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Clinical Trial

Afatinib in paediatric patients with recurrent/refractory ErbB-dysregulated tumours: Results of a phase I/expansion trial☆

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Abstract ***Aim:*** This phase I/expansion study assessed the safety, pharmacokinetics and preliminary antitumor activity of afatinib in paediatric patients with cancer.
Methods: The dose-finding part enroled patients (2– < 18 years) with recurrent/refractory tumours. Patients received 18 or 23 mg/m^2/d afatinib orally (tablet or solution) in 28-d cycles. In the maximum tolerated dose (MTD) expansion, eligible patients (1– < 18 years) had tumours fulfilling ≥2 of the following criteria in the pre-screening: *EGFR* amplification; *HER2* amplification; EGFR membrane staining (H-score > 150); HER2 membrane staining (H-score > 0). The primary end-points were dose-limiting toxicities (DLTs), afatinib exposure, and objective response.
Results: Of 564 patients pre-screened, 536 patients had biomarker data and 63 (12%) fulfilled ≥2 EGFR/HER2 criteria required for inclusion in the expansion part. A total of 56 patients were treated (17 in the dose-finding and 39 in the expansion part). DLTs were observed in one of six MTD-evaluable patients receiving 18 mg/m²/d and in two of five MTD-evaluable patients receiving 23 mg/m²/d; 18 mg/m²/d was defined as the MTD. There were no new safety signals. Pharmacokinetics confirmed exposure consistent with the approved dose in adults. One partial response (−81% per Response Assessment in Neuro-Oncology) was observed in a patient with a glioneuronal tumour harbouring a *CLIP2::EGFR* fusion; unconfirmed partial responses were observed in two patients. In total, 25% of patients experienced objective response or stable disease (95% confidence interval: 14–38).
Conclusion: Targetable *EGFR*/*HER2* drivers are rare in paediatric cancers. Treatment with afatinib led to a durable response (> 3 years) in one patient with a glioneuronal tumour with *CLIP2::EGFR* fusion.

1. Introduction

ErbB receptors have been extensively investigated as drug targets in adult cancers [1,2], but less so in paediatric indications. ErbB pathway dysregulation (e.g. ErbbB amplification or overexpression) has been described in multiple paediatric cancers [3–7], and treatment outcomes of recurrent diseases remain poor [8,9]. Studies of ErbB TKIs in paediatric patients have shown limited efficacy [10–15], potentially due to compensatory oncogenic signalling *via* non-target ErbB receptors [16]. Afatinib, an irreversible ErbB family inhibitor highly penetrant of the blood-brain barrier, blocks transphosphorylation of all ErbB dimers including those containing ErbB3 [17,18], and may therefore be effective against tumours driven by aberrant ErbB signalling, including central nervous system (CNS) tumours [19].

Validated predictive biomarkers for EGFR TKIs are rare in paediatric malignancies and have not generally been utilised in previous studies to select patients [10–14]. Our systematic biomarker analysis of 297 paediatric patients with ependymoma, high-grade glioma (HGG), medulloblastoma, recurrent/refractory low-grade astrocytoma, diffuse intrinsic pontine glioma (DIPG), neuroblastoma and rhabdomyosarcoma identified high levels of heterogeneity in terms of ErbB dysregulation [20]; 20–30% of tumour samples (except DIPG [4%] and low-grade astrocytoma [0%]) demonstrated ErbB dysregulation, defined as ≥2 of the following molecular markers based on Clinical Laboratory Improvement Amendments (CLIA)-approved assays validated in adult indications: *EGFR* amplification, *HER2* amplification, EGFR overexpression, or HER2 overexpression.

Here, we report results from the phase I/expansion study of afatinib in paediatric patients with tumours harbouring ErbB dysregulation. We presumed that simultaneous upregulation of at least two EGFR or HER2 markers might indicate ErbB pathway activation. The activity of afatinib was therefore evaluated in patients with tumours of a type likely to exhibit EGFR/HER2 amplification or overexpression, in patients selected according to ErbB pathway dysregulation as previously defined [20], or in patients with other ErbB aberrations (including mutations or gene fusions) likely to be oncogenic.

2. Materials and methods

2.1. Patients

This open label, multicentre trial (Clinicaltrials.gov identifier: NCT02372006) enroled patients with recurrent or refractory cancers for whom no curative treatment was available. For the dose-finding part (Part 1), eligible patients were aged 2– < 18 years at time of consent with a histological diagnosis of HGG, DIPG, low-grade

astrocytoma, medulloblastoma, primitive neuroectodermal tumour, ependymoma, neuroblastoma, rhabdomyosarcoma, or other solid or CNS tumours previously reported to frequently harbour ErbB aberrations. For the maximum tolerated dose (MTD) dose-expansion part (Part 2), eligible patients were aged 1– < 18 years, had measurable disease that fulfilled at least two of the following criteria determined from archived material in a prescreening procedure, as previously defined [20]: (i) EGFR or (ii) HER2 protein expression, indicated by membrane staining with immunohistochemistry (IHC) (H-score > 150 or > 0, respectively); (iii) *EGFR* gene amplification detected by fluorescence *in situ* hybridisation (FISH) (either *EGFR*/*Cen7* ≥2.0, or ≥10% of cells with ≥15 copies, or ≥40% of cells with ≥4 copies, or gene cluster in ≥10% of cells); and/or (iv) *HER2* gene amplification detected by dual-colour, dual-hapten, brightfield *in situ* hybridisation (DDISH) (*Her2*/*CEP17* ≥2.0). Patients with other confirmed ErbB alterations or ErbB alterations likely to be oncogenic were enroled into an exploratory cohort.

All patients were required to have recovered from toxicity from prior anticancer treatment, Karnofsky/Lansky performance status ≥50 (for patients > 12/≤12 years, respectively), and neurological stability ≥7 d for patients with CNS tumours. Key exclusion criteria included as follows: radiotherapy, chemotherapy, or surgery within 2, 3 or 4 weeks, respectively, prior to the start of study; and inadequate organ function (see Supplementary Methods for all criteria).

