Early Phase Drug Development Studies in Pediatric Leukemia

Clinical trials with inotuzumab ozogamicin and bosutinib

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Vroege fase geneesmiddelen ontwikkeling in leukemie bij kinderen

klinische studies met inotuzumab ozogamicine en bosutinib

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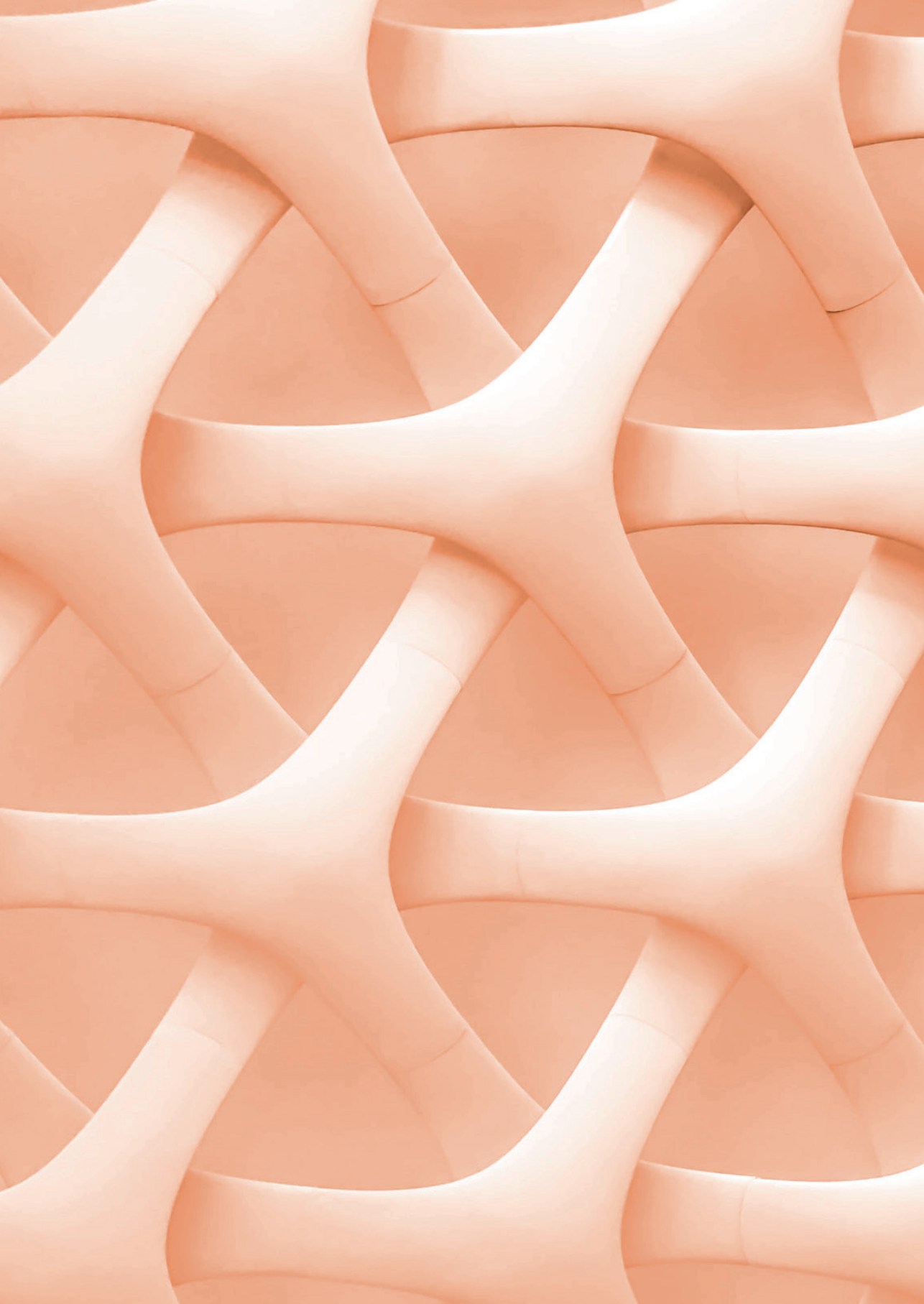
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Chapter 1

Introduction

Parts of this introduction were published in the proceedings of the 9th International Congress on Leukemia Lymphoma Myeloma organized by the Turkish Society of Hematology held on 12-13 of May 2023.

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1.1 General Overview and Thesis Scope

Haematological malignancies are the most common form of pediatric cancer with an estimated incidence of seven cases per 100.000 children.[1] This group of tumors includes an ample spectrum of diseases which are macroscopically distinguished in leukemias and lymphomas. Leukemias originate in the bone marrow and can either affect the lymphatic precursors as well as the myeloid cell lines. In both cases, they can manifest as a chronic disease, characterize by a slow-growing tendency and both immature and differentiated tumor cells; or as acute diseases, characterized by a fast-growing behavior, highly immature and undifferentiated tumor clones which aggressiveness necessitates urgent medical treatment.[2] On the other hand, lymphomas originate in the lymphatic system and tend to localize in the lymph nodes. These cancers are further divided into Hodgkin's lymphoma (HL), when multinucleated Reed–Sternberg cells are present, and non-Hodgkin lymphomas (NHL) in all other cases.[3] In children, NHLs are then classified by the WHO based on their clinical presentation, cell lineage (B-cell, T-cell and NK-cell), and histopathological features.[3] In children, almost all NHLs are aggressive, and mostly represented by mature B-cells neoplasms such as Burkitt lymphoma (BL), primary mediastinal large B-cell lymphoma (PMBCL), and diffuse large B-cell lymphoma (DLBCL), as well as anaplastic large cell lymphoma (ALCL) which is mostly of T-cell origin. This thesis is focuses on leukemic diseases only. Namely on Acute Lymphoblastic Leukemia (ALL) and Chronic Myeloid Leukemia (CML). Other forms of leukemias and lymphomas are not discussed further.

1.1.1 Acute Lymphoblastic Leukemia and Chronic Myeloid Leukemia in Children: A Brief Introduction

Leukemia accounts for approximately one third of pediatric cancers, and ALL represents by far the most prevalent form.[1] With an estimated incidence of three to four cases per 100.000 children, and a peak among three to five years of age, ALL accounts for 80% of pediatric leukemias.[4] ALL is a malignant disease affecting lymphoid progenitors in the bone marrow, more commonly of the B-cell type (85%), which accumulate in the peripheral blood and extramedullary sites.[1] The aggressiveness of the disease requires prompt treatment which usually consists of a combination of chemotherapeutic agents (such as vincristine, anthracyclines, asparaginase, methotrexate and cytarabine) and steroids (such as prednisolone and dexamethasone), given in cycles and combined in three or four phases: induction, consolidation, re-induction/intensification and maintenance. Hematopoietic stem cell transplantation (HSCT) is performed in selected cases, depending on genetics features and/or the molecular response levels (as reported later).[4,5] The treatment of childhood ALL is stratified in risk groups based on which the intensity of the treatment is chosen. In general, at the time of diagnosis, male sex, age < 1 year or ≥ 10 years, white blood cell (WBC) count ≥ 50.000/ml, and extramedullary involvement are considered factors conferring higher risk (Table 1).[6] Genetic mutations also play a relevant role, with *TP53* abnormalities, *KTM2A* rearrangements (particularly *t(4;11)(q21;q23) KMT2A/AFF1*), *iAMP21*, and *E2A/TCF3-PBX1 t(1;19)(q23;p13.3)* or *E2A/TCF3-HLF t(17;19)(q22;p13)*, among others, conferring higher risk (Table 2).[6–8] Prognostic factors are however therapy dependent and may hence lose their prognostic outcome over time when treatment outcome improves. Therefore, most modern protocols use a combination of genetics and early response (for example minimal residual disease levels after induction or consolidation as reported later).[4]

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Table 1. Pediatric ALL Risk Groups at Diagnosis[6,9]

Feature	Favorable Prognosis	Poor Prognosis
Age	1 -10 years	< 1 year or ≥ 10 years
Sex	Female	Male
Race	Caucasian, Asian	African American, Hispanic
Down Syndrome	No	Yes
CNS involvement	CNS 1	CNS 2 and 3, traumatic tap with blasts
WBC at diagnosis	< 50.000/ml	> 50.000/ml
Testicular Involvement	No	Yes
Phenotype	B-cell	T-cell

ALL: acute lymphoblastic leukemia; WBC: white blood cell; CNS: central nervous system. CNS1: presence < 5/µL WBCs and absence of blasts on cytospin preparation; CNS2: presence < 5/µL WBCs and cytospin positive for blasts, CNS3 presence of ≥ 5/µL WBCs and cytospin positive for blasts and/or clinical signs of CNS leukemia.

Table 2. Favorable and Unfavorable Genetic Abnormalities in Pediatric ALL[6]

Prognosis	Genetic Abnormality
Favorable	Any of the following:
	High Hyperdiploid (51-65 chromosomes)
	ETV6/RUNX1 t(12;21)(p13.1;q22.1)
	NUMT1 Rearrangements
Unfavorable	Any of the following:
	Hypodiploid (<44 chromosomes)
	KMT2A Rearrangement t(v;11q23.3)
	BCR::ABL1: t(9;22)(q34.1;q11.2) (Ph+)
	BCR::ABL1-like
	TCF3-HLF t(17;19)(q22;p13)
	MEF2D Rearrangements
	Intrachromosomal amplification of chromosome 21 (iAMP21)
	BCL2 or MYC Rearrangements

At the other end of the spectrum, in terms of clinical characteristics and incidence, is CML which is a very rare disease in children with an incidence of around one case per million.[10] CML is caused by a fusion between the *Abelson 1* (*Abl*) gene on chromosome 9 and the break point cluster region (*Bcr*) on chromosome 22, also referred to as the *Philadelphia (Ph)* chromosome. In CML, usually, the major breakpoint region is found (e13a2 and/or e14a2 fusion transcript and therefore p210 protein), in contrast to Ph+ ALL which is more commonly associated with the minor breakpoint region (e1a2 fusion transcript and therefore p190 protein).[11,12] The resulting *BCR::ABL* oncogene codifies a non-receptor tyrosine kinase protein that induces the aberrant proliferation of myeloid cells.

CML manifests itself with non-specific symptoms such as fatigue, malaise, weight loss, and night sweeting, but it can also be asymptomatic at the time of diagnosis in up to 50% of patients.[11,12] Typical findings include splenomegaly at physical examination, absolute leukocytosis (median 250×10^9/L), myelocyte bulge (higher proportion of myelocytes than the more mature metamyelocytes seen on the blood smear), absolute basophilia and eosinophilia, usually with normal or higher platelet count.[11,12] As per European Leukemia Net (ELN) 2020 recommendations, the diagnosis is confirmed by the morphology of a bone marrow aspirate which also evaluates the percentage of blasts and basophils.[13] Furthermore, a RT-PCR assay is recommended in order to identify the specific *BCR::ABL1* transcript and to follow the response to treatment over time.[13] Risk classifications are available for adults (e.g. Sokal-score), but usually do not apply to pediatric patients.[12]

Treatment is currently based on small molecules which selectively inhibit the BCR-ABL1 protein.[13] The fore-father of this class of compounds is imatinib, which was developed for Ph-chromosome positive patients at the beginning of the second millennium, and is now accompanied, among others, by dasatinib and nilotinib (recently also bosutinib was approved for children as reported later), which are overall referred to as Tyrosine Kinase Inhibitors (TKIs) and are already approved for pediatric use.[12] Given their young age, children diagnosed with CML may require treatment for several decades (when stopping the treatment is not possible) and in many cases also during puberty.[14] In addition, pediatric CML often tends to be more aggressive than in older patients and, more frequently than in adults, to present in an advanced-stage at diagnosis.[15,16]

The protracted duration of the treatment poses many challenges in terms of toxicities (for example regarding reduced growth velocity or cardiac toxicity), and also concerning the development of treatment resistance.[14] Indeed, compared to adults, children have a different trade-off in terms of long-term consequences of TKIs versus risk of disease progression, and in children the possibility of eradicating the disease with HSCT versus long-term TKIs exposure assumes a more relevant role than in older patients.[17] Currently, HSCT is mostly used in patients presenting with, or progressing to blastic phase while on treatment with TKIs or those cases presenting a T315I mutation (resistant to most TKIs).[15,16] On the one hand, while HSCT is associated with potentially severe and sometimes long-term toxicity (graft-versus-host disease) and potential

procedural mortality, it also has been shown to determine a long-term disease free survival in the majority of CML patients.[18] On the other hand, TKIs have not been proven curative yet, although adult studies (for example the STIM study with imatinib) provide evidence that treatment-free remission may be possible in approximately 40% of the patients, although long-term follow-up in these studies is currently limited to one or two years in most cases.[19]

It is therefore of relevance what studies testing the effect of discontinuing TKIs in children (study NCT03817398 currently performed in the US) will show in the long term: can children also stop TKIs like in adults? If TKIs are not demonstrated to yield a sustained response after their discontinuation in pediatrics, and long-term toxicities emerge, this might lead to a re-evaluation of the role of HSCT in this population. This, though, should come with high success rates of HSCT and with a reduction of the complications post HSCT, which despite improvements in terms of conditioning regimens and immunophenotype matching, is not free of risks. Indeed, recent reviews from retrospective studies, conducted before the introduction of TKIs, showed that overall survival (OS) after HSCT was significantly worse than non-transplanted patients.[20,21]

In this thesis, both CML and ALL are object of investigation with a focus on innovative treatments tested in children in the context of "*first in child*" intent-to-file phase I-II clinical trials. The thesis collects the results from a set of studies which focus ranges from antibody-drug-conjugate (ADC) therapy in ALL, namely inotuzumab ozogamicin (InO), to TKIs for CML, namely bosutinib. The common thread along this thesis is the implementation of early stage trials for innovative treatments across acute and chronic forms of leukemia, and set-up from the beginning with the purpose of obtaining marketing authorization in the content of pediatric obligations for the marketing authorization holder. In the following paragraphs, an overview of the outstanding unmet medical needs for ALL and CML patients is given together with an outline of the content of each chapter.

1.2 Acute Lymphoblastic Leukemia in Children: From Traditional Chemotherapy to the Antibody Drug Conjugate Inotuzumab Ozogamicin

1.2.1 Early Improvements

ALL represents a paradigmatic case of treatment improvement over time and exemplifies the impact that clinical research can have on patients. Fifty years ago, a child with ALL had around 20% chance of surviving.[4] A child diagnosed with ALL today has approximately 85-90% chance of being cured.[4] The introduction of rotating chemotherapy between 1950 and 1960, with vincristine and steroids in induction, followed by a second and a third cycle with antimetabolites (e.g. methotrexate and mercaptopurine), initially yielded remission rates of 80-90%.[22] Nevertheless, most patients relapsed and still had poor survival which seldomly exceeded 20% at 10 years.[4] In terms of survival, the turning point was the introduction of craniospinal irradiation and intrathecal chemotherapy to reduce the risk of central nervous system (CNS) relapse at the beginning of the 70's which, combined with rotating chemotherapy schemes with a duration of two to three years, increased survival to around 75% at two years and 60% at 10 years.[4,23] Survival curves per time period are displayed in Figure 1.

Following the rotational approach based on combining multiple agents, which was developed at the end of the 60s by St. Jude Children's Research Hospital, another milestone in treating pediatric ALL was represented by the development of intensified chemotherapy regimens (mainly the reinduction blocks) initiated by the Berlin–Frankfurt–Münster (BFM) group between 1981 and 1995, and today still used as the framework for modern chemotherapy.[25] Treatment consists of a six to eight weeks induction phase, followed by consolidation (with delayed intensification depending on risk group), and subsequently by a long maintenance phase.[25] This approach significantly improved the prognosis of these patients as demonstrated by the results of contemporary clinical trials shown in Table 3.
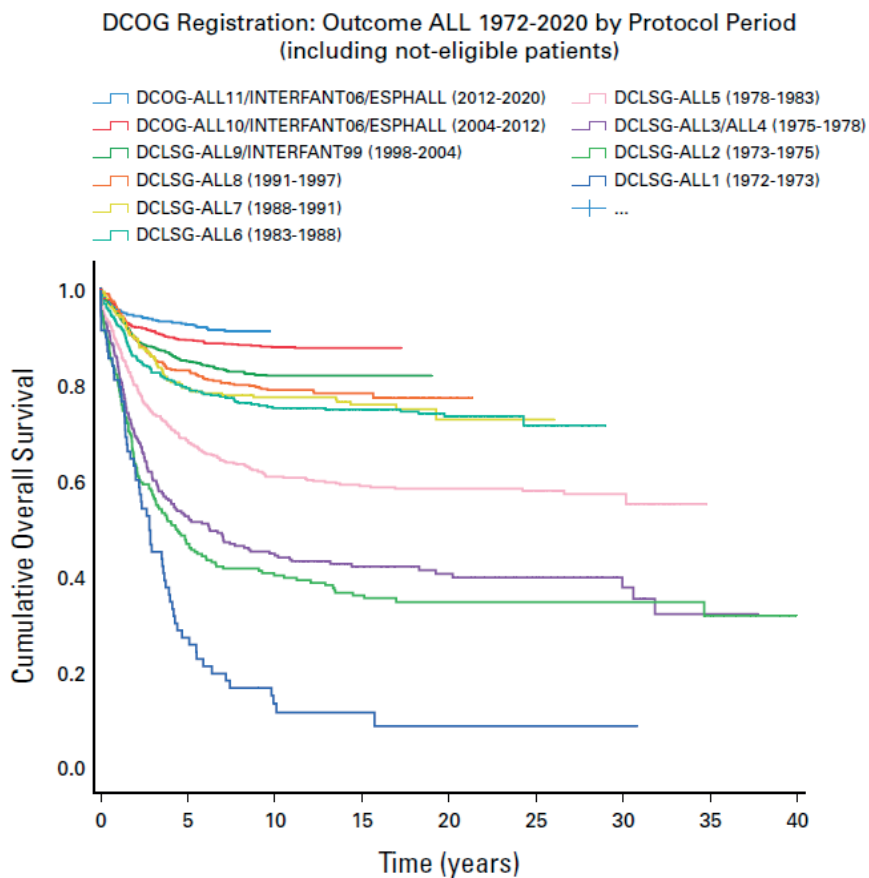
Figure 1. Outcome of Dutch children with ALL from 1972 to 2020. DCLSG: Dutch Childhood Leukemia Study Group; DCOG: Dutch Childhood Oncology Group (from R. Pieters et al, 2023)[24]

Table 3. Main Pediatric ALL Trials[24]

Trial Protocol	Period	Age Group	5-Year EFS	5-Year OS
AIEOP-BFM 2000 dexamethasone arm	2000-2006	1-17	84	90
AIEOP-BFM 2000 prednisone arm	2000-2004	1-17	81	91
SJCRH total 16	2000-2017	1-18	88	94
COALL-07-03	2003-2010	1-18	84	91
UKALL 2003	2003-2011	1-24	85	90
DFCI-05-001	2005-2011	1-18	87	93
COG	2006-2010	1-30	-	91.5
NOPHO-2008	2008-2014	1-45	85	91
MÀXIMA/DCOG-11	2012-2020	1-18	89	94

EFS: Event Free Survival Probability (%). OS: Overall Survival Probability (%).

