	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*-A	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)

TABLE A3. Clinical Characteristics of 35 Patients Treated by Immunotherapy Whose Tumors Harbored *ALK* Genetic Alterations (continued)

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.