2.2. Study design

In Part 1, patients received escalating doses of afatinib (oral, as tablet or solution including *via* feeding tube), starting with 80% of the adult recommended dose per m^2 body surface area (BSA) using allometric scaling (18 mg/m^2/d); dose escalations to 100%, 125% and 150% of the adult dose were planned *via* a rolling six design [21]. Patients who missed > 25% of their doses during Cycle 1 for reasons other than toxicity were excluded from MTD analysis. In Part 2, patients (with markers for EGFR and/or HER2 dysregulation) were recruited into expansion cohorts according to histology (DIPG, HGG, ependymoma and other solid tumours, including extracranial and CNS tumours) and received the MTD (Supplementary Fig. A1). Patients continued treatment until disease progression, undue toxicities, or discontinuation for any other reason.

The trial was performed in accordance with the principles of the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice (ICH GCP) guidelines, and was approved by ethics committees and medical authorities as per local regulatory requirements. For all patients, parent(s)/legal guardian(s)' written informed consent and age-appropriate patient assent were provided.

2.3. End-points

The primary safety end-point for Part 1 was the occurrence of dose-limiting toxicities (DLTs), defined as any of the following events considered related to afatinib: Grade (G)4 haematologic toxicity lasting for ≥7 d; G3–4 non-haematologic toxicity (except G3 fever); G ≥2 worsening of renal function, G2 nausea and/or vomiting or diarrhoea lasting for ≥7 d despite supportive treatment; G5 events. The MTD was determined as the highest dose at which no more than one in six patients in the dose-finding part (Part 1) experienced a DLT, assessed during the first treatment cycle.

Pharmacokinetic end-points included the afatinib area under the plasma concentration-time curve over the dosing interval τ at steady state ($AUC_{\tau,ss}$), the maximum measured plasma afatinib concentration at steady state ($C_{max,ss}$), AUC following the first dose (AUC_{0-24}), the maximum measured plasma concentration (C_{max}), time from (last) dosing to the maximum concentration at steady state ($t_{max[,ss]}$), and accumulation (or effective) half-life ($t_{1/2,effective}$).

For Part 2, the primary efficacy end-point was objective response (OR) by investigator assessment. Other efficacy end-points included progression-free survival (PFS; from first treatment until progression or death), duration of OR (from first documented response until disease progression or death) and overall survival (OS; from first treatment until death).

2.4. Assessments

Biomarker assays were performed centrally by TARGOS Molecular Pathology GmbH using tissue samples from archived tumour blocks, or tissue sections [20].

Adverse events (AEs) were assessed according to National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) v3.0. Hepatic injury and DLTs were considered AEs of special interest.

Tumour response was assessed every 8 weeks until progression of disease, according to the given tumour type, using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [22,23], International Neuroblastoma Response Criteria (INRC) [24], Response Assessment in Neuro-Oncology (RANO) [25], or World Health Organization (WHO) criteria [26]. For anti-tumour activity, patient data from the dose-finding part and the MTD-expansion cohorts were pooled.

Plasma pharmacokinetic sampling was performed on Days 1 and 8 of Cycle 1 (see Supplementary Methods).

2.5. Statistical analyses and sample size

Approximately 30 patients were planned to be recruited for Part 1 (Supplementary Fig. A1). Once the MTD was

Table 1
Patient characteristics.

	Dose-finding level 1	Dose-finding level 2	MTD expansion	Total
Number of patients, N	8	9	39	56
Gender, n (%)				
Male	4 (50)	5 (56)	23 (59)	32 (57)
Female	4 (50)	4 (44)	16 (41)	24 (43)
Median age, years (range)[a]	9.5 (4.0–17.0)	14.0 (2.0–16.0)	11.0 (3.0–18.0)	11.5(2.0–18.0)
Tumour histology, n (%)				
High-grade glioma	3 (38)	4 (44)	6 (15)	13 (23)
Diffuse intrinsic pontine glioma	0	0	4 (10)	4 (7)
Low-grade astrocytoma	0	0	1 (3)	1 (2)
Ependymoma	1 (13)	1 (11)	8 (21)	10 (18)
Medulloblastoma	1 (13)	0	0	1 (2)
Primitive neuroectodermal tumour	2 (25)	1 (11)	1 (3)	4 (7)
Neuroblastoma	0	1 (11)	3 (8)	4 (7)
Rhabdomyosarcoma	1 (13)	2 (22)	4 (10)	7 (13)
Other[b]	0	0	12 (31)	12 (21)
Median time from first histological diagnosis, years (range)	1.8 (0.6–4.0)	2.0 (0.5–6.1)	1.8 (0.2–10.7)	1.8 (0.2–10.7)
Baseline Karnofsky or Lansky score category				
100–80	6 (75)	7 (78)	32 (82)	45 (80)
70–50	2 (25)	2 (22)	7 (18)	11 (20)
Prior anticancer treatment, n (%)				
Chemotherapy	8 (100)	9 (100)	28 (72)	45 (80)
Radiotherapy	8 (100)	8 (89)	32 (82)	48 (86)
Surgery	7 (88)	8 (89)	31 (79)	46 (82)
Immunotherapy[c]	0	1 (11)	7 (18)	8 (14)
Other targeted therapy[d]	0	0	12 (31)	12 (21)
Number of lines of chemotherapy, n (%)				
0	0	0	11 (28)	11 (20)
1	3 (38)	2 (22)	6 (15)	11 (20)
≥2	5 (63)	7 (78)	22 (56)	34 (61)
Number of lines of radiotherapy, n (%)				
0	0	1 (11)	7 (18)	8 (14)
1	3 (38)	3 (33)	20 (51)	26 (46)
≥2	5 (63)	5 (56)	12 (31)	22 (39)