To the improvement in survival also contributed the parallel improvement in the technology used to detect the disease. The introduction of multiparametric flow-cytometry methods as well as real-time quantitative PCR (RQ-PCR) allowed the detection of the residual disease after induction at the molecular level.[26] Indeed, the levels of molecular residual disease have been correlated with disease outcome by several studies, showing that Minimal Residual Disease (MRD) levels below 0.01% (usually defined as MRD negative) after induction are associated with a lower probability of relapse and can be used to decide in which subjects to proceed with an intensified regimen.[27–30] For example, in the AIEOP-BFM ALL 2000 study, the five-year Event Free Survival (EFS) in patients who were MRD negative (two negative molecular markers with sensitivity of 10^{-4} or less) at day 33 was 92.3%, while in those still positive but inferior to 10^{-3} at day 78 EFS was 77.6%, and in those above 10^{-3} EFS was 50.1%.[31] MRD levels after induction, together with genetic features, CNS involvement, and demographic characteristics (e.g. age) are nowadays used to refine the risk stratification. See for example the classification used in Europe by the ALLTogether1 2021 protocol (Table 4).

Table 4. Risk Stratification ALLTogether1 2021 Protocol (simplified version)

Risk Group	Features	
Standard Risk	No T-cell ALL No HR-genetics* MRD negative day 29 (end of induction) No CNS3/TLP+ No ABL-class fusion	
Intermediate Risk	MRD <5% day 29	
Intermediate Risk-Low	<16 years at diagnosis + no HR genetics	
	Subgroup	**MTD cut-off**
	ETV/RUNX1	MRD d29 < 0.1%
	High hyperdiploid	MRD d29 < 0.1%
	B-other + CNA GR**	MRD d29 < 0.1%
	T-cell	No MTD signal d 78
	TLP+ ≤5 WBC/microL CSF	MRD criteria by BCP/Tcell
Intermediate Risk-High	**Subgroup**	**MTD cut-off**
	CNS3-involvement TLP+ >5 WBC/microL CSF	Any MRD <HR-criteria
	CNS2/TLP+ without clear CSF by day 15	Any MRD <HR-criteria
	Poorly responding testicular/mediastinal disease	Any MRD <HR-criteria
	CNA PR***	Any detectable MRD d29 <5%
	Genetic groups as above	MRD ≥ cut-off above
	Failed genetic work-up	Any MRD <HR-criteria
	Failed MRD work-up	All patients
	T-cell	If MRD d29 <5% MRD <0.05% d78 If MRD d29 ≥5% MRD <0.5% d 50 and undetectable d71
	≥16 years	Not SR/HR-criteria
	ABL-class fusions	Any MRD d29 MRD< 0.05% d78
High Risk	<16 years and MRD ≥5% d 29 but MRD <0.05% d 71 <16 years, NCI HR at diagnosis and MRD ≥0.01% at TP2 <16 years and remaining testicular disease/med mass ≥1/3 of initial volume after Consolidation 1	

* HR-genetics: KMT2A/MLL rearrangements, near haploidy (<30 chromosomes), low hypodiploidy (30-39 chromosomes), iAMP21, t(17;19)/TCF3-HLF. ** CAN GR: No deletions affecting, BTG1, CDKN2A/B, EBF1, ETV6, IKZF1, PAX5, RB1, and PAR1 Isolated deletion of, BTG1 or ETV6 or PAX5 deletion, Only two deletions - ETV6 and BTG1, ETV6, and CDKN2A/B, ETV6, and a PAX5 deletion. *** CAN PR: Any other deletion profile including all deletions of IKZF1, EBF1, PAR1, RB1

While these progresses improved the prognosis overall, for the 10-15% of patients that relapse or are refractory, the probability of surviving remained poor. In these cases, the OS at 10 years is close to 50%, and even less in very early relapse (< 18 months from diagnosis).[32] Patients relapsing or refractory to first induction undergo intensive chemotherapy cycles, followed by HSCT, and this is usually repeated at subsequent relapses depending on the clinical condition of the child, the relapse interval, the availability of a new donor, and the wishes of the patient or family for further treatment.[4] The acute and chronic consequences of this intense chemotherapy treatments can be severe and in some cases life threatening, including infections and sepsis, pancreatitis, osteonecrosis, cardiovascular toxicities, infertility and peripheral or central neurological impairment.[33,34]

This generated the need for alternatives which materialized in the form of immunotherapies, such as blinatumomab and Chimeric Antigen Receptor T-cell (CAR-T cell) therapy, but also ADCs such as InO. A brief overview of blinatumomab and CAR-T cell therapy is provided in section 1.2.2. While an historical account of the development of InO up to its testing in pediatric ALL is given in section 1.2.3.

1.2.2 The Experience with Blinatumomab and CAR-T

1.2.2.1 Blinatumomab

Blinatumomab is a bi-specific T-cell engaging antibody, administered in 4-week continuous infusion, which targets CD19 on leukemic cells and CD3 on T-cells inducing the latter to kill CD19-positive B cells. The early experience in adults showed a remission rate in relapsed or refractory patients treated in phase II trials of 43% and MRD negativity rates among responders of 82%.[35] The same study also found a strong dependency of the response on the initial tumor load in the peripheral blood, with remission rates of 73% in those with < 50% blasts at baseline and just 29% in those with ≥ 50% blasts.[35] Subsequently, in phase III studies, heavily pretreated relapsed or refractory adult patients were randomized 2:1 to blinatumomab versus standard chemotherapy.[36] One group received induction with up to two cycles of blinatumomab while the other up to two cycles of chemotherapy. Patients with ≤ 5% bone marrow blasts could additionally receive three cycles of consolidation with the randomized treatment, and those in continuous remission additionally received up to 12 months of maintenance chemotherapy. Failure to achieve response (>5% bone marrow blasts) after two cycles was a criterion for discontinuing the treatment in both groups. The complete remission rate in the blinatumomab arm was 34% versus 16% in the standard chemotherapy arm (p <0.001), together with a median OS of 7.7 months versus 4 months (p = 0.01), respectively, and a similar percentage of grade 3 adverse events in both groups (92% and 87%, respectively).[36] The experience was replicated in a phase I/II study in children by the COG and BFM groups which published a remission rate with blinatumomab of almost 40%, of which around 50% MRD negative.[37] These results granted accelerated approval also in pediatrics for relapse and refractory patients by the Food and Drug Administration (FDA). Blinatumomab also

proved its higher efficacy, when compared to chemotherapy, as a consolidation treatment (which represent now the main indication for this drug) in two randomized trials conducted in first relapse pediatric B-ALL, which tested the drug either alone or intercalated between chemotherapy blocks. Brown et al (2021) randomized first-relapse patients to either two cycles of consolidation with blinatumomab or two cycles with multiagent chemotherapy (UKALLR3), after a common reinduction chemotherapy, based on vincristine, dexamethasone, peg-asparaginase, and mitoxantrone. The two-year OS probability in the blinatumomab arm was superior (54% vs 39%).[38] In the European trial testing blinatumomab versus chemotherapy, high-risk first-relapse ALL children received induction and two blocks of consolidation with chemotherapy and then were randomized to either blinatumomab or further chemotherapy according to the IntReALL HR 2010 protocol. Data reported by Locatelli et al (2021) showed a higher probability of survival in the blinatumomab arm, with the OS hazard ratio being 0.43 (95% CI: 0.18-1.01).[39] Different from the adult studies, febrile neutropenia, infection, and sepsis, were significantly lower in the blinatumomab groups, at the expense of more frequent neurological adverse events and Cytokine Release Syndrome (CRS), which however was clinically manageable.[39–41] These results led to the incorporation of blinatumomab as an effective consolidation treatment after re-induction, especially as a possible bridge to HSCT in those with persistent MRD positive levels (> 0.01%); but also in protocols for newly diagnosed patients with either high-risk features or slow MRD clearance as performed by several research groups such as COG (AALL1731), AIEOP-BFM (NCT03643276) and the St. Jude Children's Research Hospital Consortium (NCT031177510).

Of interest, recently published data from the phase III COG trial AALL1731, in which blinatumomab was added to chemotherapy in 255 low-risk first-relapse ALL pediatric (and young adults) patients and compared to chemotherapy alone, showed mixed results. Overall, no statistically significant differences were observed between the two group, particularly for those with isolated extra medullary relapse (N=81; 4-years OS: 76.5% vs 68.8%, with and without blinatumomab respectively, p=0.53); while in those with combined bone marrow relapse, the addition of three blinatumomab blocks after re-induction seems to significantly improve survival (N=174; 4-years OS: 97.1% vs 84.8%, with and without blinatumomab respectively, p=0.02).[42]

Finally, blinatumomab has also been tested in combination with chemotherapy in infants with ALL. Blinatumomab, at 15 µg/m^2/day, has been combined with the Interfant-06 chemotherapy scheme in newly diagnosed ALL *KMT2A*-rearranged infant patients and administered in the post-induction phase (hoping to prevent early relapse) in 30 patients below one year of age (EudraCT number 2016-004674-17).[43] Recently published data reported a two-year disease-free survival of 81.6% as compared to 49.4% in the Interfant-06 trial.[43]

1.2.2.2 CAR-T

Traditionally, patients achieving a second remission are inevitably consolidated with HSCT to reduce the risk of further relapse. Both, the intense chemotherapy and the HSCT are associated

with a risk of morbidity and mortality.[27,44] With the intent to achieve remission in patients resistant to chemotherapy, CAR-T cell therapy was developed. Given the persistence of CAR-T cells, the treatment was also hypothesized as an alternative to HSCT with the intent of limiting the potential sequelae of transplant in heavily pretreated patients. The studies of CAR-T therapy in children with ALL started with phase I and phase II trials conducted by Maude et al. (ELIANA trial) testing autologous anti-CD19 CAR-T cell (CTL019) in children and young adults with relapsed or refractory B-cell ALL with centralized production by a pharmaceutical company.[45] The reported remission rate of 81% and prolonged response, with EFS of 50% (95% CI, 35 to 64) and OS of 76% (95% CI, 63 to 86) at 12 months, led to its approval in refractory and second or greater relapse children in 2017 by FDA and by the European Medical Agency (EMA) in 2018.[45,46] Nine out of 10 patients treated in the ELIANA trial had CRS, of which at grade 4 in 25% of the cases, reflective of the high tumor load at infusion in most patients.[45,46] Another frequent toxicity with CTL019 is neurological, including tremor, confusion, delirium, hallucinations and focal deficits, now known as ICANS.[34,45–47] Despite occurring frequently, CRS has been controlled, in most cases, by lowering the tumor load prior to infusion or by using steroids and targeting the cytokines or their receptors with antibodies. To the latter approach belongs tocilizumab, an antibody directed against the receptor of the interleukin-6 which have been shown efficacious in the management of this adverse event.[48] The reasons of failure of CAR-T therapy are multiple. Antigen escape with the loss of CD19 and high levels of leukemia in the bone marrow before infusion have been identified as risk factors by several retrospective studies.[49,50] Furthermore, previous treatments with other immunotherapies, including blinatumomab, might also correlate with impaired expansion of the CAR-T product. For example it has been observed that non-response to blinatumomab and high-disease burden are independently associated with worse EFS (6-month EFS 27.3% vs 72.6%) and higher probability of relapse after CAR-T infusion.[51] It remains object of debate whether or not previous immunotherapies administered before the harvesting can damage the T cells, as evidence in this domain is limited. It also remains an open question whether CAR-T alone can be considered a final consolidation for relapsing ALL patients or it should be anyway considered a bridge to HSCT. The first approach might spare the toxicities of HSCT, while the second might have the advantage of reducing the risk of relapse further while also allowing a longer interval between the end of the induction and HSCT.[52] It seems unlikely, however, that a randomized trial will be performed in this area given that the mid-term results (median follow-up of 38.8 months) of the ELIANA Trial (NCT02435849) showed that the relapse-free EFS dropped from 59% at 1 year to 48% at three years.[53] It also becomes imminent to develop a new generation of CAR T-cells therapies, for example addressing more than one epitope, or use decentralized production with 'fresh' autologous T-cells (as reported in Chapter 6).

1.2.3 Inotuzumab Ozogamicin: From adults to Children With Acute Lymphoblastic Leukemia

While blinatumomab improved the outcome of children with relapsed and refractory ALL which managed to achieve a second remission with chemotherapy, the re-induction remission rate remains unsatisfactory. For example, Stackelberg et al (2016) reported the efficacy of blinatumomab in relapsed and refractory ALL pediatric patients with more than 25% bone marrow blasts at screening.[37] The response rate among the 70 subjects treated at the recommended phase II dose was 39% (95%CI: 27% to 51%) and the MRD negativity rate among responders was 52%.[37] Among the other molecules available, ADCs loaded with calicheamicin became available and were initially tested in adults.

Calicheamicin derivatives are a family of antitumor antibiotics known since the 80's for their cytotoxic effect mediated by the cleavage of the DNA double strand.[54,55] The testing of this compound as a possible conjugate for antibodies started with gemtuzumab ozogamicin, a CD33 directed ADC loaded with a semisynthetic and more stable calicheamicin derivative (N-acetyl-γ calicheamicin 1,2-dimethyl hydrazine dichloride) for the treatment of Acute Myeloid Leukemia (AML).[56,57] Later, CD22 was also identified as a possible target for B-cell lymphoid malignancies given its characteristics by the same company, and InO was synthesized. Indeed, CD22 is expressed on the surface of 60% to >90% of B-lymphoid malignancies, but not on hematopoietic stem cells, non-lymphoid cells, and memory B cells.[58,59] InO is an IgG4 targeting CD22 and loaded with an average of six molecules of calicheamicin.[55] Preclinical models, pioneered by DiJoseph et al, both in-vitro and in- vivo, with systemic disseminated B-cell lymphoma, showed a remarkable and dose-dependent cytotoxicity of InO, superior to chemotherapy.[60–62] Importantly, it was also shown that the combination of InO with chemotherapy was synergetic with vincristine, steroids, and cyclophosphamide.[62] The preclinical findings also showed the high sensitivity of ALL blasts harvested from both adult as well as pediatric patients, even higher than in NHLs cells.[63,64] In addition, in-vitro studies conducted on pediatric ALL cells, showed that the complex InO and CD22 receptor is rapidly internalized in the cytoplasmic space and then transferred in the lysosomes where the cytotoxic payload is released from the antibody.[64] The study also showed how both the binding and internalization of InO correlate with calicheamicin accumulation in the intracellular space.[64] The leukemic cell apoptosis induced by InO was also shown to be time-dependent with a relatively wide range of 50% inhibitory concentration (IC_{50}) from 0.15 to 4.9 ng/mL.[64]

The first in human trial was conducted in adults with relapsed or refractory B-cell NHLs. Advani et al. reported a response rate of 68% for patients with follicular NHL treated at the Maximum Tolerated Dose (MTD) of 1.8 mg/m² given as non-fractionated bolus, but much lower (15%) for patients with DLBCL.[65] Transient thrombocytopenia was reported as the main adverse event, together with severe cytopenia for those treated at 2.4 mg/m². InO combined with rituximab versus either rituximab plus bendamustine or rituximab plus gemcitabine was further

tested in a large randomized phase III trial (NCT01232556) enrolling 338 adult patients with aggressive NHL CD22+. The trial was terminated prematurely due to futility and final results reported a response rate of 44% in the arm treated with InO vs 41% in the control arm.[66] InO might still have a role in specific NHL populations, such as those with DLBCL not suitable for anthracycline based chemotherapy (NCT01679119), but it may be difficult for ADCs to penetrate solid masses.

However, the evidence summarized in the previous sections supported the testing of InO in ALL. Forty-nine patients (including five pediatric patients) with refractory or relapsed ALL already heavily pretreated with chemotherapy were enrolled in a phase II trial (NCT01134575) testing InO as single agent therapy.[67] The dose selected for adults with ALL was based on the data previously obtained from lymphoma patients and, after testing 1.3 mg/m^2/cycle in the first three patients, the dose was increased to 1.8 mg/m^2/cycle as intra-venous infusion over 1 h. every 3–4 weeks, then reduced to 1.5 mg/m^2/cycle once in remission. This dose level yielded an overall response rate of 57% (95% CI: 42% to 71%) and the median OS was 5.1 months (95% CI: 3.8 to 6.4).[67]

These results paved the way the pivotal phase III trial INO-VATE ALL.[67] As reported above, InO was initially administered in a single dose every three to four weeks in NHL. In vitro evidence produced by de Vries et al. supported the fractionated approach now used in ALL and based on three administrations per cycle (21 days), with a loading dose on day one, which might reduce the toxicities while increasing the efficacy of the regimen.[64] Indeed, continuous exposure to the drug over time was shown more effective than pulse exposure (in bolus) in preclinical studies.[64] A fractionated regimen with three weekly infusion on day 1, 8 and 15 of each cycle was therefore tested in further trials. The INO-VATE ALL trial was a 1:1 randomized study, testing InO at 1.8 mg/m^2 per cycle (0.8 mg/m^2 on day 1; 0.5 mg/m^2 on day 8 and 0.5 mg/m^2 on 15) or 1.5 mg/m^2 per cycle (0.5 mg/m^2 on day 1; 0.5 mg/m^2 on day 8 and 0.5 mg/m^2 on 15) once in remission; against chemotherapy chosen as per investigator discretion among three regimens (FLAG: cytarabine, fludarabine and granulocyte-colony stimulating factor; cytarabine plus mitoxantrone; or high-dose cytarabine). The remission rate was 80.7% in the experimental arm (InO) versus 29.4% of the control group (p<0.001), median Progression Free Survival (PFS) was significantly longer in the InO group (median: 5.0 months, 95%CI: 3.7 to 5.6) compared to the standard chemotherapy arm (median: 1.8 months, 95%CI: 1.5 to 2.2); while the median OS was 7.7 months (95% CI: 6.0 - 9.2) versus 6.7 months (95% CI: 4.9 to 8.3) respectively.[68] The drug was granted priority review, and, in 2017, the FDA approved InO for adults with relapsed or refractory ALL.

While a very limited population of children had already been treated with InO in the context of adult trials, the experience in pediatric ALL patients initiated in the form of compassionate use studies, such as the one conducted by Bhojwani et al. on 51 children with relapsed or refractory ALL.[69,70] Data reported that 67% achieved bone marrow remission, of which 71% also achieved MRD negativity.[70] The study also highlighted potential hepatic toxicities in the

form of transaminases elevation and, most importantly, hepatic sinusoidal obstructive syndrome (SOS). Indeed, 52% of patients who underwent HSCT post-treatment developed SOS.[70] In 2016, the first formal trial testing InO in children opened, and it was the trial ITCC-059 (EudraCT Number:2016-000227-71). The trial consisted of two main strata (later amended). Stratum I included ALL pediatric patients refractory to previous induction or that relapsed. Stratum II included other CD22+ B cell malignancies (NHLs) as an exploratory cohort. Stratum I was further divided into a Phase IA, testing InO as single agent, and a Phase IB/IB-ASP testing the combination of InO with a UKALLR3 modified chemotherapy scheme (based on vincristine plus steroids) with and without asparaginase (ASP) (IB-ASP never opened). In addition, a phase II for the single agent regimen in ALL patients was planned. The trial was developed in collaboration with Pfizer Inc. in the context of a Pediatric Investigation Plan with *intent-to-file* by both FDA and EMA. In **Chapter 2**, the results from the phase II cohort of the ITCC-059 trial are presented, while the results from the Phase IA were published previously by Brivio et al in 2021.[71]

As mentioned above, the traditional approach to treat subjects with ALL has been based on intensive chemotherapy. Among the most widely used chemotherapy regimen, the UKALL-R3, and more precisely the mitoxantrone arm of the trial NCT00967057, has been proven effective, particularly in first relapse ALL patients, showing a three-year OS probability of 69.0% (95%CI: 58.5-77.3) and a three-year PFS probability of 64.6% (95%CI: 54.2–73.2).[72–74] Therefore, the question was whether the replacement of the most toxic chemotherapeutic agent (mitoxantrone) with InO might decrease toxicities while preserving or even improving the efficacy of the re-induction.[33] This is the research question answered in **Chapter 3**, where the results from the phase IB of the ITCC-059 trial are presented.

To complete the suit of trials investigating InO in children, **Chapter 4** outlines the pharmacokinetic (PK) behavior of InO as single agent in children. When moving from adults to children, it is important to understand how the metabolic system in children differs from adults. Genetic factors, food intake, and concomitant medications are all factors that can alter the drug disposition particularly for those administered orally. Furthermore, the pediatric population is actually a rather heterogenous group in terms of absorption, distribution, metabolism and elimination of drugs given the differences in the maturation of kidneys and liver observed in neonates (0-28 days), infants (>28 days to 12 months) and older children. For oral compounds, the bioavailability might also be affected by age-dependent enzyme expression in the gastro-intestinal tract such as cytochrome P-450 1A1 (CYP1A1), which tends to increase with age.[75] The percentage of drug unbounded to proteins in the blood stream is also a potentially age dependent parameter. As infants have lower albumin and a1-acid glycoprotein, the disposition of compounds with high protein affinity might change significantly with age.[75] Glomerular filtration rate is usually comparable to adults after 12 months of age, but in infants, and especially those born pre-mature, it is significantly lower.[75] Similarly, the liver enzymes system undergoes changes during pediatric age. Patients below 10 years of age have shown a higher plasma clearance of drugs with intense liver metabolization and might therefore need higher weight-based or body-surface-area

(BSA)-based dose.[75] Finally, and specifically in the context of InO, it has been shown in models from adults that the clearance of this ADC can be described by a fixed term and a time-varying term.[76] It has been speculated that this might relate to the change in tumor burden over time as responders usually exhibit a rapid drop in the percentage of peripheral blasts already after the first or second administration (one or two weeks of treatment). Nevertheless, PK studies using tumor burden indicators, such as the percentage of CD22+ blasts in the peripheral blood, were tested as a time dependent covariate are limited and, to the best of our knowledge, not existing in pediatrics. Therefore, despite plausible from a clinical viewpoint, more evidence is needed to characterize this relationship. All these factors justify the necessity to conduct dose-finding trials in children separately from adults. The goal of **Chapter 4** is to add a piece to the puzzle, by expanding the population PK model of InO which was previously developed using only adult data and now including also pediatric data collected within trial ITCC-059.

1

1.3 Chronic Myeloid Leukemia in Children: the Role of Tyrosine Kinase Inhibitors

Since the discovery of imatinib, TKIs have become the standard treatment for CML, and replaced the approach based on interferon and/or chemotherapy, followed by HSCT. In the last 20 years, the landscape of TKIs has expanded significantly, and, at the time this thesis project started, pediatric patients could benefit from a portfolio of at least three TKIs, namely imatinib, and two second generation compounds, dasatinib and nilotinib (Table 5).[12,14] TKIs have become the standard treatment for CML, and also among the second generation, some (such as dasatinib) have become the first choice in newly diagnosed patients in some countries.[77] A child diagnosed with CML today has an estimated five-year probability of survival of around 94% (95%CI: 66% to 99%) with second generation TKIs, and a PFS at five years between 75% and 90%.[78,79]

Table 5. TKIs Approved for Chronic Myeloid Leukemia in Pediatrics*

TKI	Indication	Year	Recommended Dose	With Food	Ref.
Imatinib	R/I	2003‡	340^a mg/m²/day q.d. (max 600 mg/day)	Y	80,81
Imatinib	ND	2013	340^a mg/m²/day q.d. (max 600 mg/day)	Y	81,82
Dasatinib	R/I	2017	40-100^b mg/day q.d.	Y	79,83,84
Dasatinib	ND	2017	40-100^b mg/day q.d.	Y	79,83
Nilotinib	R/I	2018	230 mg/m² b.i.d (maximum single dose 400 mg)	N^c	78,85
Nilotinib	ND	2018	230 mg/m² b.i.d (maximum single dose 400 mg)	N^c	78,85

* Bosutinib was approved in 2023 (see chapter 5)
‡ Preliminary approval for Imatinib was granted in 2001 and converted in full approval in 2003
a. 260 mg/m²/day for children with Ph+ chronic phase CML recurrent after stem cell transplant or who are resistant to interferon-alpha therapy
b. pediatric dosages (as reported in the respective labels) are based on body weight: 10-19 kg 40mg, 20-29 kg 60mg, 30-45 kg 70mg, ≥ 45 kg 100mg
c. fasting 2 hours before administration and 1 hour after is required
TKI: Tyrosine Kinase Inhibitor; ND: Newly Diagnosed; R/I: Resistant or Intolerant; b.i.d: bis in die. q.d.: quaque die; Y: Yes, N: No. Ref.: References

Despite the demonstrated efficacy of TKIs (Table 6), the alternatives for children are limited when compared to adults. Approximately 30% of pediatric patients either develop resistance or intolerance to imatinib, and for them the possibilities of treatment are currently limited to dasatinib and nilotinib (bosutinib has been recently approved by FDA thanks to the evidence generated in the trial reported in this thesis).[82,86] On the other hand, adults can already benefit from other TKIs such as bosutinib, ponatinib, and more, recently asciminib (a STAMP inhibitor specifically targeting the ABL myristoyl pocket and exerting a non-competitive allosteric inhibition of BCR-ABL1 protein), which are only available in the context of clinical trials in children.[87]

CML is a rare condition in children and the recruitment in pediatric trials is often slow and necessitates large international multi-center studies and cooperation among multiple research groups. Consequently, the management and study protocols for children are based on adult data, where the disease is much more common.[88] For example pediatric studies often strive to mimic adult exposure, whereas the risk-benefit ratio in children is only supported by limited data despite potential biological differences between adults and children.[15] Another issue faced by children with CML, and different from adults, is the fact that they will be exposed to the treatment during the development age and potentially for longer periods (depending on whether or not the TKI can be discontinued without risk of relapse) than adults. Therefore, long-term toxicities, and those related to growth development, become a primary concern. Finally, pediatric CML is usually more aggressive than in adults, showing a different breakpoint distribution pattern of *BCR::ABL1* fusion gene, and higher frequency negative prognostic factors at the time of diagnosis (e.g. higher baseline WBC count).[89] This requires to address the pediatric population specifically and with tailored treatments which account for these differences.

Table 6. Frontline TKIs Efficacy in Pediatric CML

TKI	CCyR (time)	MMR (time)	PFS (time)	EFS (time)	Trial	Reference
Imatinib	61% (12 mts)	31% (12 mts)	98% (3y)	n/a	NCT00845221	[90]
Dasatinib	92% (12 mts)	52% (12 mts)	93% (4y)	n/a	NCT00777036	[79]
Nilotinib	64% (12 mts)	64% (12 mts)	n/a	91.2% (2y)	NCT01844765	[91]

mts: months; y: years

Therefore, despite the drastic improvement in survival of pediatric CML, the problem of finding manageable drugs that can help compliance (e.g. one daily administration only, available drinking solution or *mini-tabs*) and that can mitigate the toxic effects of TKIs during the growth spurt and adolescent phase remains only partially addressed. For example, nilotinib requires twice daily administration with fasting, and all TKIs currently approved in children have shown a certain level of off-target binding that may for example impact on longitudinal growth.[92–94] Animal models have highlighted that bosutinib might have a more tolerable profile from this point of view, and could therefore be advantageous for pediatric patients as the experience in adults demonstrate an efficacy comparable to the other second generation TKIs.[95] Bosutinib is a Src and ABL tyrosine kinases dual inhibitor, but differently from imatinib, dasatinib and nilotinib, it does not inhibit grow factor receptors such as insulin-like growth factor I receptor, fibroblast growth factor receptor, epidermal growth factor receptor, platelet-derived growth factor receptor, and serine–threonine kinases (e.g Akt and Cdk4).[96] In addition, each TKI tends to have a specific tolerability profile, which might therefore meet the individual sensitivity or preference of each patient (Table 7).[14,97] For example, muscle cramps, musculoskeletal pain, and myalgia are observed in more than 30% of patients treated with imatinib, but are less common with nilotinib, at the expenses of more frequent increased bilirubinemia (53%) and transaminases (36%).[98,99] This might be attributable, at least partially, to the specificity of the binding to the molecular target which also differs among TKIs, as reported in Table 8. As tolerance is a very important factor in determining the adherence to therapy in children, expanding the landscape of TKIs becomes particularly relevant.

For all these reasons, bosutinib is being tested in children for the first time in the context of an international collaboration between Europe, the USA, the UK, Switzerland and Israel and supported by regulatory incentives from the FDA and EMA. The results from this *first-in-child* registrational phase I study, testing bosutinib for patients with CML which are resistant or intolerant to previous lines of therapy, are presented in **Chapter 5**.

Table 7. Main Adverse Events per TKI and Their Characteristics[14]

TKI	Adverse Events	Other characteristics
Imatinib	Muscle cramps Edema Diarrhea	
Dasatinib	Pleural/pericardial effusions Pulmonary hypertension Gastro-intestinal bleeding QTc prolongation	Crosses the blood brain barrier
Nilotinib	QTc prolongation Arterial occlusion Metabolic imbalance (glucose/lipids)	
Bosutinib (adult data)	Diarrhea Hepatic enzyme increase	
Ponatinib	Arterial and venous thrombosis Pancreatitis	Effective against T315I mutation
Asciminib (adult data)	Myelosuppression Pancreas enzyme elevation, pancreatitis Hypertension	Effective against T315I mutation

Table 8. Main Molecular Targets of TKIs Used in CML

TKI	Targets	References
Imatinib	BCR-ABL, c-KIT, , PDGFR-β/α, CSF1R	[100–102]
Dasatinib	BCR-ABL, SRC family (SRC, LCK, YES, FYN), c-KIT, EPHA2, and PDGFRβ	[103–105]
Nilotinib	BCR-ABL, c-kit and PDGFβ/α	[106,107]
Bosutinib	BCR-ABL, SRC family (SRC, LCK, YES, FYN), TEC family kinases, STE20 family of kinases, CAMK2G	[96,108]
Ponatinib	BCR-ABL, FGFR I-IV, PDGFα, , VEGFR II, c-SRC, c-KIT, RET and FLT3	[109]
Asciminib	ABL Myristoyl Pocket	[110,111]

PDGFR: platelet-derived growth factor receptor; CSF1R: colony stimulating factor 1 receptor; CAMK2G: calcium/calmodulin dependent protein kinase II gamma; VEGFR: Vascular endothelial growth factor receptor; FGFR: Fibroblast growth factor receptors.

1.4 Conclusions

Several challenges remain to be addressed. First, developing drugs in adults and then in children in a consecutive manner often leads to a delay in the pediatric space which lags behind the fore-front of scientific innovation. Second, statistical designs for phase I and II trials are still commonly based on assumptions deriving from the chemotherapeutic era, but might not be valid when applied to current compounds based on molecular targets and immunological therapy. Third, despite improved over the last decades, international cooperation might still be harmonized and the partnerships between public and private players further consolidated. How can we shorten the development of pediatric drugs? How can we identify and share a common vision of future research globally? And how can we improve the methodology we rely on in pediatric trials? A more elaborate discussion on these problems is provided in **Chapter 6** together with an outlook on the treatment landscape for ALL and CML.

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Chapter 2

Inotuzumab Ozogamicin as Single Agent in Pediatric Patients with Relapsed and Refractory Acute Lymphoblastic Leukemia: Results from a Phase II Trial

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Abstract

Inotuzumab ozogamicin is a CD22-directed antibody conjugated to calicheamicin and approved in adults with relapsed or refractory (R/R) B cell precursors acute lymphoblastic leukemia (BCP-ALL). Patients aged 1-18 years, with R/R CD22+ BCP-ALL were treated at the RP2D of 1.8 mg/m^2/cycle. Using a single-stage design, with an overall response rate (ORR) ≤ 30% defined as not promising and ORR > 55% as expected, 25 patients needed to be recruited to achieve 80% power at 0.05 significance level. Thirty-two patients were enrolled, 28 were treated, 27 were evaluable for response. The estimated ORR was 81.5% (95%CI: 61.9%-93.7%), and 81.8% (18/22) of the responding subjects were minimal residual disease (MRD) negative. The study met its primary endpoint. Median follow up of survivors was 16 months (IQR: 14.49-20.07). One year Event Free Survival was 36.7% (95% CI: 22.2%−60.4%), and Overall Survival was 55.1% (95% CI: 39.1%−77.7%). Eighteen patients received consolidation with hematopoietic stem cell transplant (HSCT) or CAR T-cells therapy. Sinusoidal obstruction syndrome (SOS) occurred in seven patients. MRD negativity seemed correlated to calicheamicin sensitivity in vitro, but not to CD22 surface expression, saturation, or internalization. InO was effective in this population. The most relevant risk was the occurrence of SOS, particularly when InO treatment was followed by HSCT.

2.1 Introduction

In pediatric patients with acute lymphoblastic leukemia (ALL), relapse still occurs in 10-15% of the subjects.[1,2] Overall survival (OS) after relapse plateaus at 50-60%, while event free survival (EFS) after second and third relapse are approximately 25% and 15%, respectively.[3-5] Novel therapies are changing the treatment of children with relapsed/refractory (R/R) B-cell precursor ALL (BCP-ALL), with the approval of blinatumomab, a CD19-antigen directed T-cell engager, and Tisagenlecleucel, a CD19 chimeric antigen receptors (CAR) T-cell therapy. However, cases in which these agents are no longer effective were reported.[6,7]

Inotuzumab ozogamicin (InO) consists of an anti-CD22 monoclonal antibody linked to the cytotoxic agent calicheamicin.[8,9] InO is approved for adults with CD22-positive R/R BCP-ALL, based on the INO-VATE ALL trial.[10] CD22 is an antigen on the cell surface of most normal B-cells (60–90%), and is expressed on the leukemic blasts in more than 90% of childhood BCP-ALL.[11-13] Two retrospective studies of InO in pediatric BCP-ALL patients showed a remission rate of approximately 67%.[14,15] The results of the phase I pediatric study from our group reported an Overall Response Rate (ORR) of 80% (95% CI: 59-93%) and, among the responders, 84% (95% CI: 60–97%) achieved minimal residual disease (MRD) negativity.[16] Recently, the Children's Oncology Group (COG) reported a remission rate of 58.3% (90% CI: 46.5-69.3%) from 48 patients enrolled in a phase II trial using 1.8 mg/m^2/cycle.[17]

A correlation between clinical response and CD22 alternative splicing and expression has been hypothesized. A study in Acute Myeloid Leukemia (AML) patients treated with gemtuzumab ozogamicin, a CD33 conjugate to calicheamicin, showed a link between alternative splicing of the CD33 antigen and clinical response, while an increased expression of a CD19 isoform with intraexonic splicing of exon 2 was found associated with treatment failure on blinatumomab.[18,19]

Moreover, a trial in adults treated with the combination of chemotherapy and InO, with or without blinatumomab, identified baseline CD22 expression level <70% as predictor of poor outcome.[20] In this paper, we report the clinical results from the phase II InO single-agent ITCC-059 clinical trial and elaborate on the pharmacodynamic (PD) investigations on potential causes of intrinsic resistance to InO.

2.2 Materials/Subjects and Methods

ITCC-059 (EUDRACT nr 2016-000227-71; NTR5736) is a phase I-II, multicenter, international, single-arm, open-label study conducted in accordance with the International Ethical Guidelines for Biomedical Research Involving Human Subjects, International Council for Harmonization Guidelines for Good Clinical Practice, and the Declaration of Helsinki. The protocol received Ethics Committee review and approval at all participating centers. Patients were treated under protocol version 2 and 3 following an amendment unrelated to this cohort. Informed consent was obtained from all patients or their parents (as applicable) before enrolment. The study was sponsored by the Erasmus MC and funded by Pfizer inc. in the context of a Pediatric Investigational Plan.

2.2.1 Patients and Treatment

Criteria for enrollment (Supplementary Table 1) included age ≥1 to <18 years, diagnosis of CD22-positive R/R BCP-ALL with an M2 or M3 bone marrow (BM) status and either refractory disease or ≥2nd relapse, or any relapse after hematopoietic stem cell transplant (HSCT). Exclusion criteria included isolated extramedullary disease, active infections, and any history of prior or ongoing hepatic sinusoidal obstruction syndrome (SOS). Subjects started InO at the recommended phase II dose (RP2D) of 1.8 mg/m^2/cycle fractionated in three weekly administrations, or 1.5 mg/m^2/cycle once remission was achieved. Intrathecal prophylaxis was administered depending on the central nervus system (CNS) status. A maximum of six cycles were allowed, except for patients proceeding to HSCT for which the recommended number of cycles was two, or three if still MRD positive. Patients attaining an M1 BM with absolute neutrophil count (ANC) ≥ 0.5 x 10^9/L and platelets count ≥ 50 x 10^9/L, and those with M3 BM at study entry attaining an M2 BM irrespective of hematological criteria, could proceed to the subsequent treatment cycles.