MTD, maximum tolerated dose.
[a] Patients who reached 18 years after signing consent for pre-screening but before treatment start were permitted in this paediatric trial.
[b] This group included patients with tumours of the following histology: adenoid cystic carcinoma, fibrolamellar hepatic carcinoma, osteosarcoma, nasopharyngeal carcinoma, adrenocortical carcinoma, choroid plexus carcinoma, undifferentiated high-grade intracranial tumour, atypical teratoid rhabdoid tumour, glioneuronal tumour, neuroectodermal neoplasm (n = 1 each) and hepatocellular carcinoma (n = 2).
[c] Immunotherapies include as follows: monoclonal antibodies Ch.14.18; (n = 3); anti-GD2 antibodies + interleukin 2 (n = 2); nivolumab (n = 3).
[d] Other targeted therapies include as follows: nimotuzumab (n = 3); lapatinib with bevacizumab; imetelstat; tazemetostat; retinoic acid (n = 1 each); bevacizumab monotherapy (n = 3; the patient who received tretinoin also received bevacizumab in a later line); sorafenib (n = 3).

determined, at least five patients with confirmed ErbB dysregulation were to be accrued into each expansion cohort. A total of 20 patients was originally planned for the MTD-expansion cohort. However, following a written request from the FDA, trial recruitment was changed to instead include ≥5 patients in each cohort according to histology (DIPG, HGG, ependymoma and other solid tumours), in order to confirm a safe RP2D and to assess tumour response. An exploratory cohort of patients with proven genomic, transcriptomic or proteomic alterations which were not defined in the biomarker prevalence study was also included. Assuming a response rate of 20% or 30%, the estimated probability of observing at least one response per cohort would be 67% or 83%, respectively. Assuming a biomarker prevalence of 10–15%, 322–485 patients were to be screened to recruit 38 patients into the expansion cohorts. All analyses were descriptive and exploratory.

2.6. Preclinical evaluations

Animal experiments were executed in compliance with appropriate institutional guidelines and regulations and after approval from the Regierungspräsidium Freiburg (protocol approval number Az. 35-9185.81/G-20/163).

To assess the impact of the newly-discovered *CLIP2::EGFR* fusion on EGFR activation and inhibitory action of afatinib, preclinical assessments were undertaken in addition to the clinical evaluations within the trial. *CLIP2::EGFR* (based on the patient DNA sequence) and *CLIP2::EGFR D837R* (negative control) constructs were generated on a pMSCV-PGK-Puro-IRES-GFP backbone. Transduced NIH-3T3 cells were cultured for *in vitro* EGFR activation assays and 3D proliferation assays, and were implanted into 9- to 11-week-old female nude mice (NMRI nu/nu mice; CRL:NMRI-*Foxn1*$_{nu}$, Charles River Laboratories) for

Table 2
Summary of biomarker characteristics of patients in the treated set of the MTD-expansion cohort.

	HGG (n = 6)	DIPG (n = 4)	EP (n = 8)	Other[a] (n = 19)	Exploratory (n = 2)	Total (N = 39)
EGFR FISH, n (%)						
Positive	5 (83)	4 (100)	2 (25)	11 (58)	0	22 (56)
Negative	1 (17)	0	6 (75)	8 (42)	2 (100)	17 (44)
HER2 DDISH, n (%)						
Positive	2 (33)	2 (50)	1 (13)	4 (21)	0	9 (23)
Negative	4 (67)	2 (50)	7 (88)	13 (68)	2 (100)	28 (72)
Missing	0	0	0	2 (11)	0	2 (5.1)
EGFR IHC, n (%)						
H-score > 150	4 (67)	2 (50)	8 (100)	14 (74)	0	28 (72)
H-score ≤ 150	2 (33)	2 (50)	0	5 (26)	2 (100)	11 (28)
HER2 IHC, n (%)						
H-score > 0	1 (17)	0	8 (100)	12 (63)	1 (50)	22 (56)
H-score = 0	5 (83)	4 (100)	0	7 (37)	1 (50)	17 (44)
***CLIP2::EGFR* fusion, n (%)**						
Positive	0	0	0	0	1 (50)	1 (3)

CLIP2, CAP-Gly domain containing linker protein 2; DDISH, dual-colour, dual-hapten, brightfield *in situ* hybridisation; DIPG, diffuse intrinsic pontine glioma; EGFR, epidermal growth factor receptor; EP, ependymoma; FISH, fluorescence *in situ* hybridisation; HER2, human epidermal growth factor receptor 2; HGG, high-grade glioma; IHC, immunohistochemistry; MTD, maximum tolerated dose.
[a] HER2 DDISH data missing for two patients.

in vivo xenograft assays (see Supplementary Materials for detailed methods).

3. Results

3.1. Patients

Between May 2015 and August 2020, 564 patients were pre-screened across 28 sites in 11 countries, including 519 patients pre-screened for inclusion in the MTD-expansion cohorts. Of these, 381 were ineligible based on negative biomarker assessment, 107 patients did not meet other inclusion/exclusion criteria and 20 were not treated for other reasons (Supplementary Fig. A2). Of 536 patients with biomarker data, 63 (11.8%) were positive for ≥2 biomarkers (Supplementary Table A1). Fifty-six patients received afatinib (median age of 11.5 years [range 2–18], 57% male; Table 1): 17 patients in the dose-finding part (eight and nine patients received 18 mg/m^2/d and 23 mg/m^2/d, respectively) and 39 in the MTD-expansion cohorts. Afatinib was administered orally as tablet or solution or using a feeding tube in 22 (39%), 32 (55%) and four (7%) patients, respectively (one patient switched from liquid solution to tablet, and one from solution to feeding tube). In the expansion cohorts, 28 (72%) patients had EGFR H-scores > 150, 22 (56%) patients had positive *EGFR* FISH, 22 (56%) patients had HER2 IHC scores > 0, and nine (23%) patients had positive *HER2* DDISH (Table 2, Fig. 1A); 79% of patients had two biomarkers and 15% had three (Fig. 1B).