2.2.2 Endpoints and Statistical Design

The primary objective was to establish the preliminary activity of InO. Secondary objectives included safety, other measures of antileukemic activity, PD analysis and pharmacokinetic (PK) parameters. PD analysis was performed on patients from phase I and II for whom laboratory material was available. The primary endpoint of the study was the overall response rate (ORR), defined as the combined Complete Remission (CR), CR with insufficient platelet recovery (CRp) and without recovery of counts (CRi) rate (Supplementary Table 2); and measured as best response during the entire treatment. Secondary endpoints included ORR after cycle one, EFS, OS, duration of response (DOR), MRD negativity rate and safety (Supplementary Table 3). MRD negativity was defined as either a PCR result below 10^{-4}, or a flow cytometry result below 0.01% when the PCR was negative, but the quantitative range (QR) was above 10^{-4} (Supplementary Text 2).[21,22] PD parameters included the relationship between clinical response (MRD-negativity rate) and CD22 expression, saturation kinetics, CD22 clonal evolution, alternative splicing of the CD22 transcript, calicheamicin sensitivity, and the percentage of patients who exhibited anti-drug

antibodies (ADAs). The statistical design consisted of a single-stage design; an ORR of ≤ 30% was considered not promising (null hypothesis, H_0) and an ORR of > 55% was expected (alternative hypothesis, H_1); 25 patients evaluable for response provided 80% power at a significance level of 0.05 (one-sided) based on exact binomial distribution.

2.2.3 CD22 Expression Levels

Flow cytometry was used to evaluate CD22 expression levels, by measuring both the mean fluorescent intensity (MFI) of leukemic blasts and the percentage of CD22-positivity at diagnosis on peripheral blood (PB) and BM samples at the Erasmus MC central Immunology laboratory in Rotterdam. Leukemic blast cells were gated based on expression of CD45, CD10, CD20, CD19, CD38, CD81, and CD34. The CD22 antibody RFB4® MHCD2204 (Thermo Fisher, Waltham, Massachusetts) was used for flow cytometry. In addition, CD22 saturation (Eq.1 in Supplementary) and internalization (Eq.2 in Supplementary) were measured on PB samples taken at day one and day eight of cycle one. The methods for the analysis of CD22 saturation and internalization of InO have been described previously.[9]

2.2.4 In-Vitro Drug Response

In vitro drug response to calicheamicin (MedChemExpress, Monmouth Junction, New Jersey) was assessed with MTT assays in U-bottom 96-well plates. Patient samples from BM or PB were enriched to at least 80% leukemic blasts, based on morphology with a May-Grunwald-Giemsa staining, using a negative magnetic bead enrichment. The concentrations of calicheamicin on the MTT assay plates were tested in duplicates and ranged from 0.4 ng/ml to 400 ng/ml. MTT assays were performed over four days at 1.6 million cells/ml density using medium containing RPMI 1640 Dutch Modified with 20% Fetal calf serum, Penicilline, Streptavidine, and Fungizone. After four days >70% leukemic blast had to be present in the no-drug control wells to construct dose response curves. The absorbance was read on a spectrophotometer at wavelengths of 562 nm and 720 nm and analyzed with Softmax Pro software. Optical density (OD) values of >50, after correction for blank wells, were required. Metabolic activity was calculated at each drug concentration relative to control wells after correction for the background OD values of the blank wells. IC50 values represent the concentration of the drug which inhibits 50% of the leukemic cells.[23]

2.2.5 RNA Sequencing, CD22 Splice Variants

The GENCODE reference annotations version 29 for GRCh38 and the GRCh38.p12 compliant Ensembl human genome reference were provided by the CTAT resource bundle (release: 27th of March 2019). Paired-end RNA-sequencing reads were aligned to this human genome reference and, subsequently, read counts per gene were calculated using STAR 2.6.0c. Split-reads were used to evaluate alternative splicing of the CD22 transcript. Only splice variants with at least 10 split reads were considered. Differential gene expression was assessed using TMM normalized counts

and the generalized linear model from the EdgeR package in R statistics. Anti- and pro-apoptotic genes were selected based on the hallmark geneset of the GeneSet Enrichement Analysis software.

2.2.6 Anti-Drug Antibody Analysis

Blood samples were collected during the screening, prior to each course of treatment, and at the end of treatment study. Samples were tested for ADA using a validated electro chemiluminescent bridging assay.

2.2.7 Statistical Analysis

The response analysis set included all enrolled patients who received at least one dose of InO and completed at least one baseline and one post-baseline disease assessment. The full analysis set consisted of all enrolled patients who received at least one dose of study therapy and was used for the safety analysis. Detailed definitions of outcome measures are provided in Supplementary Table 4. EFS and OS were estimated using the Kaplan–Meier method. Events defined as non-response (not achieving CR, CRi or CRp, considered as event at day 0), relapse, death or second malignancy.

For the analysis of PD parameters, patients were categorized into three groups: CR and MRD negative; CR and MRD positive; and no CR. For RNA sequencing analysis and calicheamicin sensitivity, subject not in CR and subject with CR MRD positive were grouped together, due to limited sample size. The Kruskal-Wallis test was used to test the association between the three response groups and the following PD parameters: CD22 surface expression (as MFI and percentage positive cells), CD22 saturation and InO internalization. The Wilcoxon rank-sum test was used to test calicheamicin sensitivity.

As a post-hoc analysis, Fisher's exact test and Mann-Whitney U test were used to test the association between clinical characteristics (eg. sex and age) and MRD response and between potential risk factors (eg. number of InO cycles received, time to HSCT) and SOS occurrence in post-InO transplanted patients. For all hypothesis tested, p values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using R statistical software, version 4.1.3 (the code is available on request).

2.3 Results

Results are based on a data cut-off date of 12 October 2021.

2.3.1 Patients and Treatment

Overall, 32 patients consented and were screened for inclusion from 03 June 2019 to 24 April 2020 at 16 sites of the ITCC consortium. In total, 30 patients were enrolled (two screening failures, both with inadequate liver function), 28 started treatment (two patients did not start treatment due to rapidly progressive disease), and 27 were evaluable (disease response not assessed in one patient, who discontinued due to SOS). Patient characteristics are reported in Table 1. A total of 147 doses of InO were given to 28 patients (median six doses/patient, range: 1-12). Thirteen (46.4%) subjects received one cycle, nine (32.1%) received two cycles, five (17.9) received three cycles and one (3.6%) received four cycles.

2.3.2 Efficacy

Twenty-two patients achieved response (ORR 81.5%; 95%CI: 61.9%-93.7%), in all cases after the first cycle; 14 were in CR, one in CRp and seven in CRi. MRD negativity, as best response, was achieved by 18 out of 22 (81.8%) responding subjects; after the first cycle by 13 (59.1%) patients, and after the second cycle by the other five. All patients were CNS negative at the end of cycle one and maintained the response at end of treatment. A total of 18 patients (66.7%) proceeded to consolidation therapy, 14 with HSCT (one after subsequent therapy with blinatumomab due to loss of response), two with CAR T-cell therapy (supplementary figure 1), and two with CAR T-cell therapy followed by HSCT. Three patients received blinatumomab as bridging therapy before HSCT; one patient received chemotherapy; two received CAR-T as mentioned above; and the others did not receive additional treatment between the last InO administration and transplant. Median time between last InO dose and HSCT was 45 days (IQR:26.5-70.5).

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Table 1. Patient Characteristics

Patients' Characteristics	Total (n=28)
Male	19 (67.9%)
Female	9 (32.1%)
Median age in years at enrollment (IQR)	7.5 (4 - 13)
Age at enrollment breakdown	
>1 & ≤ 2 years	2 (7.1%)
>2 & ≤ 6 years	10 (35.7%)
>6 years	16 (57.1%)
Extramedullary Disease (screening)*	
CNS1	21 (75%)
CNS2	4 (14.3%)
CNS3	2 (7.1%)
Testicular involvement	0
Lymph nodes enlarged	1 (3.6%)
Other locations (excluding spleen and liver)	0
Diagnosis	
first relapsed BCP-ALL post allogeneic HSCT	6 (21.4%)
second or greater relapsed BCP-ALL	16 (57.1%)
refractory BCP-ALL	6 (21.4%)
first HSCT prior to study treatment	14 (50.0%)
second HSCT prior to study treatment	1 (3.6%)
WBC (109/L) at screening, median (IQR)	3.1 (2.3 - 9)
CD22 PB Blast (%), median (IQR)	96.7 (86.7 - 99.9)
MFI - CD22+ expression, median (IQR)	2296.9 (1025.5-3709.2)
Prior antibody therapy	
Blinatumomab	7 (25%)
Karyotype abnormalities	
Normal	4 (14.3%)
Not Assessed/Available	9 (32.1%)
Hypodiploid (40-45 chromosomes)	2 (7.1%)
Low Hypodiploid (<40 chromosomes)	2 (7.1%)
Hyperdiploid (47-50 chromosomes)	2 (7.1%)
High hyperdiploid (51-65 chromosomes)	2 (7.1%)
Pseudodiploid	7 (25.0%)
t(9;22)(q34;q11.2) and variants	0
t(4;11)(q21;q23)	0
t(12;21)p13;q22	1 (3.6%)
t(11;v)(q23;v)	1 (3.6%)
t(1;19)(q23;p13)	1 (3.6%)
dic(9,20)(p11;q11)	1 (3.6%)
Down syndrome	0

*in one patient the sample was not evaluable due to red blood cells contamination. IQR: Interquartile range.

Median time between last InO dose and CAR-T therapy was 53.5 days (IQR:46.5-260.75). In two cases lymphocyte apheresis for CAR T-cell therapy was performed before initiating InO and in the other two cases after. Of the other four responding subjects, one

proceeded to blinatumomab (in CCR after seven months), one received maintenance therapy with 6-mercaptopurine and intrathecal triple therapy (then relapsed after seven months), one relapsed within one month after end of treatment, and one patient died while in CR due to neurological deterioration attributable to previous therapy and CNS leukemic involvement. The median follow for survival was 16 months (IQR: 14.49-20.07). At six months, EFS probability was 55.6% (95% CI: 39.6-77.8) and OS was 66.7% (95% CI: 51.1-87.0). At 12 months, EFS probability was 36.7% (95% CI: 22.2-60.4) and OS probability was 55.1% (95% CI: 39.1-77.7) (Figure 1). Median DOR was 7.74 months (95% CI: 5.65-not reached). The cumulative incidence of non-response or relapse was 29.63% (95% CI 13.77-47.42) at six months and 40.74% (95% CI 22.03-58.69) at 12 months. Combining phase I (n=25) and II (n=27) results, 52 patients received InO, of which 40 were treated at the RP2D (13 in phase I and 27 in phase II) [92]. Considering the combined response data from patients treated at the RP2D, 33 achieved response (ORR 82.5%; 95%CI: 67.2% - 92.7%), and 27 (81.8%) of the responders were MRD negative. EFS at 12 months was 41.3% (95%CI: 28.3%-60.1%); and OS at 12 months was 56.3% (95%CI: 42.6%-74.3%) (Supplementary Figure 2). In patients treated at RP2D (N= 40), age, gender, previous treatments, WBC, and CD22 MFI were not found to impact the ORR, MRD-negative response (Supplementary Table 5) or EFS (Table 2). In total, 17 patients relapsed (11 in phase I and six in phase II) and for 10 of them CD22 expression data were available. CD22 expression turned negative in three cases, and partially negative in two.
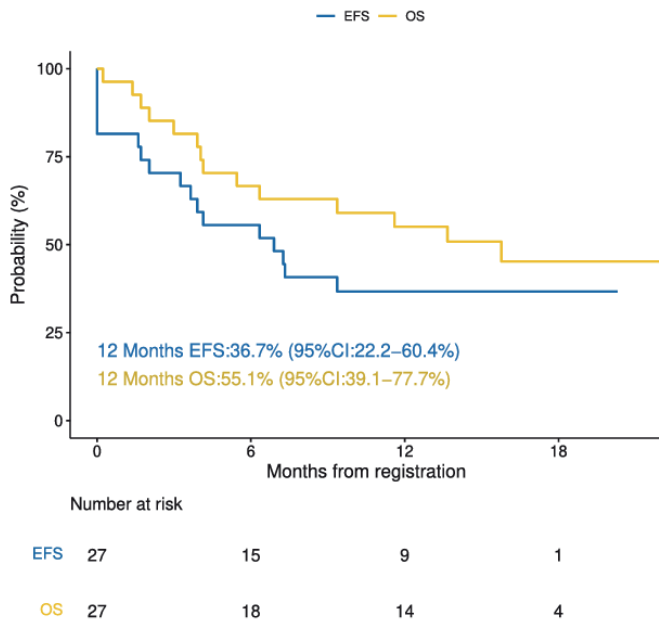
Figure 1. Event Free Survival and Overall Survival of Patients Treated in Phase II. Blue line: Event Free Survival (EFS); Yellow Line: Overall Survival (OS). CI Confidence Interval.

Table 2. Univariable Cox Model for EFS of all Patients Treated at RP2D (N = 40 Patients, Phase I and II Combined, 25 Events)

Variable name	Events	Number patients	HR	Lower 95% CI	Upper 95% CI	Level P	P value
Age at enrolment (per year)	25	40	1.191	0.542	2.617	0.6643	0.66
Sex							0.47
male	18	29	1				
female	7	11	1.377	0.574	3.305	0.4735	
Diagnosis							0.17
first relapse post HSCT	6	10	1				
2nd or greater relapse	13	23	1.052	0.399	2.775	0.9179	
refractory	6	7	2.452	0.783	7.681	0.1236	
Prior HSCT							0.19
no	15	20	1				
yes	10	20	0.589	0.264	1.314	0.1964	
Prior antibody therapy (blinatumomab)							0.22
no	18	31	1				
yes	7	9	1.713	0.714	4.108	0.2279	
PB WBC at screening (10^9/L)	25	40	0.821	0.374	1.806	0.6246	0.62
MFI - CD22+ expression	24	38	0.681	0.304	1.526	0.3505	0.35

PB: Peripheral Blood; WBC: White Blood Cells; MFI: Mean Fluorescence Intensity; HR: Hazard Ratio.

2.3.3 Safety

All patients (n=28) had at least one adverse event (AE), 20 (71.4%) at least one grade 3-4 AE. The most common AE was fever (n=16, 57.1%). Five (17.6%) patients had infection of grade ≥ 3, and six (21.4%) had febrile neutropenia. All patients had at least one grade 3-4 hematologic laboratory test abnormality (neutropenia being the most common, n=26, 92.9%). Only four patients still had thrombocytopenia grade 3/4 at day 22 of cycle one, of which one after day 42. Details are provided in Supplementary Table 7-9. A total of 26 serious adverse events were observed in 17 (60.71%), patients (Supplementary Table 10). Seven (25%, n=28) cases of SOS were reported; one grade 2, four grade 3 and two grade 4 (the latter six classified as "severe" according to the EBMT criteria).[24] Six cases occurred after HSCT post-treatment with InO, four resolved after treatment with defibrotide, and two might have contributed to death due to multi organ failure and infection. Another case of SOS occurred after one dose of InO in a patient treated due to relapse three months after HSCT. SOS resolved completely following administration of defibrotide. Including the phase I part of the study, nine SOS cases occurred in 52 treated patients (17.3%), of which six in the 23 transplanted patients (26.1%) (Supplementary Table 6). In a post-hoc analysis, when considering patients who developed SOS subsequently to transplant post-InO and those who did not, the median time interval between the last dose of InO and HSCT was shorter in patients developing SOS (24.5 (n=6) vs 54.5 days (n=17), p=0.01). The number of InO cycles received, previous HSCT, defibrotide prophylaxis, conditioning regimen with total body irradiation and dose level, were not statistically significant (Supplementary Table 6). No cases of toxic death considered related to InO were observed during study treatment. One patient died of encephalopathy considered by the local investigators attributable to prolonged intrathecal chemotherapy due to CNS leukemic involvement. Five additional non-relapse deaths occurred after HSCT due to multiple complications. The cumulative incidence of relapse was 29.63% (95% CI 13.77-47.42) at six months, and 40.74% (95% CI 22.03-58.69) at 12 months. The cumulative incidence of non-relapse death was 14.81% (95% CI 4.47-30.94) at six months, and 22.59% (95% CI 8.8-40.23) at 12 months, including post-HSCT follow-up (Supplementary Figure 3).

2.3.4 Pharmacodynamics

In vitro sensitivity to calicheamicin was available for 11 patients, of which 10 had MRD data available. In the latter group, MTT assays were performed, nine from BM and one from PB. IC_{50} values for calicheamicin ranged from 0.035 to 27.27 ng/ml. The median was twelve times higher in the five MRD-positive patients compared to MRD-negative patients (3.12 ng/ml vs 0.26 ng/ml, p=0.032, n=10; Figure 2). Furthermore, patients with IC_{50} values above the median seemed to have a poorer EFS (Supplementary Figure 4), despite not statistically significant (n= 5, p=0.19). Nevertheless, four of the five poor responders were treated at 1.4 mg/m²/cycle in phase I. The association of CD22 expression on leukemic blasts in BM samples obtained at baseline with response to InO was not statistically significant, neither the mean fluorescence intensity (MFI, range= 479-9619, p=0.37, n=49, Figure 3A), nor the percentage of CD22-positive cells

(range=53-100%, p=0.47, n=49, Figure 3B). Additionally, neither the level of saturation of CD22 antigens on PB leukemic blasts after the InO dose (samples taken prior and after infusion at day one) (range=23-100, p=0.52, n=32, Figure 3C), nor the level of internalization of InO after the first InO dose (range=0-90, p=0.55, n=32, Figure 3D) were associated to response. Alternative splicing was assessed by the number of split reads that included or excluded particular exons. The most prevalent alternative splicing variants of CD22, observed in our samples, were further analyzed. Multiple splicing isoforms involving the skipping of at least exon 2, which contains the start-codon for CD22 translation, were observed in all patients (Supplementary Figure 5). The splice variant of the CD22 transcript with exclusion of exons two to six (Δex2to6-CD22), encoding part of the extracellular domain, was seen most frequent with variable expression levels among patients. No correlation between alternative splicing of exon 2 and response was observed. Additionally, we did not find any association between the skipping or inclusion of exon 2 in the CD22 transcript and the expression of CD22 antigen on leukemic blasts, the saturation levels of CD22 on leukemic blasts with InO, or the internalization levels of InO (Supplementary Figure 6). Skipping of exon 5 and 6 was seen in all patients (supplementary figure 7), but did not influence the expression of CD22 antigen on leukemic blasts, the saturation levels of CD22 on leukemic blasts with InO, or the internalization levels of InO (Supplementary Figure 8).

Skipping of exon 12, suggested to negatively affect internalization of InO, was found in all patients (n=9, supplementary figure 9), but did not seem to affect internalization levels (Supplementary Figure 10).[25] RNA sequencing of leukemic cells was performed on nine patients with available material. Since calicheamicin acts by causing DNA double-strand breaks, leading to apoptosis of the cells, we looked at the expression of various anti- and pro-apoptotic genes, including *BCL2* gene family members.[9] No significantly different expression of apoptotic genes was seen in the leukemic cells of patients MRD negativity and those MRD positive (Supplementary Figure 11). Among the 52 patients treated in phase I and II, one (1.9%) patient had positive ADA (titer ≥ 2.30) against InO at baseline. The patient was treated at DL1 in Phase I and did not respond to InO. The presence of positive ADA at baseline was likely due to pre-existing host antibodies that were cross-reactive with InO and seemed not to impact on the PK. No treatment-boosted ADA responses were identified.
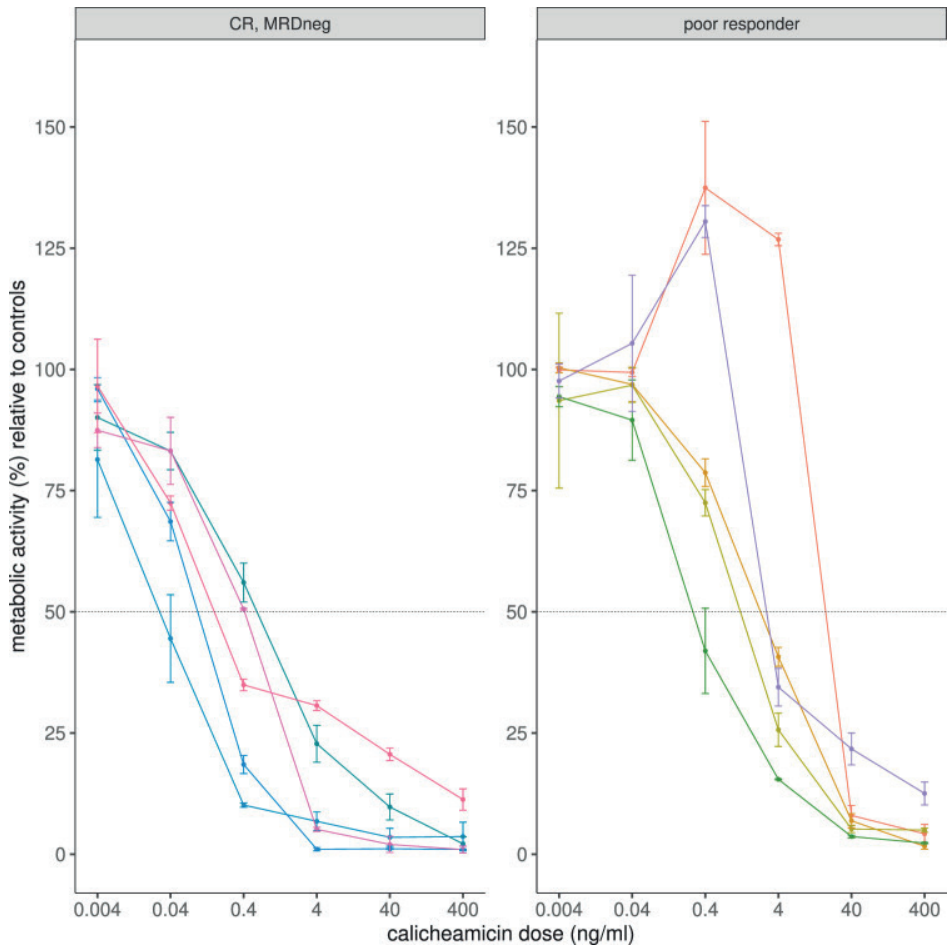
Figure 2. Dose-response Curves for Calicheamicin Based on MTT Assays. Each color represents a different patient. The intersection with the black line at 50% represents the IC50 value. IC50: concentration of drug required for 50% inhibition; MRD neg: minimal residual disease <10−4; CR: complete response; poor responders: no CR and/or MRD ≥ 10−4. The median IC50 value for all patients was 0.75 ng/ml (range 0.035−27.27; n = 10), median 0.26 ng/ml (range 0.035−1.05; n = 5) in the good responders (left panel) and median 3.12 ng/ml (range 0.34−27.27; n = 5) in the poor responders (right panel) (p = 0.032). From the literature, the median calicheamicin sensitivity in AML cells was 4.8 ng/ml, ranging between 0.1−1000 ng/ml (de Vries JF, et al.; 2012).
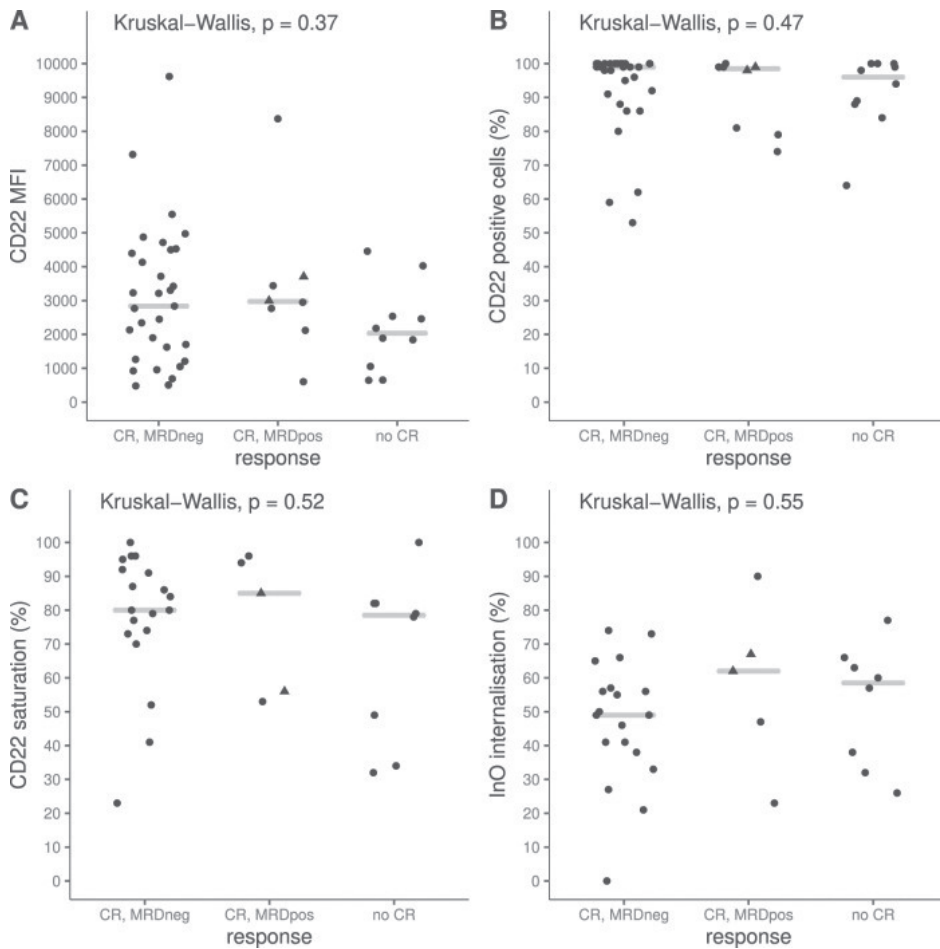
Figure 3. CD22 Expression on BM Blasts at Baseline, Saturation and InO Internalization on Leukemic PB Blasts Post Infusion on Day One. Presented as Mean Fluorescence Intensity (MFI) (A), percentage CD22-positive cells (B), saturation (C) and internalization (D). Grey horizontal lines represent the median value per group. In all four parameters, there were no statistically significant differences between the response groups as defined in the statistical methods for the PD analysis. Triangles represent patients with PCR-MRD quantitative range >10^{-4}.

2.4 Discussion

This phase II study provides further evidence for the activity of InO in R/R BCP-ALL pediatric patients. No clinical characteristic were found related to ORR or EFS (Supplementary Table 5). InO was generally well tolerated, with a low incidence of infections during treatment (17.8%). SOS remains the most serious AE (25%, n=7), although mostly occurring after subsequent HSCT and only occasionally while on treatment. Most SOS cases (5/7) resolved completely and no cases of toxic death considered related to InO or deaths in CR due to infections were observed during study treatment. Combining data from phase I (all dose levels) and II cohorts, SOS occurred in 26.1% of the patients transplanted post-InO, which is significantly lower than reported by Bhojwani et al. (52%, 11/21) but in line with data from O'Brien et al. (28.6%, 6/21), all treating patients at 1.8 mg/m². [14,17] Median time since the last InO dose appeared to be statistically significantly shorter in patients who developed SOS post HSCT. A possible explanation might be the long half-life of InO (12 days), and the inverse relationship between tumor load and the time dependent component of its clearance, generally longer after few weeks of treatment. [26] Therefore, InO might still be circulating during the first month after treatment, particularly in patient achieving CR early on treatment. The use of prophylactic defibrotide was left at investigators' discretion, therefore not uniformly performed. This makes difficult to assess its impact as a protective factor for SOS in this small cohort. Taken together, the relationship between risk factors and SOS occurrence should be investigated in larger series.

As suggested in other studies, the sensitivity of ALL cells to calicheamicin might contribute to the achievement of MRD negativity. [9] When considering all patients from phase I and II, including those treated at 1.4 mg/m²/cycle, MRD negativity was found to correlate to calicheamicin sensitivity in vitro but not to CD22 surface expression, saturation, or internalization. Although CD22 expression is needed for binding of InO, high levels of CD22 expression on leukemic blasts, saturation of CD22 with InO and internalization of InO do not appear to be crucial for clinical response to InO. Our findings are in line with previous *in vitro* studies from our group on BCP-ALL cells which showed that, although CD22 expression was essential for InO binding, efficacy was not dependent on CD22 expression levels, while a clear correlation with calicheamicin sensitivity was noticed. [9] In contrast, COG reported lower baseline CD22 density (measured as antibody bound per cell), and a reduction of CD22 percentage over time in the poor responders. [17] Nevertheless, they did not find CD22 percentage at baseline as significant, but only four subjects had a CD22 expression < 90%. Instead, CD33 expression on leukemic blasts does affect clinical response of AML patients to Gemtuzumab Ozogamicin. [18,27,28] This may be due to AML cells being less sensitive to calicheamicin, higher levels of CD33 on leukemic blasts, higher levels of CD33 saturation, and a continuous loop of internalization and renewed expression of CD33 antigens might be required for a sufficient accumulation of calicheamicin inside AML cells; whereas in ALL cells lower levels of accumulated calicheamicin might be sufficient to cause apoptosis. [9] In adults treated with chemotherapy and InO, baseline CD22 expression <70% was

independently associated with worse survival, while ORR and MRD negativity rate could not be identified as significantly different in the two groups.[20] Moreover, a significant correlation between higher median baseline CD22 levels and achievement of MRD-negative CR was found in patients treated with anti-CD22 CAR-T cells.[29]

In our cohort, no correlation between CD22 expression and ORR and MRD was found, but only a limited number of patients (n=4) had a CD22 expression <70%. CD22 exon 2 seems to be crucial for both initiating RNA translation into a protein product and for the binding of CD22-directed antibody.[30] One patient with only the Δex2 CD22 splice isoform has been reported resistant to InO.[30] In our study, alternative splicing of CD22 was observed in all patients, especially the Δex2-6 variant, but did not correlate with response, probably because all patients had at least some normal CD22 expression. Skipping of exon 5-6 (binding region of RFB4 antibody) was observed only in a limited percentage which might explain why it did not correlated to CD22 MFI. Similarly, skipping of CD22 exon 12, previously reported as potentially reducing InO internalization, occurred in all patients samples in our cohort (n=9) at various extent, but did not correlate to lower internalization levels.[25] These findings underscore that high levels of full-length CD22 might not be necessary to respond to InO. Nevertheless it is worth noting that CD22 surface expression was tested before study inclusion by using anti-CD22 RFB4 antibody and only CD22-positive cases were included. Our findings confirmed evidence of CD22 negative/ dim relapses after treatment with anti-CD22 CAR T-cells, suggesting CD22 downmodulation as possible mechanism of acquired resistance.[29] A RNA sequencing analysis pre- and post-relapse, which could better highlight the mechanism of CD22 downmodulation, was not performed. Previous studies suggested gene expression as possible cause of resistance to calicheamicin, but we did not find significant differences between the response groups in the expression of known genes of the apoptotic pathway such as BCL2, albeit in a small sample size.[31,32]

InO is currently tested in front-line treatment by the COG in a phase III randomized trial for high-risk CD22-positive BCP-ALL (NCT03959085), and in the ALLTogether1 protocol, for patients stratified to the intermediate-high risk group (NCT03911128). The ITCC-059 study is ongoing, testing InO in combination with chemotherapy in R/R pediatric ALL and as single agent in very high risk first relapse ALL.

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Supplementary Material

Supplementary Table 1. Inclusion and Exclusion Criteria

Inclusion Criteria	
Age	≥1 and <18 years at time of enrollment The first three patients on dose level 1 must be ≥6 and <18 years Then ≥2 additional patients ≥1 year and <6 years at the same dose level
Diagnosis	First relapse of BCP-ALL post allogeneic HSCT Second or greater R/R BCP-ALL Refractory disease (newly diagnosed patients who had induction failures after ≥2 previous regimens without attainment of remission, or patients with refractory first relapse after one previous reinduction regimen without attainment of remission) **<u>AND</u>**: M2 or M3 marrow status (≥5% blasts by morphology) Malignant clone CD22 surface antigen positive (in either bone marrow or peripheral blood) by institutional standards and measured by the routine diagnostic method of the local laboratory and reported as positive or negative according to the local interpretation of the data (no specific cut-off was used). The first six patients must have M3 marrow status (≥25% blasts by morphology)
Performance level and life expectancy	Karnofsky >60% (>16 years) or Lansky >60% (≤16 years) Life expectancy of ≥6 weeks

	Patients must have recovered from the acute toxic effects of all prior therapy, defined as resolution of non-hematologic toxicities to ≤Grade 2 per the CTCAE 4.03 prior to entering the study
	Chemotherapy ≥7 days since the completion of cytotoxic therapy (exceptions: hydroxyurea, 6-mercaptopurine and steroids which are permitted up until 48 hours prior to initiating protocol therapy)
	Radiotherapy ≥28 days since any prior radiation therapy
	Hematopoietic stem cell transplant ≥90 days since previous allo-HSCT No evidence of active graft vs host disease No GVHD prophylaxis or treatment
Prior therapy	Hematopoietic growth factors ≥7 days since the completion of therapy with GCSF or other growth factors, or ≥14 days since completion of therapy with pegfilgrastim (Neulasta®) Immunotherapy ≥42 days after the completion of any type of immunotherapy, e.g. CART therapy. Patients may not have received prior CD22-targeted therapy (immunotoxin or CART therapy)
	f. Monoclonal antibodies ≥3 half-lives of the antibody must have elapsed after the last dose of a monoclonal antibody (rituximab = 66 days, epratuzumab = 69 days) Exclusion of blinatumomab: patients must have been off blinatumomab infusion for ≥14 days and all drug-related toxicity must have resolved to ≤Grade 2
	g. Investigational drugs ≥7 days or five drug half-lives (whichever is longer) since prior treatment with any experimental drug (with the exception of monoclonal antibodies) under investigation. No residual toxicities should be observed following previous treatment
	h. Prior calicheamicin exposure Patient has not received prior treatment with a calicheamicin conjugated antibody (e.g. gemtuzumab ozogamicin)
Renal and hepatic function	Serum creatinine ≤1.5 x institutional ULN according to age AST and ALT ≤2.5 x institutional ULN Total bilirubin ≤1.5 x institutional ULN unless the patient has documented Gilbert syndrome
Cardiac function	Shortening fraction ≥30% by echocardiogram or an ejection fraction >50% by MUGA.
Reproductive function	Female patients of childbearing potential: negative urine or serum pregnancy test confirmed prior to enrollment Female patients with infants must agree not to breastfeed on study Male and female patients of child-bearing potential must agree to use a *highly effective* method of contraception (≥8 months for females and for ≥5 months for males after the last dose of InO)

Exclusion Criteria	
Isolated extramedullary relapse	Patients with isolated extramedullary disease are excluded
VOD/SOS	Any history of prior or ongoing VOD/SOS as per modified Seattle criteria, or prior liver-failure [defined as severe acute liver injury with encephalopathy and impaired synthetic function (international normalized ratio of ≥1.5)]
Infection	Systemic fungal, bacterial, viral or other infection that is exhibiting ongoing signs/symptoms The patient may not have: A requirement for vasopressors Positive blood culture within 48 hours of study enrollment Fever above 38.2 degrees Celsius within 48 hours of study enrollment with clinical signs of infection. Fever that is determined to be due to tumor burden is allowed, with documented negative blood cultures for ≥48 hours prior to enrollment and no concurrent signs or symptoms of active infection or hemodynamic instability A positive fungal culture within 30 days of study enrollment Active fungal, viral, bacterial, or protozoal infection requiring intravenous or oral treatment. Chronic prophylaxis therapy to prevent infections is allowed
Other anti-cancer therapy	Patients will be excluded if there is a plan to administer non-protocol anti-cancer therapy during the study period
Allergic reaction	Patients with prior Grade 3/4 allergic reaction to a monoclonal antibody are excluded
Concurrent disease	Significant concurrent disease, illness, psychiatric disorder or social issue that would compromise patient safety or compliance with protocol therapy, interfere with consent, study participation, followup, or interpretation of study results Children with Down syndrome are excluded from participation in the dose finding parts of the study

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; CART, chimeric antigen receptor T cell; GCSF, granulocyte-colony stimulating factor; GVHD, graft versus host disease; HSCT, hematopoietic stem cell transplant; InO, inotuzumab ozogamicin; MUGA, multiple gated acquisition scan; R/R, relapsed/refractory; SOS, sinusoidal obstruction syndrome; ULN, upper limit of normal; VOD, veno-occlusive disease.

Supplementary Table 2. Definitions of Response

Response Category	Criteria*
Complete Response	No evidence of circulating blasts or extramedullary disease; including CNS-1 status; absence of splenomegaly, lymphadenopathy, skin/gum infiltration, testicular mass A bone marrow with <5% blasts (M1 marrow) Recovery of peripheral counts (platelets >50,000/μL and transfusion independent, and ANC >500/μL)
Complete Response with Insufficient Platelet Recovery	No evidence of circulating blasts or extramedullary disease A bone marrow with <5% blasts (M1 marrow) An ANC > 500/μL but Platelets ≤ 50,000/μL
Complete Response without Recovery of Counts	No evidence of circulating blasts or extramedullary disease A bone marrow with <5% blasts (M1 marrow) An ANC ≤ 500/μL and / or Platelets ≤50,000/μL
Partial Response	Greater than 50% relative reduction (with a minimum of 10% absolute reduction) in the bone marrow aspirate leukemic cell count, irrespective of recovery of the peripheral blood counts
Stable Disease/ No Response	Stable disease is present when the patient fails to qualify for CR, CRi, PR, or PD
Progressive Disease	Progressive disease is defined as an increase of at least 25% of the absolute number of bone marrow or circulating leukemic blasts, development of extramedullary disease, or other laboratory or clinical evidence of PD, with or without recovery of ANC or platelets
Relapse	After documentation of remission, a bone marrow aspirate and/or biopsy showing ≥5% leukemic blasts using morphology with flow cytometric confirmation, and/or pathological/radiological evidence of extramedullary disease, including development of CNS3 status or clinical CNS-involvement with radiological confirmation (MRI).
Refractory	Any patient not achieving CR, CPp or CRi after induction therapy (cycle 1 and/or cycle 2).