The median duration of treatment with afatinib until cutoff was 53 d (range 2–337). Reasons for treatment discontinuation were progressive disease (n = 49, 88%), AEs other than DLT (n = 3, 5%), refused trial medication (n = 2, 4%), and DLT, (n = 1, 2%). One patient remained on study treatment for 255 d without progression before trial close (5th August 2020) and was transferred to a compassionate use program.

3.2. Safety

In Part 1, 11 of 17 patients were evaluable for MTD determination (five patients received < 80% of the 28 doses in Cycle 1 and were replaced; one patient was treated [18 mg/m^2/d] after the MTD had been exceeded). At the starting dose, one of six evaluable patients experienced DLT (G3 diarrhoea) during treatment Cycle 1. At 23 mg/m^2/d, two of five evaluable patients experienced DLT (G3 decreased appetite considered serious due to hospitalisation and associated with moderate diarrhoea); G4 hypernatremia, dehydration, diarrhoea, G3 decreased appetite, hypokalemia, cheilitis, rash). Afatinib once daily at 80% of the recommended adult dose per m^2 BSA using allometric scaling (18 mg/m^2/d) was therefore identified as the MTD for paediatric patients. Out of 56 patients overall, 6 patients (11%) experienced DLT events during the first treatment cycle, and 11 patients (20%) experienced DLT at any time during the trial.

Overall, 56 patients received a total of 144 cycles of therapy. Any-grade AEs and G ≥3 AEs were experienced by 56 (100%) and 35 (63%) patients, respectively. Treatment-related AEs were experienced by 52 (93%) patients, most commonly diarrhoea, stomatitis, dry skin, and vomiting (Table 3). G3 and G4 treatment-related AEs were reported in eight (14%) and two (4%) patients, respectively.

Treatment-related AEs leading to dose reduction were experienced by eight (14%) patients. AEs leading to discontinuation of afatinib were experienced by six (11%) patients. AEs of special interest and serious AEs were experienced by 11 (20%; all were DLT events as reported above; hepatic injury was not reported) and

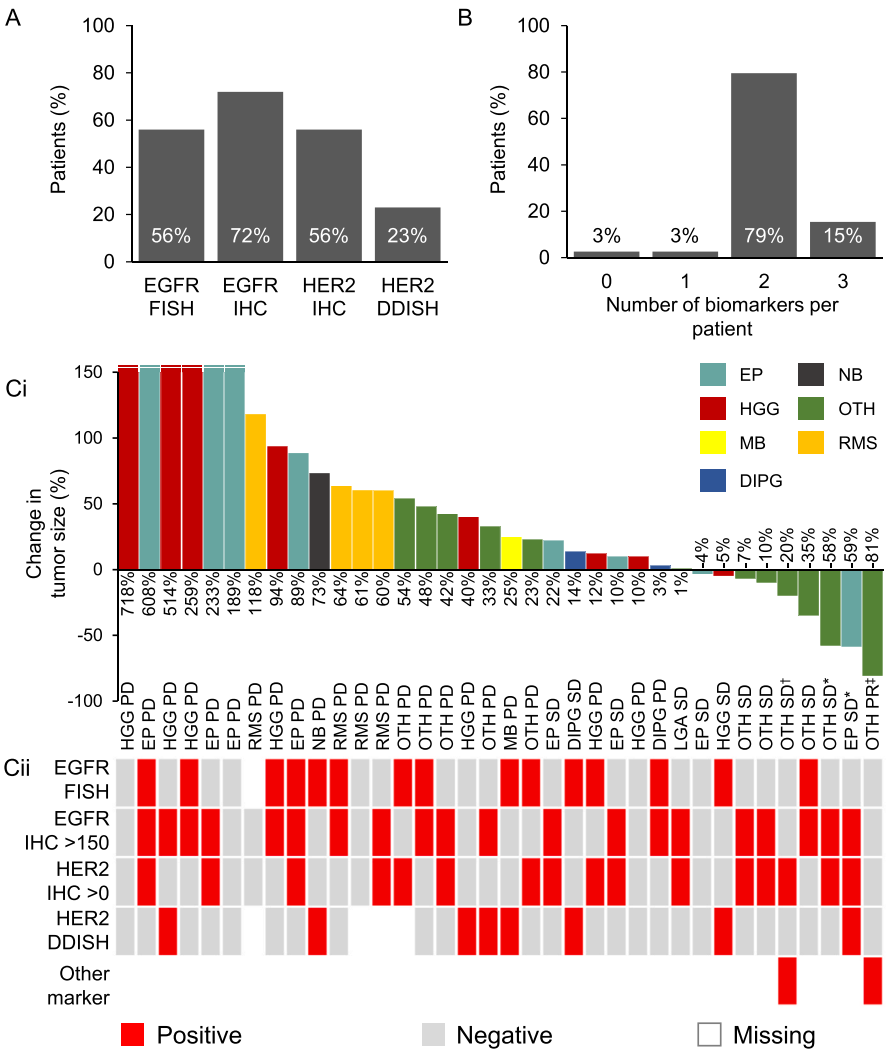

Fig. 1. Biomarker characteristics in patients treated in the expansion cohorts, and best-recorded change in tumour size among patients in the treated set with measurable disease. A. Proportion of patients treated in the expansion cohorts (n = 39) with each biomarker. B. Proportion of patients treated in the expansion cohorts with either 0, 1, 2, or 3 biomarkers present. Ci. Waterfall plot of best-recorded change in tumour size from baseline in patients in the treated set with available best change in tumour size data (n = 36). Colour coding indicates histology. Axis cutoff at 150%; tumour volume changes > 150% are indicated in text on the plot. Cii. Biomarkers present for patients in Ci (MTD-expansion cohorts and dose-finding cohorts). *Patient with partial response (unconfirmed in two patients). †Patient had *ERBB3* V104L mutation in addition to HER2 receptor expression and was thus enroled in the study. ‡Patient was negative for all four selection biomarkers but had a *CLIP2::EGFR* fusion, considered to be the tumour driver, and was thus enroled in the study. The patient also demonstrated an EGFR IHC H-score of 85 (therefore below the threshold of 150). No other *ErbB* mutations were recorded in this patient cohort. DDISH, dual-colour, dual-hapten, brightfield *in situ* hybridisation; DIPG, diffuse intrinsic pontine glioma; EGFR, epidermal growth factor receptor; EP, ependymoma; FISH, fluorescence *in situ* hybridisation; HER2, human epidermal growth factor receptor 2; HGG, high-grade glioma; IHC, immunohistochemistry; LGA, low-grade astrocytoma; NB, neuroblastoma; OTH, other; PD, progressive disease; PR, partial response; RMS, rhabdomyosarcoma, SD, stable disease. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

33 (59%) patients, respectively. Three fatal AEs were reported (hydrocephalus, respiratory distress, respiratory arrest), none considered treatment-related.

3.3. Pharmacokinetics

The pharmacokinetics analysis set included 17 patients from Part 1 (18 mg/m^2/d: n = 8; 23 mg/m^2/d: n = 9) and 39 patients from Part 2. At 18 mg/m^2/d, the geometric mean (gMean) $AUC_{\tau,ss}$ was 758 ng*h/ml (geometric coefficient of variance [gCV] 56.8%); the gMean C_{max} at steady state was 52.6 ng/ml (gCV 57.5%); AUC_{0-24} was 364 ng*h/ml and t_{max}, $t_{max,ss}$ and $t_{1/2,effective}$ were 3.5 h, 4.0 h and 27.2 h, respectively (Supplementary Table A2). Steady state was reached at Day 8 (Supplementary Fig. A3).

3.4. Antitumor activity

Of 56 patients treated, one experienced a partial response (PR) (ORR: 1.8%, 95% confidence interval [CI]:

Table 3
Summary of adverse events and treatment-related adverse events, maximum CTCAE grade.

Patients, n (%)	Any grade	Grade ≥3
Any AE	56 (100)	35 (63)
AEs leading to afatinib dose reduction	8 (14)	5 (9)
AEs leading to discontinuation of afatinib	6 (11)	3 (5)
Protocol-defined AESIs[a]	11 (20)	9 (16)
SAEs	33 (59)	25 (45)
Treatment-related AEs leading to afatinib dose reduction	8 (14)	5 (9)
Treatment-related AEs[b]	52 (93)	10 (18)
Gastrointestinal		
Diarrhoea	41 (73)	3 (5)
Stomatitis	14 (25)	1 (2)
Vomiting	13 (23)	0
Abdominal pain	11 (20)	0
Nausea	9 (16)	0
Mucosal inflammation	7 (13)	0
Constipation	4 (7)	0
Dermatological/skin/eye		
Dry skin	14 (25)	0
Paronychia	11 (20)	2 (4)
Cheilitis	10 (18)	1 (2)
Acneiform dermatitis	10 (18)	0
Rash	5 (9)	1 (2)
Maculo-papular rash	5 (9)	0
Pruritus	4 (7)	0
Dry eye	4 (7)	0
Conjunctivitis	3 (5)	0
Dry lip	3 (5)	0
Xerosis	3 (5)	0
General symptoms		
Decreased appetite	9 (16)	2 (4)
Weight decreased	8 (14)	0
Fatigue	7 (13)	0
Epistaxis	6 (11)	0
Headache	3 (5)	0
Laboratory alterations		
Anaemia	4 (7)	0
Hypokalemia	4 (7)	2 (4)
Hyponatremia	3 (5)	1 (2)
Alanine aminotransferase increased	3 (5)	1 (2)
White blood cell count decreased	3 (5)	0

AE, adverse event; AESI, AE of special interest; CTCAE, National Cancer Institute Common Terminology Criteria for Adverse Events; SAE, serious AE.
[a] All AESIs observed were dose-limiting toxicities; no hepatic toxicity was observed.
[b] Treatment-related AEs listed affected ≥3 patients.

0.05–9.6) and 13 had stable disease as best response (disease control rate: 25.0% (95% CI: 14.4–38.4). Best change in tumour size and biomarker data are presented in Fig. 1Ci and Cii for patients with measurable disease.

One patient in the ErbB-alteration expansion cohort had a confirmed PR (RANO; Fig. 2A and B). This patient, who had a glioneuronal tumour with a *CLIP2::EGFR* gene fusion (Fig. 2C and D), had an 81% reduction from baseline and resolution of all clinical symptoms. As of February 2023, the patient was still benefiting from continuous afatinib treatment for > 3 years (David S. Ziegler, personal communication). One patient with ependymoma (EGFR IHC score > 150; *HER2* DDISH+; HER2 IHC > 0) and one with choroid plexus carcinoma (EGFR IHC score > 150; HER2 IHC > 0) experienced PR at Cycle 2 but progressed at the subsequent assessment (PFS: 110 and 173 d, respectively). Additionally, one patient carrying an *ERBB3* V104L mutation experienced 20% reduction in tumour size. Overall, median PFS was 1.8 months (95% CI: 1.5–1.9) and median OS was 4.6 months (95% CI: 3.8–8.7). Further details of response-evaluable patients are presented in Supplementary Table A3.