ANC: Absolute Neutrophile Count; CNS 1/2/3: Central Nervous System Disease Status; CR: Complete Response; CRi: Complete Response without Recovery of Counts; CRp: Complete Response with Insufficient Platelet Recovery; PD: Progressive Disease; SD: Stable Disease.

* All criteria must be present to define each response category

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Supplementary Table 3. Study Endpoints

Primary endpoints

ORR, defined as the percentage of patients with CR, CRi, CRp, measured as best response during InO treatment

Secondary endpoints

1. Safety:
- AEs, as characterized by type, frequency, severity (as graded using CTCAE v4.03), timing, seriousness, and relation to study therapy, during the first and subsequent cycles of therapy.
- Occurrence of toxic death; i.e., death attributable to InO therapy.
- Occurrence of VOD/SOS during or after therapy with InO.
- Laboratory abnormalities as characterized by type, frequency, severity and timing.
- The cumulative incidence of non-relapse mortality, defined as the cumulative probability of non-relapse mortality, with time calculated between start of study treatment and death due to other causes than relapsed or refractory leukemia or lymphoma, accounting for competing events.

2. Other measures of anti-leukemic activity:
- ORR after cycle 1.
- Minimal residual disease levels, including the percentage of patients who become MRD-negative (complete MRD response defined as an MRD-level $< 1 \times 10^{-4}$), after cycle 1, as well as the best response (MRD-negativity) over multiple cycles.
- Duration of response, defined as the time between achieving response (CR, CRi or CRp) after starting study treatment and documented relapse or death.
- Number and percentage of patients being transplanted and those receiving CAR T-cell therapy after treatment with InO.
- EFS, defined as the time between start of study treatment and first event including failure to achieve CR/CRp/CRi (calculated as an event on day 0), relapse, death of any cause and second malignancies.
- Survival, defined as time to death following start of study treatment.
- The cumulative incidence of non-response or relapse, defined as the cumulative probability of non-response or relapse, with time calculated between start of study treatment and relapse and with non-responders included as an event on day 0. Non-relapse death is considered a competing event.

3. Serum pharmacokinetic parameters of InO and unconjugated calicheamicin.

4. Pharmacodynamics parameters[*]
- Relationship between response (ORR) and CD22 expression levels and WBC.
- Relationship between response (ORR) and CD22 saturation kinetics.
- Relationship between response (ORR) and calicheamicin sensitivity.
- Clonal evolution (CD22-negativity) and relation to loss of response.

Other endpoints

The percentage of patients responding to InO (ORR) without adequate recovery of CD19-positive B-cells (below LLN for age) or immunoglobulins (below LLN for age) following 4 weeks, 10 weeks, 3, 6 and 12 months after treatment with InO, excluding patients who have been transplanted from the date of HSCT or have received CAR-T cells therapy.

Percentage of patients who exhibit ADA

LLN: lower limit for normality; ADA: anti-drug antibodies; WBC: with blood cells; ORR: overall response rate; MRD: Minimal residual disease; SOS/VOD: Sinusoidal occlusive syndrome/veno-occlusive disease; EFS: event free survival; HSCT: Hematopoietic stem cell transplantation. * in post hoc analysis we also investigated the relationship between PD parameters and achievement of MRD negativity as defined in the main paper.

Supplementary Table 4. Definitions of Outcome Measurements

Term	Definition
Overall response rate (ORR)	defined as complete remission (CR), CR with incomplete hematologic recovery (CRi), or CR with incomplete platelet recovery (CRp; response criteria followed standard procedures in leukemia, as designed by the National Comprehensive Cancer Network, with slight modifications in relation to bone marrow regeneration [CR = platelets >30,000/μL rather than >100,000/μL; and ANC >500/μL rather than >1000/μL]).
Minimal Residual Disease (MRD) status:	bone marrow negative if MRD $<1\times10^{-4}$ with real-time quantitative-PCR or <0.01% with multi-parameter flow cytometry according to EuroFlow protocols when PCR was negative but the QR was $> 10^{-4}$.[1,2]
Event-free survival (EFS)	defined as time from start of treatment to first event including failure to achieve CR/CRp/CRi, relapse, death, and second malignancies.
Cumulative incidence of non-response or relapse	defined as the cumulative probability of non-response or relapse, with time calculated between start of study treatment and relapse and with non-responders included as an event on Day 0. Non-relapse death is considered a competing event.
Cumulative incidence of non-relapse mortality	defined as the cumulative probability of non-relapse mortality, with time calculated between start of study treatment and death due to other causes than relapsed or refractory leukemia or lymphoma, accounting for competing events.
Duration of response	defined as the time between achieving response (CR, CRi or CRp) after starting study treatment and documented relapse or death
Overall survival (OS)	defined as time to death following start of study treatment.

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Additional Specifications on the Methodology

Supplementary Methods 1. CD22 Saturation and Internalization

Saturation was defined as (Eq. 1)

$$\frac{\text{(specific fluorescence intensity of bound Ino)}}{\text{(specific fluorescence intensity maximal Ino binding)}} \cdot 100\%$$

Internalisation was defined as (Eq. 2)

$$1 - \left(\frac{\text{specific fluorescence intensity of bound Ino}_{\tau+1}}{\textit{specific fluorescence intensity maximal Ino binding}_{\tau}}\right) \cdot 100\%$$

Where τ stands for time. Data were acquired on a FACSCanto flow cytometer (BD Biosciences) using EuroFlow instrument settings and analyzed using DIVA (BD Biosciences) and Infinicyt (Cytognos) software.

Supplementary Methods 2: Determination of MRD Levels by PCR and Flowcytometry

Molecular MRD levels were centrally determined by RQ-PCR of leukemia-specific rearranged immunoglobulin (IG) and T-cell receptor (TR) genes (van der Velden and Van Dongen, 2009). Quality control and standardized interpretation of RQ-PCR data were achieved following the guidelines of the European Study Group on MRD detection in ALL (EuroMRD) (Van der Velden et al, Leukemia 2007). For flowcytometric MRD analysis, also centrally performed, bone marrow samples were bulk-lysed and subsequently stained using 8 color stainings according to EuroFlow protocols (Theunissen et al, Blood 2017; Kalina et al, Leukemia 2012). Four million cells (if available) were acquired and MRD positivity was defined if at least 20 ALL cells could be detected. Flow MRD negativity was defined as MRD < 0,01% using an assay with a sensitivity of at least 0,01%. MRD negativity was defined as PCR below 10^{-4} or flow cytometry below 0.01% when PCR was negative but the Quantitative Range was above 10^{-4}.

Supplementary Table 5. Risk Factors Analysis for MRD Response of all Patients Treated at RP2D (N = 40 patients, phase I and II combined)

	MRD neg	MRD pos	P value
Age at enrollment			0.73
<10 years	13	7	
>=10 years	14	5	
Sex			0.26
male	21	7	
female	6	5	
Diagnosis			0.27
first relapse post allogeneic HSCT	7	2	
second or greater relapsed	17	6	
refractory	3	4	
Prior HSCT			0.73
no	13	7	
yes	14	5	
Prior antibody therapy (blinatumomab)			0.42
no	22	8	
yes	5	4	
PB WBC at screening			0.73
<median	13	7	
>=median	14	5	
IC50 Calicheamicin (N=10)			0.21
<median	4	1	
>=median	1	4	

HSCT: Hematopoietic Stem cell transplant; PB WBC: Peripheral Blood With Blood Count; IC50: values represent the concentration of the drug which inhibits 50% of the leukemic cell

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Supplementary Table 6. SOS Cases in Patients Receiving Transplantation after InO for Combined Cohort of Phase I and Phase II

Age at enrollment	Conditioning regimen	Defibrotide prophylaxis	Previous HSCT	Days since last InO dose	Number of InO courses	Dose level* (mg/m²)	SOS	Grade	Outcome SOS
6	Etoposide; TBI	no	no	125	1	1.8	no		
4	Fludarabine; Thiotepa; treosulfan	no	no	83	1	1.4	no		
14	Fludarabine; busulfan; Thiotepa	na	no	99	1	1.8	no		
12	Etoposide; TBI	no	no	26	2	1.4	no		
11	Fludarabine; treosulfan; Thiotepa	no	no	23	2	1.4	no		
15	Etoposide; TBI	na	no	39	2	1.8	no		
16	Fludarabine; melphalan; Thioptepa	na	yes	51	1	1.8	no		
4	Etoposide; TBI	yes	yes	55	2	1.8	no		
13	Etoposide; TBI	yes	no	47	3	1.8	yes	3	Resolved
6	Fludarabine; Thiotepa; TBI	no	no	72	4	1.8	no		
12	Fludarabine; busulfan; Thiotepa	no	yes	54	3	1.8	no		
2	Busulfan; fludarabine; Thiotepa	yes	no	27	2	1.8	yes	3	Resolved

Age at enrollment	Conditioning regimen	Defibrotide prophylaxis	Previous HSCT	Days since last InO dose	Number of InO courses	Dose level* (mg/m²)	SOS	Grade	Outcome SOS
1	fludarabine; treosulfan; thiotepa	yes	no	35	2	1.8	no		
8	treosulfan; fludarabine; thiotepa	no	yes	20	1	1.8	yes	3	Ongoing at time of death (due to MOF)
14	fludarabine; TBI	yes	no	22	2	1.8	no	3	Resolved
14	fludarabine; TBI	yes	no	22	2	1.8	yes	2	Resolved
13	etoposide; TBI	yes	yes	110	1	1.8	no		
5	etoposide; TBI	no	no	25	3	1.8	no		
7	thiotepa; fludarabine	no	no	70	2	1.8	no		
7	etoposide; TBI	no	no	182	2	1.8	no		
13	etoposide; TBI	no	yes	78	2	1.8	no		
4	etoposide; TBI	yes	yes	35	1	1.8	yes	4	Resolved
8	etoposide; TBI	yes	yes	43	1	1.8	no		
17	etoposide; TBI	yes	no	22	3	1.8	yes	4	Ongoing at time of death (due to MOF)

To identify the risk factors for SOS, Fisher's exact test (for categorical variables) and Mann-Whitney U test (for continuous variables) were used to compare the patients who received transplantation after Inotuzumab Ozogamicin (InO) (6 who later developed SOS vs 18 who did not). Days from last InO dose were found to be statistically significant with a p value of 0.014. The median value of days from last InO dose in patients with SOS was 24.5 (IQR: 21.5-38) while the median in patients with no SOS was 54.5 (IQR: 30.5-75). Dose level 1.4 mg/m2 refers to phase I only. Conditioning regimen with TBI (p=1.0), age at enrollment (p=0.84), defibrotide prophylaxis (p=0.06), previous HSCT (p=1.0), and number of InO courses received (p=0.69) were not statistically significant. SOS: sinusoidal obstruction syndrome; HSCT hematopoietic stem cell transplantation; TBI: total body irradiation; MOF: multi organ failure. SOS was diagnosed based on Seattle criteria and was graded according to the CTCAE v. 4.03 under hepatobiliary disorders, and AE term: Other, specify.

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Supplementary Table 7. AEs listing Grade 3 and 4 during phase 2 (N=28) reported by the local investigators as clinically significant (worst grade per term per patient)

| AE term Grade	3	4	Total
Alkaline phosphatase increased	1	0	1
Anal mucositis	1	0	1
Anal pain	1	0	1
Arthralgia	1	0	1
Aspartate aminotransferase increased	5	0	5
Blood bilirubin increased	1	1	2
Cardiac disorders - Other specify: Hypotension	1	0	1
Catheter related infection	1	0	1
Constipation	1	0	1
Cytokine release syndrome	1	0	1
Depressed level of consciousness	0	1	1
Febrile neutropenia	6	0	6
Fever	2	0	2
gamma-glutamyl transferase increased	1	0	1
Hematoma	4	2	6
Hepatobiliary disorders - sinusoidal obstruction syndrome	1	0	1
Hypokalemia	2	0	2
Hypotension	2	0	2
Immune system disorders - engraftment syndrome	1	0	1
Infections and infestations - infection CMV	1	0	1
Infections and infestations - Septicemia from Pseudomonas plecoglossicida	1	0	1
Left ventricular systolic dysfunction	0	1	1
Lung infection	2	0	2
Mucositis oral	2	0	2
Rash maculo-papular	1	0	1
Sepsis	2	0	2
Sinus tachycardia	0	1	1
Tumor lysis syndrome	3	0	3
Upper gastrointestinal hemorrhage	1	0	1
Vascular disorders - Other specify: hematoma legs	1	0	1
Vomiting	1	0	1
Weight gain	1	0	1

Supplementary Table 8. List of Chemistry Laboratory Abnormalities and Grade (highest toxicity grade per patient) as Compared to Local Normal Ranges

Grades	0	1	2	3	4	NA	Total
Test Abnormality							
Albumin (low)	5	13	9	0	0	1	28
Alkaline phosphatase (high)	22	6	0	0	0	0	28
ALT (high)	2	16	5	5	0	0	28
Amylase (high)	23	2	2	0	0	1	28
AST (high)	3	16	5	4	0	0	28
Calcium (low)	12	12	3	0	1	0	28
Creatinine	21	7	0	0	0	0	28
GGT (high)	6	8	8	3	0	1	26
Lipase (high)	20	2	4	2	0	0	28
Phosphate (low)	19	3	3	3	0	0	28
Potassium (low)	16	9	0	3	0	0	28
Sodium (low)	15	12	0	1	0	0	28
Total Bilirubin (high)	22	4	1	1	0	0	28
Uric acid (high)	19	7	0	0	0	2	28

The highest toxicity grade per patient was counted only once per patient. This table includes also abnormalities not reported by the local investigator as AEs.

Supplementary Table 9. List of Hematologic laboratory abnormalities and grade (highest toxicity grade per patient) as compared to local normal ranges

Grade	0	1	2	3	4	Total
Test Abnormality						
Absolute neutrophil count decrease	1	1	0	2	24	28
Hemoglobin decrease	1	3	16	8	0	28
Platelet count decrease	0	3	3	5	17	28
White blood cell count decrease	1	1	1	7	18	28

The highest toxicity grade per patient was counted only once per patient. This table includes also abnormalities not reported by the local investigator as AEs.

Supplementary Table 10. Serious Adverse Events (SAE) observed in the phase II

SAE Term	SAE Category	Grade	Outcome
Intestinal massive hemorrhage	Life threatening	4	Ongoing
Febrile neutropenia	Prolongation of hospitalization	3	Resolved
Sepsis	Prolongation of hospitalization	3	Resolved
Febrile neutropenia	Prolongation of hospitalization	3	Resolved
Febrile neutropenia	Prolongation of hospitalization	3	Resolved
Febrile neutropenia	Prolongation of hospitalization	3	Resolved
Catheter related infection	Prolongation of hospitalization	3	Resolved
Hematuria	Prolongation of hospitalization	2	Resolved
Engraftment syndrome	Life threatening	3	Resolved
Mycosis pneumopathy	Life threatening	4	Death
Sinusoidal obstruction syndrome	Prolongation of hospitalization	3	Resolved
Encephalopathy	Death	5	Death
Maculo-papular rash	Prolongation of hospitalization	2	Resolved
Upper respiratory tract infection	Prolongation of hospitalization	2	Resolved
Sepsis	Prolongation of hospitalization	4	Resolved
Sinusoidal obstruction syndrome	Life threatening	4	Resolved
Pain	Prolongation of hospitalization	3	Resolved
Sinusoidal obstruction syndrome	Life threatening	4	Ongoing
Febrile neutropenia	Prolongation of hospitalization	2	Resolved
Bacteremia	Other medically important condition	2	Ongoing
Sinusoidal obstruction syndrome	Other medically important condition	3	Resolved
Multi organ failure	Death	5	Death
Sinusoidal obstruction syndrome	Other medically important condition	3	Ongoing
Sinusoidal obstruction syndrome	Other medically important condition	2	Resolved
Disease progression	Death	5	Death
Sinusoidal obstruction syndrome	Other medically important condition	3	Resolved