3.5. Validation of the oncogenic *CLIP2::EGFR* fusion and its sensitivity to afatinib

During screening, the *EGFR::CLIP2* fusion was considered to have oncogenic potential and the patient was therefore included in the exploratory cohort. As the oncogenic function of the novel *CLIP2::EGFR* gene fusion was unknown, in parallel with the patient's treatment, we constructed a preclinical model based on the patient's gene fusion sequence to explore its oncogenic role. In *CLIP2::EGFR*-expressing NIH/3T3 cells, phosphorylated CLIP2::EGFR (CLIP2::pEGFR) was observed, as measured by antibody staining on two different tyrosines, showing tyrosine kinase activation (Fig. 3A). These data are corroborated by the absence of phosphorylation observed when using the kinase-impeded construct *CLIP2::EGFR D837R*. Parental cells (negative control) did not show a band at the corresponding position. In *CLIP2::EGFR* NIH/3T3 cells, incubation with afatinib dose-dependently reduced CLIP2::pEGFR immunoreactivity (Fig. 3B) and showed long-lasting inhibition. Afatinib inhibited cell proliferation in *CLIP2::EGFR*-expressing 3D cultures (half maximal inhibitory concentration [IC_{50}]: 0.81 nmol/l) but had no effect on the proliferation of NIH/3T3 parental cells (Fig. 3Ci and Cii). Tumour growth was faster in mice implanted with NIH/3T3 *CLIP2::EGFR* cells (n = 26) than NIH/3T3 parental cells (n = 10; Fig. 3D), even when grown under permissive conditions for the parental cells [27]. Tumour volumes were significantly larger at Day 21 for mice transplanted with *CLIP2::EGFR* cells versus parental ($P < 0.0001$, Welch's t-test; Fig. 3D inset). When NIH/3T3 *CLIP2::EGFR*-implanted mice were randomised to afatinib or vehicle treatment, afatinib (10 mg/kg/d) induced tumour growth regressions in all animals (n = 9), whereas vehicle (water; n = 8; Fig. 3E) treatment showed no effect on tumour growth.

4. Discussion

This study established the MTD of afatinib in paediatric patients and assessed the activity of afatinib in paediatric patients selected according to the presence of hypothesis-guided potential biomarkers.

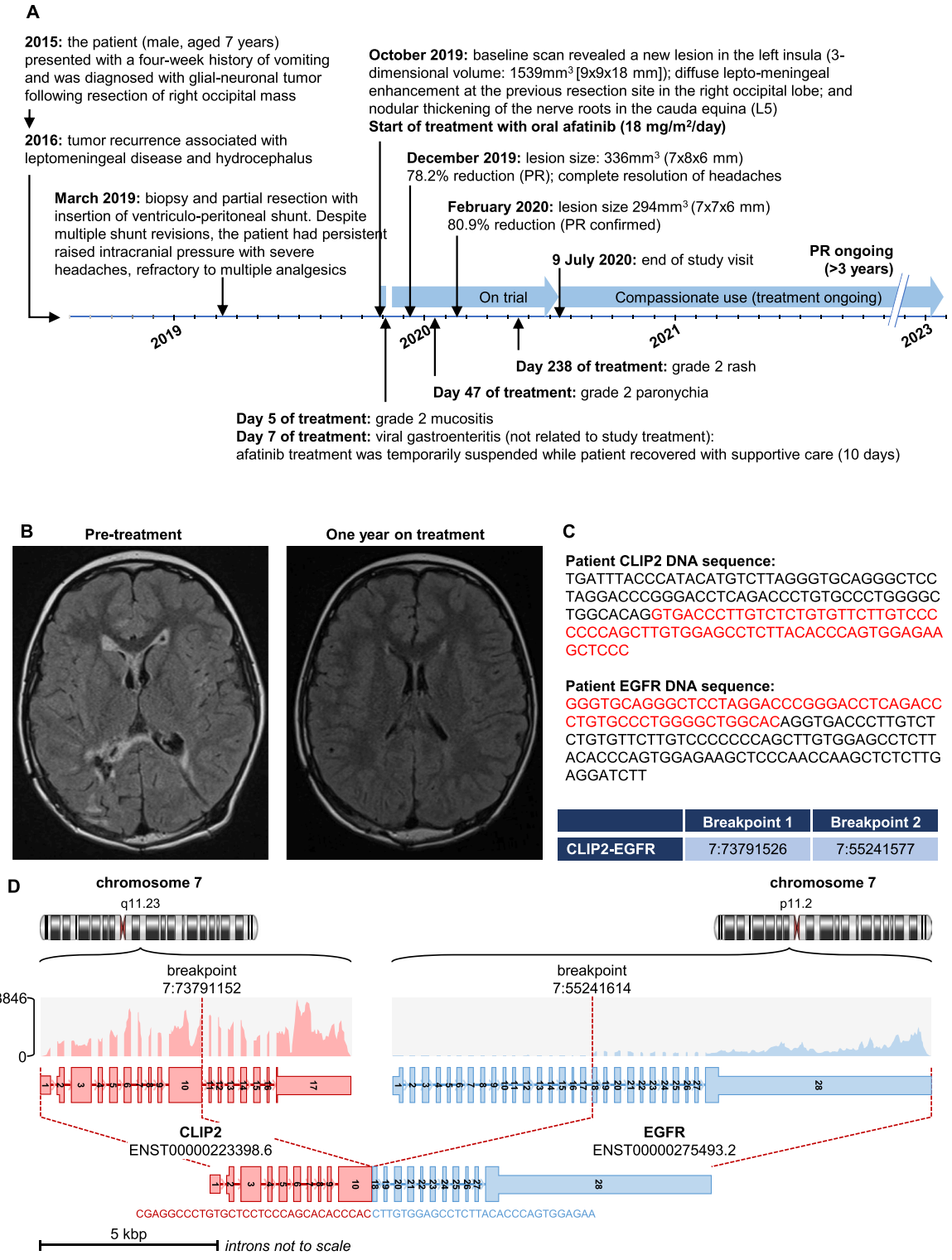

Fig. 2. *CLIP2::EGFR* gene fusion and tumour response in a patient with a glioneuronal tumour. A. Timeline of patient case history. B. MRI scans of patient pretreatment and after approximately 1 year on afatinib treatment. C. *CLIP2::EGFR* fusion DNA sequence and breakpoints. D. Breakpoints and schematic of *CLIP2::EGFR* fusion coding RNA. *CLIP2*, CAP-Gly Domain Containing Linker Protein 2; *EGFR*, epidermal growth factor receptor; PR, partial response.