Duration of response in months
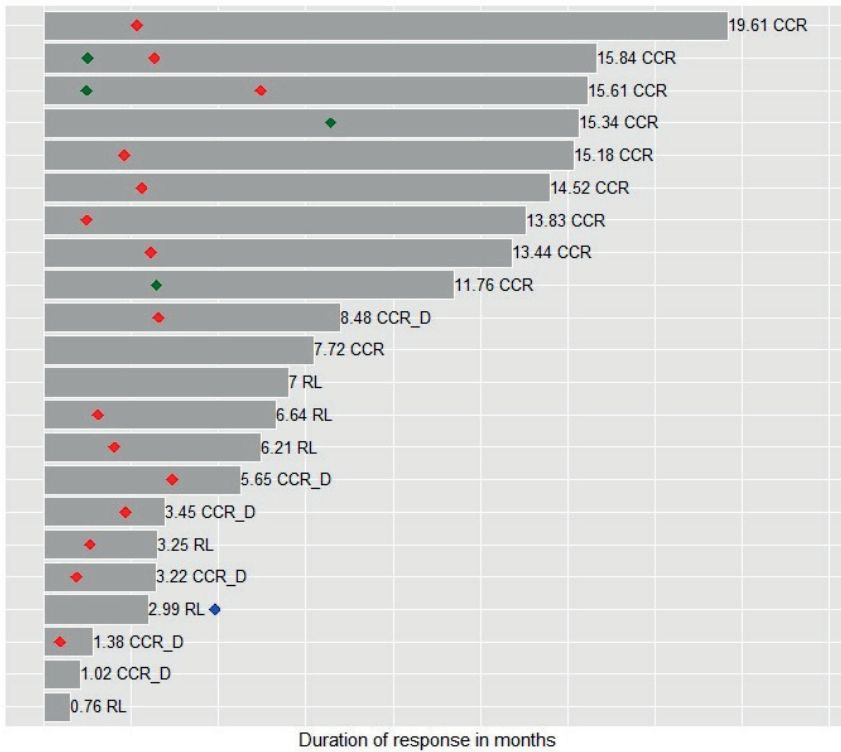
Supplementary Figure 1. Swimmer plot (n=22). CCR: Continuous Complete Remission; RL: Relapse; CCR_Death: Death while in Continuous Complete Remission. Red squares: HSCT treatment; Blue square: in this patient HSCT was performed after additional treatment with blinatumomab due to loss response; Green squares: CAR-T treatment. Numbers at the end of each bar represent the duration of response in months. Four patients were non-responders (not reported here).
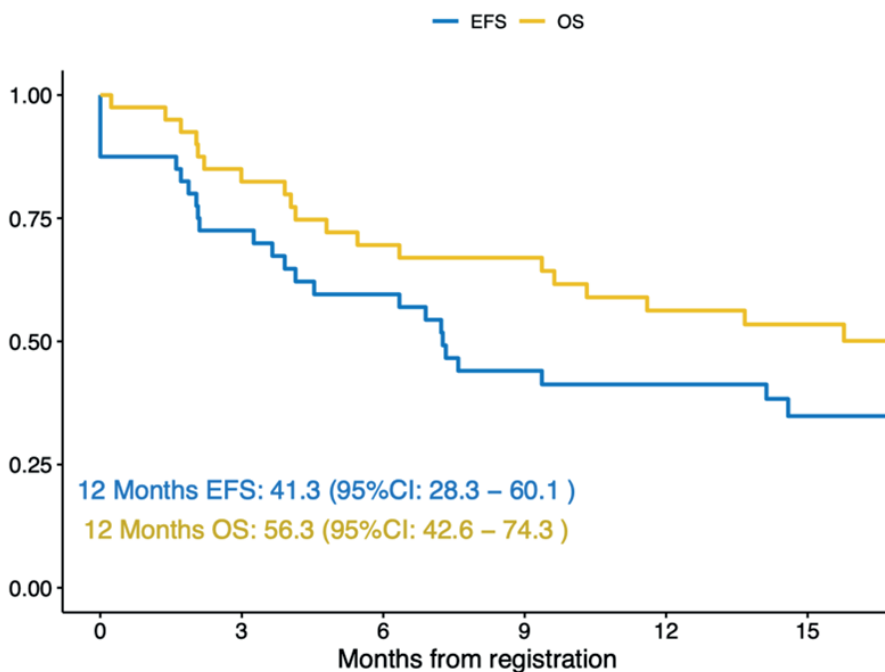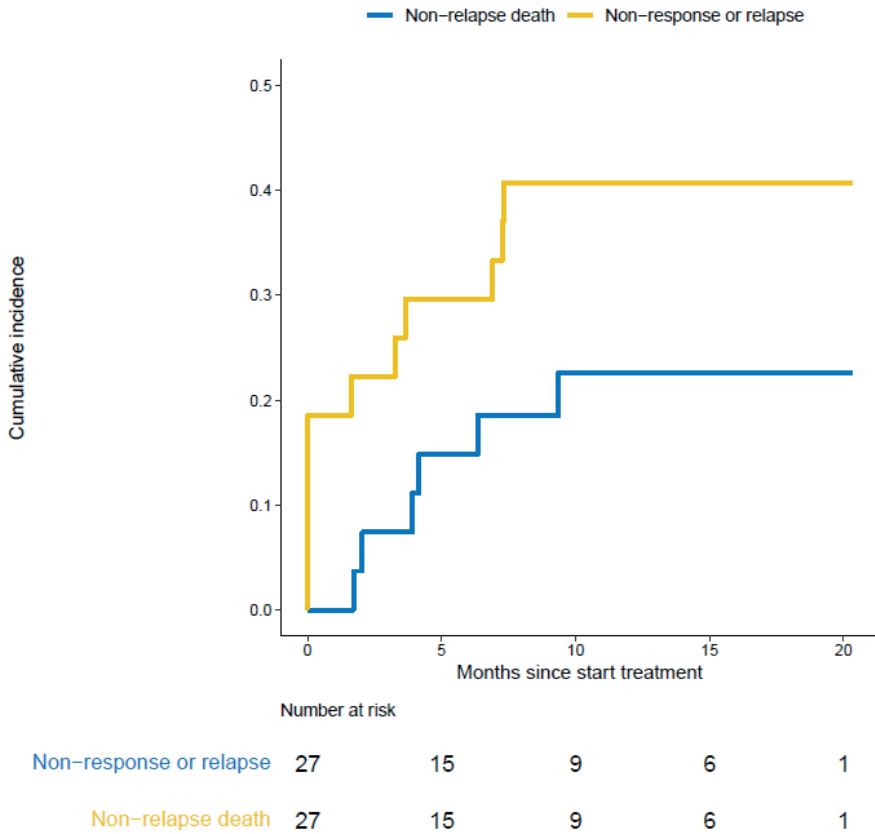
Supplementary Figure 2. EFS and OS for all patients from phase I treated at the RP2D, and phase II patients (n=40). Number at risk is presented below the graph. Blue line: Event Free Survival. Yellow line: Overall Survival. Non responders are added as event on day 0. RP2D: Recommended Phase II dose = 1.8 mg.m²

Supplementary Figure 3. Cumulative incidence of refractory/relapse and non-relapse death. The number at risk is presented below the graph. Blue line: refractory/relapse cumulative incidence. Yellow line: non-relapse death cumulative incidence. Non responders are added as event on day 0. Five non-relapse death occurred after hematopoietic stem cell transplant procedure.
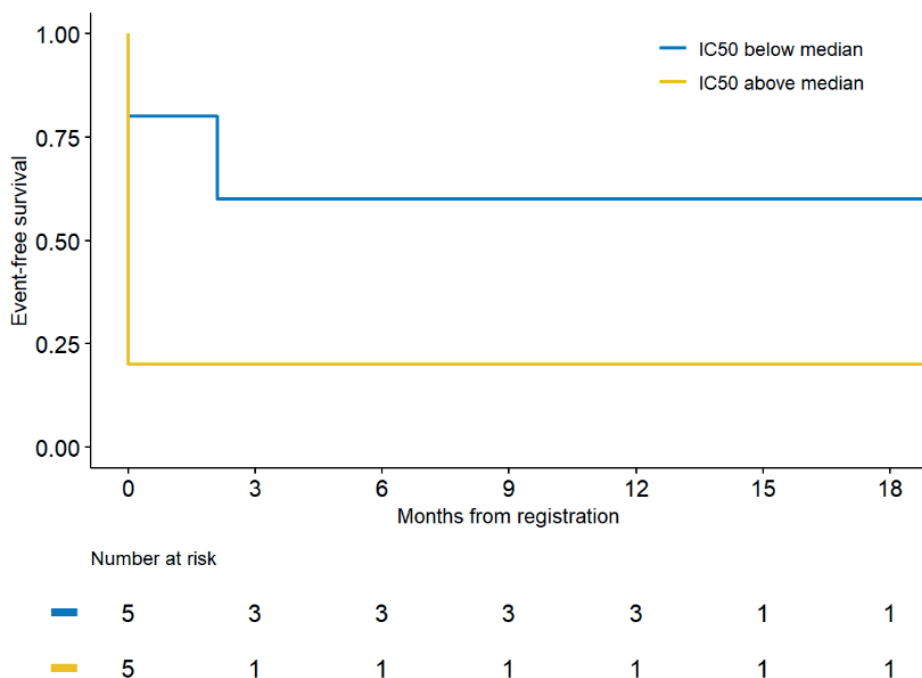
Supplementary Figure 4. EFS in patients with calicheamicin IC$_{50}$ above the median (yellow line) and in those with calicheamicin IC$_{50}$ below the median (blue line), n=10. The event definition is this graph differs in that not achieving MRD neg CR is calculated as an event at day 0. Number at risk is presented below the graph. Patients with in vitro calicheamicin sensitivity (IC50) below the median have a better one year EFS than patients with an IC50 above the median (63% (95%CI 29.3-100%) versus 20% (95%CI 3.46-100%)), although the difference is not statistically significant (p=0.19).
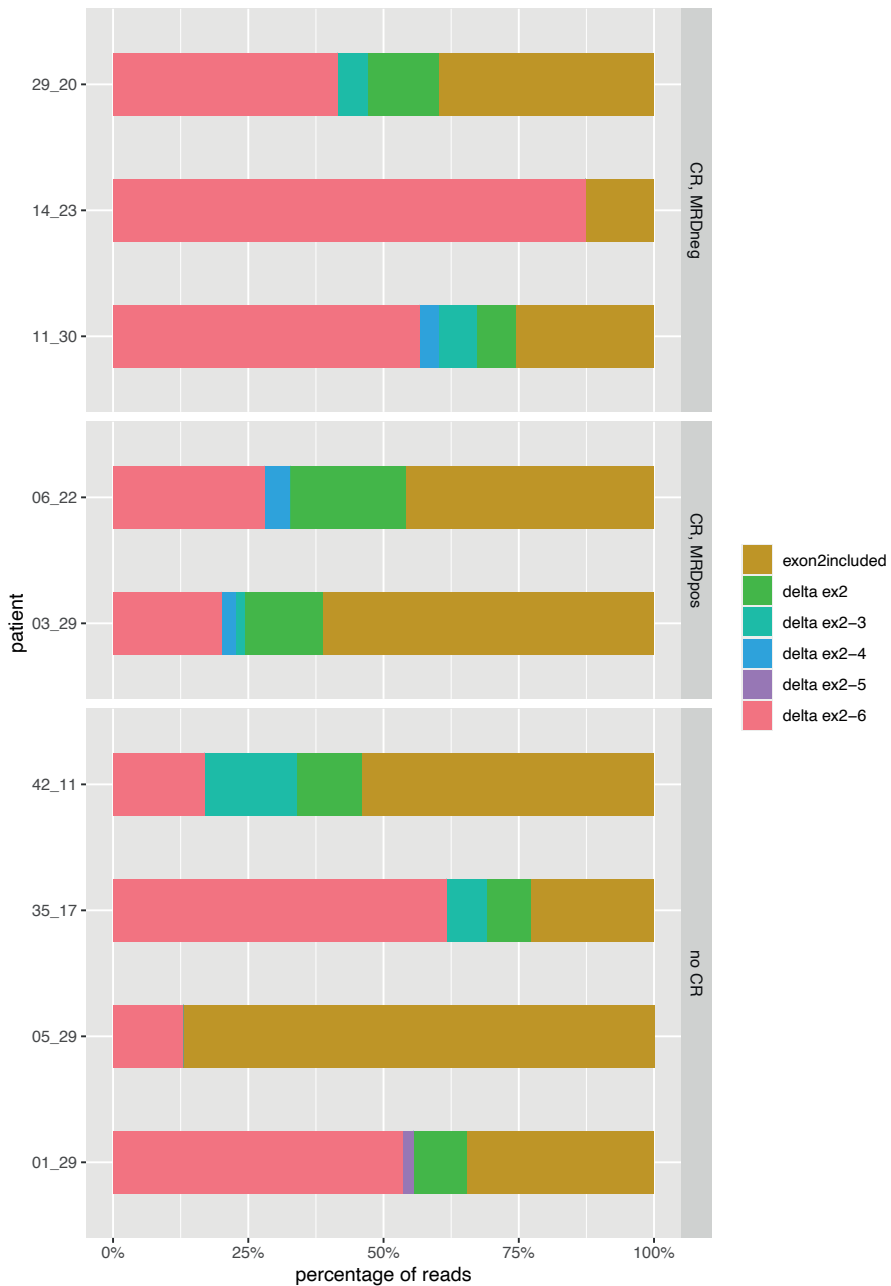
Supplementary Figure 5. Splice variants concerning exon 2 of the CD22 transcript in the 9 patients with RNA sequencing data available. Percentages represent the number of split reads either in- or excluding the exons as percentage of the total number of split reads. All patients had at least some RNA copies including exon 2. The variant delta ex2-6 seems the most prevalent in this sample.
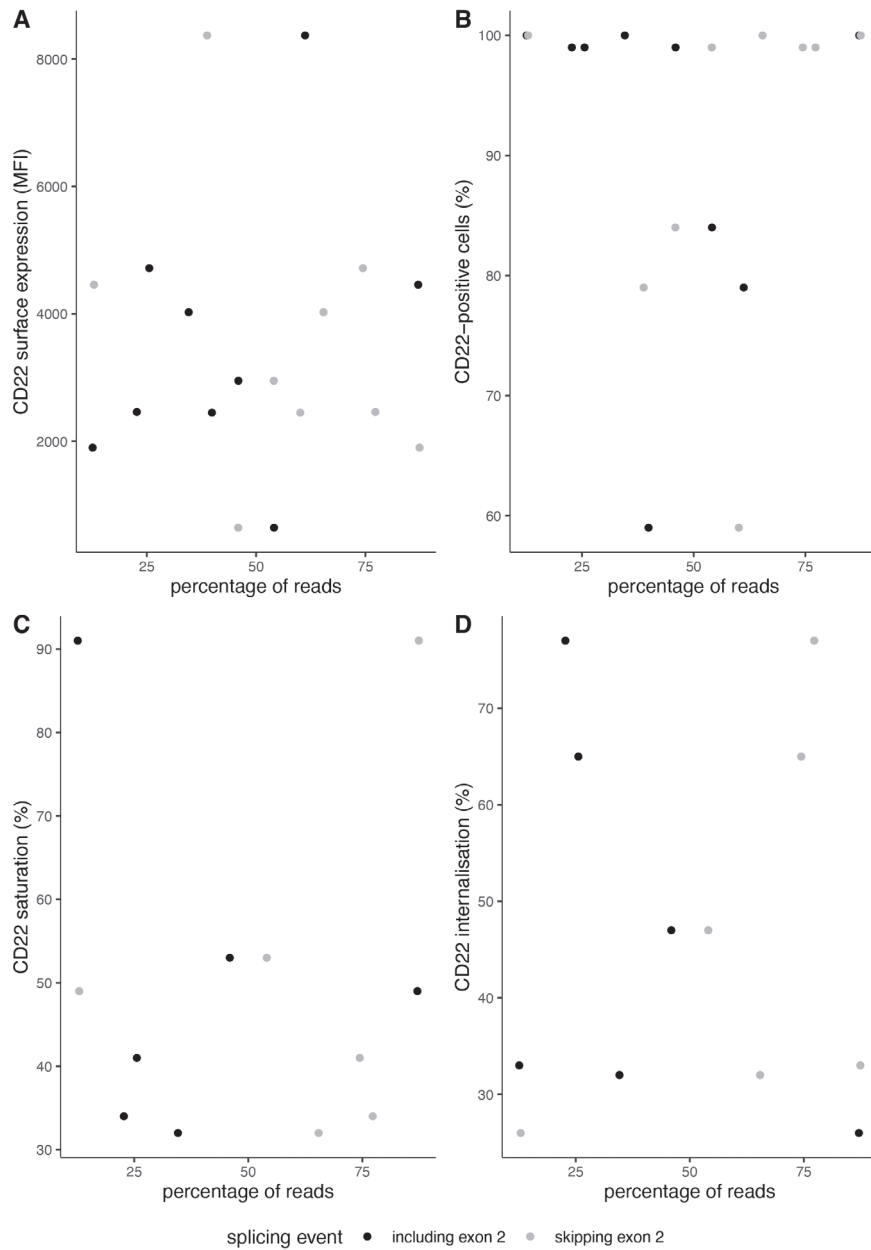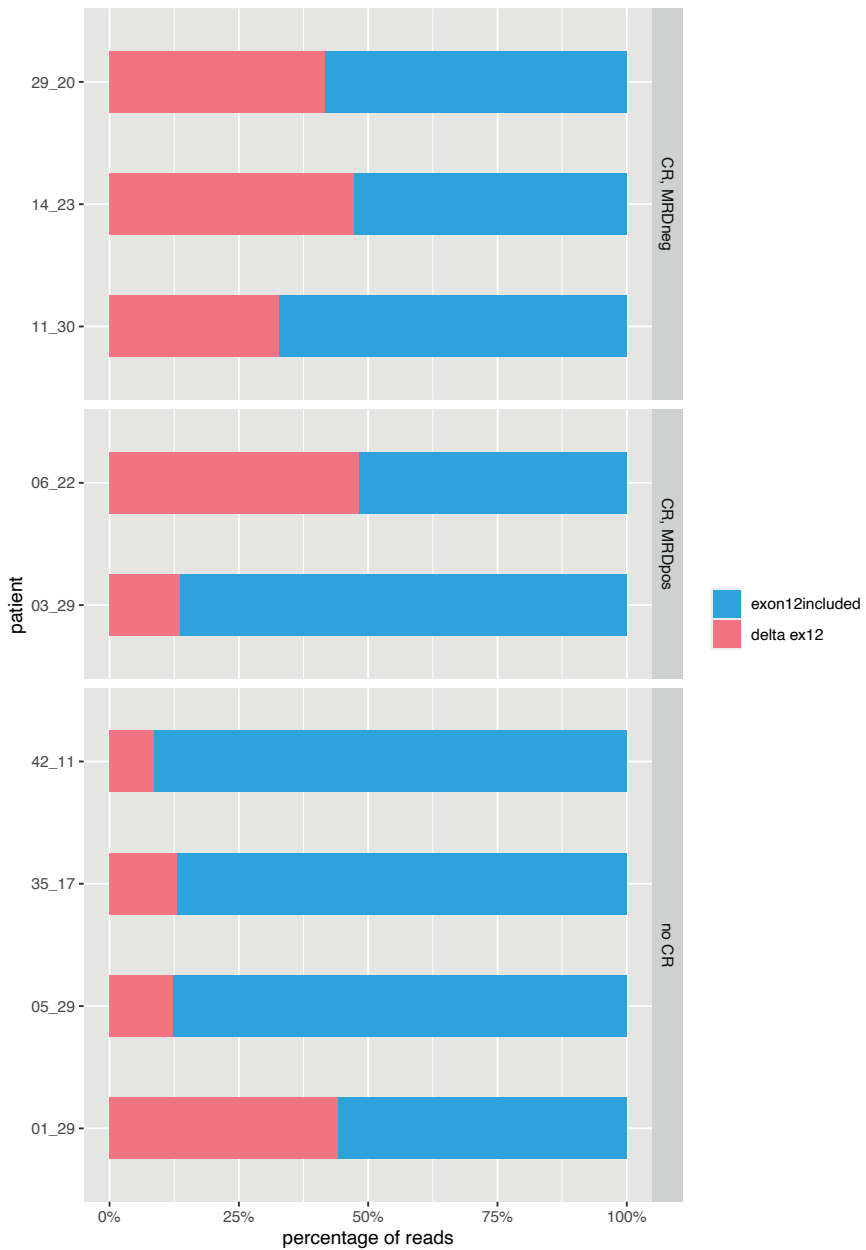
Supplementary Figure 6. Correlation between all splice variants skipping or including exon 2 and CD22 on leukemic blasts as MFI (A), and percentage CD22-positive cells (B), saturation (C) and internalization (D). No trend of association between the in- and exclusion of exon 2 and the expression of CD22 on leukemic blasts, the saturation levels of CD22 on leukemic blasts with InO, or the internalization levels of InO was found.
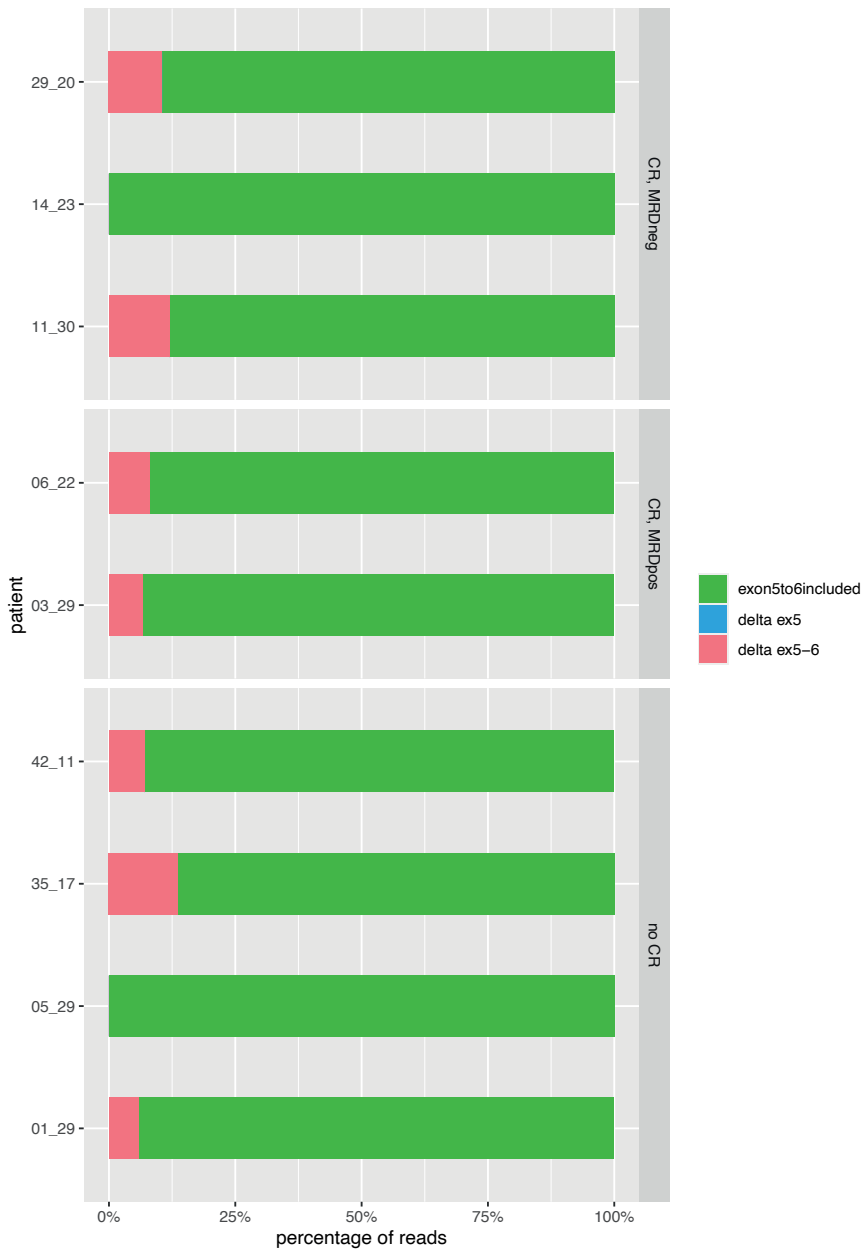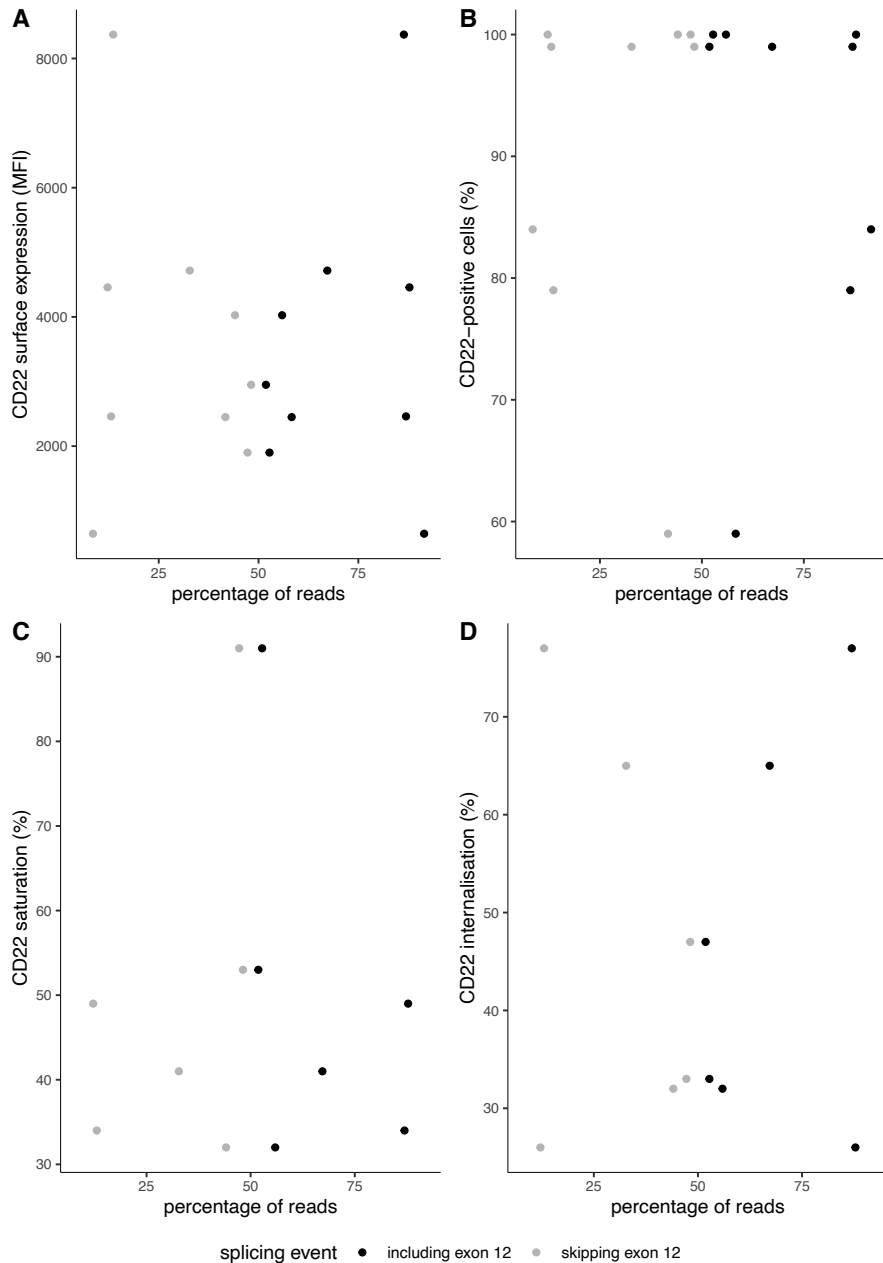
Supplementary Figure 7. Splicing variants concerning exon 5 and 6 of the CD22 transcript in the 9 patients with RNA sequencing data available. Percentages represent the number of split reads either in- or excluding the exons as percentage of the total number of split reads. All patients had at least some RNA copies including exon 5 and 6.
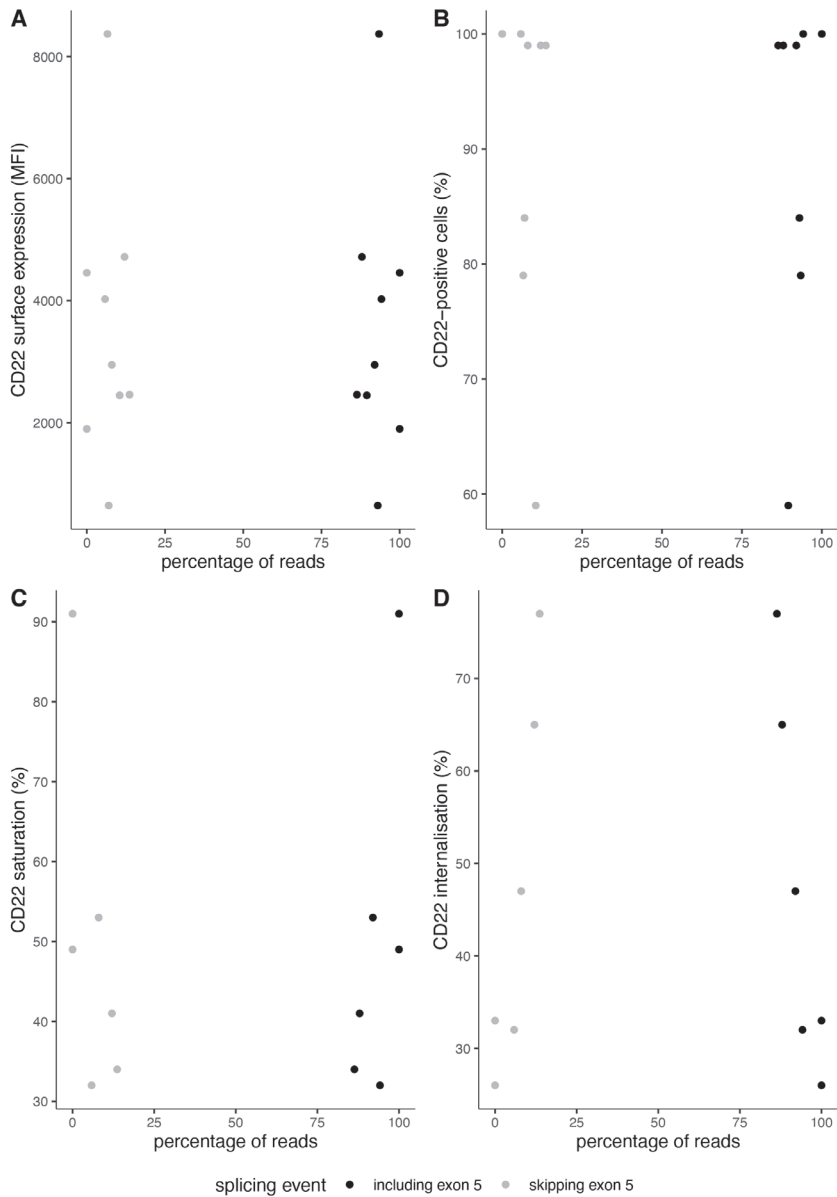
Supplementary Figure 8. Correlation between splicing variants skipping or including exon 5-6 and CD22 on leukemic blasts as MFI (A), and percentage CD22-positive cells (B), saturation (C) and internalization (D). No trend of association between the in- and exclusion of exon 5-6 and the expression of CD22 on leukemic blasts, the saturation levels of CD22 on leukemic blasts with InO, or the internalization levels of InO was found.