At the established MTD for paediatric patients (18 mg/m²/d), afatinib exposure in children was similar to that in adults receiving 40 mg/d, an effective adult dose. Consistent with reports in adults [28,29], moderate interpatient variability in exposure was observed and the most common treatment-related AEs were diarrhoea, dry skin, stomatitis, and vomiting. G ≥3 treatment-related AEs affected 18% of patients (a

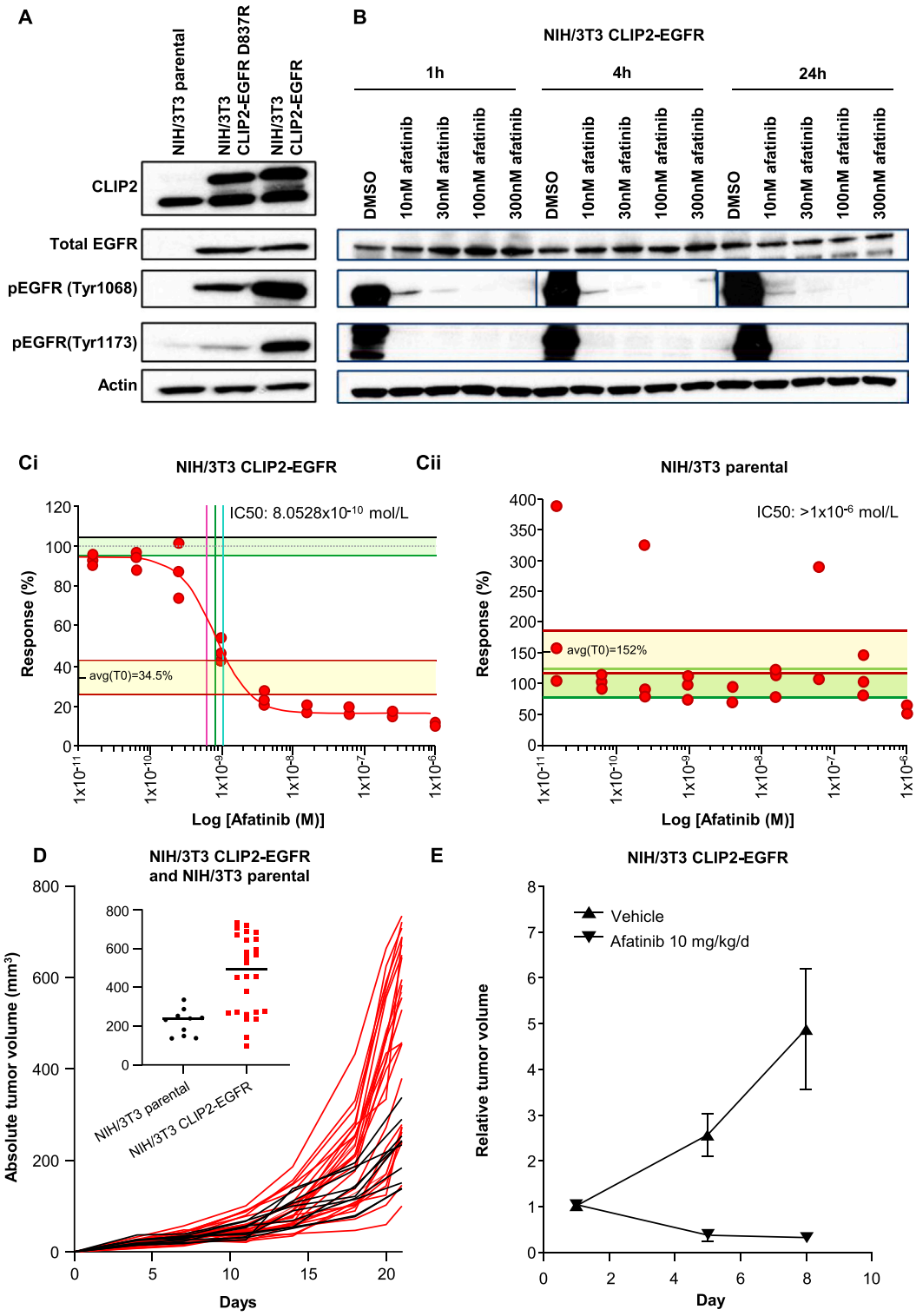

Fig. 3. Afatinib in preclinical models expressing CLIP2::EGFR fusion protein. A. EGFR and pEGFR immunoreactivity in NIH/3T3 cells expressing CLIP2::EGFR and CLIP2::EGFR D837R. B. pEGFR immunoreactivity in NIH/3T3 cells expressing CLIP2::EGFR incubated with afatinib (10–300 nM) or vehicle (DMSO) for 1 h, 4 h, or 24 h. C. Afatinib sensitivity of NIH/3T3 cells (Ci: CLIP2::EGFR; Cii: parental) using CellTiter-Glo® 3D Proliferation assays. D. Tumour growth volume in mice implanted with NIH/3T3 cells (red: CLIP2::EGFR; black: parental). Inset: tumour volume on Day 18. E. Relative tumour volumes with corresponding standard deviations in mice implanted with NIH/3T3 CLIP2::EGFR cells, treated with vehicle (water; n = 8) or afatinib (n = 9). CLIP2, CAP-Gly Domain Containing Linker Protein 2; d, day; DMSO, dimethyl sulfoxide; EGFR, epidermal growth factor receptor; h, hour; NIH/3T3, National Institute of Health, 3T3 fibroblast cell line, inoculum 3×10^5 cells; pEGFR, phosphorylated EGFR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lower proportion than in the LUX-Lung studies) [30,31].

In the MTD-expansion part, we implemented criteria from our recent biomarker study [20] to select for ErbB-driven malignancies and thus provide the most likely setting to observe responses in paediatric cancers associated with afatinib, an ErbB-targeting drug. The most common positive results were *EGFR* FISH, EGFR IHC, and HER2 IHC (15–20% each of pre-screened patients); *HER2* amplification/polysomy was observed in only 5% of patients. One patient had an *EGFR* gene fusion, and none had EGFR-activating mutations, illustrating the rarity of ErbB mutations in paediatric cancers. Despite the presumed biomarker-driven selection criteria, afatinib did not demonstrate substantial anti-tumour activity in any of the prespecified indications or in children with recurrent or refractory malignant tumours with ErbB pathway dysregulation as defined in the biomarker study. The present analysis did not stratify according to type of *EGFR* FISH test result (e.g. amplification versus high polysomy). There is some evidence that tumours with *EGFR* amplification may be sensitive to afatinib [32–35]. Focal *EGFR* amplification is observed less frequently in paediatric cancers than forms of polysomy [20]. It is possible that the low ORR in our study is, in part, attributable to a low proportion of *EGFR* amplification-positive tumours. The only confirmed OR was in the patient with a *CLIP2*::*EGFR* gene fusion, the first known occurrence in paediatric oncology. The durable response in this patient provides strong evidence supporting the effective penetrance of afatinib in CNS tumours, and reinforces the importance of targeting fusions, as this important class of somatic alterations can drive cancers sensitive to targeted agents [36–39]. Functional measures of oncogenic signalling, for example, EGFR signalling-associated protein complexes, may represent another biomarker type that could predict afatinib sensitivity [40]. There are few reports of ErbB fusions in paediatric cancers of the CNS [41]; however, our comprehensive preclinical experiments demonstrated that the *CLIP2::EGFR* fusion caused afatinib-sensitive activation of the EGFR kinase and resulted in fast-growing tumours, illustrating its transforming and oncogenic addiction potential in human tumours.

5. Conclusion

Although afatinib was generally not active in this assumed biomarker-preselected paediatric population, afatinib represents a valuable salvage treatment option for patients with certain rare ErbB-activating mutations.

Clinical trial information

Clinicaltrials.gov identifier: NCT02372006.

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CRediT authorship contribution statement

Birgit Geoerger: Conceptualisation, Methodology, Resources, Formal Analysis, Writing – Original Draft, Writing – Review & Editing; **Lynley Marshall:** Resources, Writing – Original Draft, Writing – Review & Editing; **Karsten Nysom:** Resources, Writing – Original Draft, Writing – Review & Editing; **Guy Makin:** Resources, Writing – Original Draft, Writing – Review & Editing; **Eric Bouffet:** Resources, Writing – Original Draft, Writing – Review & Editing; **Anne-Sophie Defachelles:** Resources, Writing – Original Draft, Writing – Review & Editing; **Loredana Amoroso:** Resources, Writing – Original Draft, Writing – Review & Editing; **Isabelle Aerts:** Resources, Writing – Original Draft, Writing – Review & Editing; **Pierre Leblond:** Resources, Writing – Original Draft, Writing – Review & Editing; **Paulette Barahona:** Resources, Data Curation, Writing – Original Draft, Writing – Review & Edit; **Kim Van Vlerken:** Project Administration, Data Curation Resources, Writing – Original Draft, Writing – Review & Editing; **Eric Fu:** Conceptualisation, Methodology, Formal Analysis, Writing – Original Draft, Writing – Review & Editing; **Flavio Solca:** Conceptualisation, Methodology, Project Administration, Resources, Data Curation, Formal Analysis, Writing – Original Draft, Writing – Review & Editing; **Robert M. Lorence:** Data Curation, Writing – Original Draft, Writing – Review & Editing; **David S. Ziegler:** Resources, Data Curation, Formal Analysis, Writing – Original Draft, Writing – Review & Editing. **All authors** gave final approval of the manuscript and are accountable for all aspects of the work.

Data availability

To ensure independent interpretation of clinical study results and enable authors to fulfil their role and obligations under the ICMJE criteria, Boehringer Ingelheim grants all external authors access to clinical study data pertinent to the development of the publication. In adherence with the Boehringer Ingelheim Policy on Transparency and Publication of Clinical Study Data, scientific and medical researchers can request access to clinical study data when it becomes available on https://vivli.org/, and earliest after publication of the primary manuscript in a peer-reviewed journal, regulatory activities are complete, and other criteria are met. Please visit https://www.mystudywindow.com/msw/datasharing for further information.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Birgit Geoerger reports being paid for a consulting/advisory role by Boehringer Ingelheim, AstraZeneca, and Novartis. Lynley Marshall reports being paid for a consulting/advisory role, honoraria, and participating in a speakers' bureau by Bayer; and a consulting/advisory role by Illumina, BMS and Tesaro, and participation in independent data monitoring committees for studies run by Eisai and Merck. Karsten Nysom reports being paid for a consulting or advisory role and honoraria by Y-mAbs and Bayer; being paid for a consulting/advisory role by EUSA Pharma and for participation in independent data monitoring committee for a study run by Lilly; and has received travel/accommodation/expenses from Bayer. Eric Bouffet reports being paid for a consulting or advisory role by Novartis, Bayer, and Roche, and has received institutional research funding grants from Roche and BMS. Isabelle Aerts reports being paid for a consulting/advisory role by AstraZeneca. Kim Van Vlerken reports being an employee of Boehringer Ingelheim and has been compensated for a leadership role on clinical trials for Boehringer Ingelheim. Eric Fu and Flavio Solca report being employees of Boehringer Ingelheim. Robert M. Lorence reports both being an employee of Boehringer Ingelheim and being paid for a consulting/advisory role by Boehringer Ingelheim. David S. Ziegler reports being paid for a consulting/advisory role by Bayer, AstraZeneca, Accendatech, Novartis, Day One, FivePhusion, Amgen, Alexion and Norgine. Guy Makin, Anne-Sophie Defachelles, Loredana Amoroso, Pierre Leblond and Paulette Barahona report no conflicts of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.04.007.

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