Supplementary Figure 9. Splicing variants concerning exon 12 of the CD22 transcript in the 9 patients with RNA sequencing data available. Percentages represent the number of split reads either in- or excluding the exons as percentage of the total number of split reads. All patients had at least some RNA copies including exon 12.

Supplementary Figure 10. Correlation between the splice variant skipping or including exon 12 and CD22 on leukemic blasts as MFI (A), and percentage CD22-positive cells (B), saturation (C) and internalization (D). No trend of association between the in- and exclusion of exon 12 and the expression of CD22 on leukemic blasts, the saturation levels of CD22 on leukemic blasts with InO, or the internalization levels of InO was found.
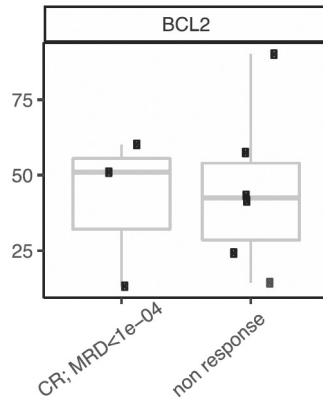
Figure 10. BCL2 gene expression per response group. Presented as fragments per kilobase per million (FPKM), which correct for library size and gene length. No difference in *BCL2* gene expression between response groups was observed.

References for Supplementary Material

1. *Theunissen P, Mejstrikova E, Sedek L, et al. Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood. 2017;129(3):347-357.*

2. *van der Velden VH, Panzer-Grümayer ER, Cazzaniga G, et al. Optimization of PCR-based minimal residual disease diagnostics for childhood acute lymphoblastic leukemia in a multi-center setting. Leukemia. 2007;21(4):706-713.*

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4. *Theunissen P, Mejstrikova E, Sedek L, et al. Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood. 2017;129(3):347-357.*

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6. *van der Velden VH, Panzer-Grümayer ER, Cazzaniga G, et al. Optimization of PCR-based minimal residual disease diagnostics for childhood acute lymphoblastic leukemia in a multi-center setting. Leukemia. 2007;21(4):706-713.*

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3

Chapter 3

Inotuzumab Ozogamicin Combined with Chemotherapy in Pediatric B-Cell Precursor CD22+ Acute Lymphoblastic Leukemia: Results of the Phase 1B ITCC-059 Trial

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Abstract

Inotuzumab ozogamicin (InO) is a CD22-directed antibody conjugated with calicheamicin. The Phase 1B of the ITCC-059 trial tested InO combined with chemotherapy in pediatric B-cell precursor Acute Lymphoblastic Leukemia (BCP-ALL). Relapsed/refractory CD22+ BCP-ALL pediatric patients were enrolled. The primary objective was to establish the Recommended Phase 2 Dose (RP2D). Secondary objectives included preliminary efficacy and tolerability. InO was combined with 1.5 mg/m^2 of vincristine (days 3, 10, 17, 24), 20 mg/m^2 of dexamethasone (two 5-day blocks, then amended), and intrathecal therapy. A rolling-6 design was used testing InO from 0.8 to 1.8 mg/m^2/cycle. Between May-2020 and Apr-2022, 30 patients were treated, and 29 were evaluable for dose limiting toxicities (DLTs). At 1.1 mg/m^2/cycle, two out of four patients had DLTs (liver toxicity). InO was de-escalated to 0.8 mg/m^2/cycle (n=6) without DLTs while awaiting a protocol amendment to reduce dexamethasone dose to 10 mg/m^2. Post amendment, InO was re-escalated to 1.1 mg/m^2/cycle (n=6, 1 DLT), then to 1.4 mg/m^2/cycle (n=3, no DLTs), and finally to 1.8 mg/m^2/cycle (n=7, 1 DLT). Three additional patients were treated in an expansion cohort. The pooled response rate was 80% (24/30; 95%CI: 61.4% to 92.3%) and, among responders, 66.7% achieved minimal residual disease (MRD) negativity. The RP2D of InO combined with vincristine, dexamethasone and IT therapy was declared at 1.8 mg/m^2/cycle (1.5 mg/m^2/cycle after remission) in a fractionated schedule. This combination showed an ORR similar to the single agent cohorts of this study, with liver toxicity issues at the initial higher dexamethasone dose.

3.1. Introduction

Approximately 10-15% of pediatric patients with acute lymphoblastic leukemia (ALL) experience disease relapse.[1] Following relapse, the estimated 10-year overall survival (OS) probability is around 50%, depending on the risk group.[2,3] The traditional treatment for relapsed patients is based on intensive chemotherapy.[4] A randomized trial in relapsed and refractory (R/R) patients, comparing the two most used treatment strategies in Europe, the ALL-REZ BFM 2002 and the UKALL-R3, showed no significant differences in the 5-year probability of event-free survival (EFS) and OS .[5] Nevertheless, a subgroup analysis showed that patients with isolated bone marrow (BM) relapse had a significantly lower relapse rate (RR) if treated within the R3 arm (5-year cumulative incidence 6.5%, n=153) compared to the BFM arm (5-year cumulative incidence 12.5%, n=146); while the BFM approach resulted in superior outcome in patients experiencing isolated Central Nervous System (CNS) relapse (5-year EFS 81.6% , n=40 vs 43.3%, n=45).[5]

Increasing the intensity of chemotherapy to treat R/R patients is constrained by toxicity. For example, the UKALL-R3 reinduction block 1 (vincristine, mitoxantrone, dexamethasone, and asparaginase) results in non-negligible adverse events, especially in terms of severe infections (23.7%) and induction death (3%). [5]In B-cell precursors(BCP) ALL, toxicity can be reduced by using the CD19-directed T-cell engager blinatumomab, which proved efficacious in high-risk first relapse patients, while the reinduction remission rate in overt relapse ranged between 34% and 60%.[6–8] Moreover, CD19-specific chimeric antigen receptor (CAR) T-cells therapies showed high complete remission rates in multiple relapsed BCP-ALL patients, and may be considered definitive therapy without allogeneic hematopoietic stem cell transplantation (HSCT) in some cases. Indeed, a 3-year EFS of 44% was reported for patients enrolled in the ELIANA trial.[9,10]

Despite improvements, new options for effective salvage of pediatric R/R ALL patients and for increasing the overall cure rates in this cancer are still needed.In the context of targeted chemotherapy, inotuzumab ozogamicin (InO) is a CD22-directed antibody-drug conjugate loaded with the cytotoxic agent calicheamicin. InO is approved for adults with CD22-positive R/R BCP-ALL, based on the INO-VATE ALL trial.[11,12] The safety and preliminary efficacy of InO as single agent in pediatric R/R BCP-ALL have been tested in phase I and phase II trials conducted by The Innovative Therapies for Children with Cancer (ITCC) consortium in Europe and by the Children's Oncology Group (COG) in the USA.[13–15] Namely, the estimated Overall Response Rate (ORR) in the phase II trials from COG and the ITCC ranged from 58.3% (90% CI: 46.5 - 69.3) to 81.5% (95% CI: 61.9%-93.7%), respectively, with approximately 70% MRD negativity rate in responding patients.[14,16]Overall, InO appeared well-tolerated in children with R/R BCP-ALL and was associated with high response rates, potentially higher than with blinatumomab, despite no trial compared the two treatments in this population.

Studies in adults have investigated InO combined with chemotherapy, for examples with mini-hyper-CVD (cyclophosphamide, vincristine and dexamethasone in cycles 1, 3, 5, 7, and methotrexate plus cytarabine in cycles 2, 4, 6 and 8) or CVP (cyclophosphamide, vincristine and

prednisone), and showed it is safe to combine these agents .[17,18] By contrast, in pediatrics, the safety of InO in combination with chemotherapy has not been assessed yet. Herein, we report the results from the phase 1B of the trial ITCC-059 in the R/R setting,in which InO was combined with a modified UKALL-R3 regimen containing vincristine, dexamethasone and intrathecal (IT) therapy. This combination was developed with the aim to replace mitoxantrone with InO in the UKALL-R3 reinduction regimen, aiming at increasing efficacy while reducing toxicity.

3 .2. Methods

Trial ITCC-059 is a phase I-II, multicenter, international, open-label clinical trial conducted in accordance with the International Council for Harmonization Guidelines for Good Clinical Practice, and the Declaration of Helsinki. The protocol received Ethics Committee review and approval at all participating centers. Patients were treated under protocol version 3 and 4 following an amendment, after the single-agent recommended phase II dose (RP2D) was established in the single agent phase I part. Informed consent was obtained from all patients or their parents (as applicable) before enrolment. The study was sponsored by the Erasmus MC and funded by Pfizer inc. in the context of a Pediatric Investigational Plan. Netherlands Trial Registry nr NL5629 (EudraCT:2016-000227-71).

3.2.1 Patients and Treatment

The main criteria for enrollment (Supplementary Table 1) included age ≥1 to <18 years, signed written informed consent, diagnosis of CD22-positive R/R BCP-ALL with an M2/M3 bone marrow status, and either refractory disease, ≥2nd relapse, or any relapse post-HSCT. Exclusion criteria included isolated extramedullary disease, active infections, and any history of hepatic sinusoidal obstruction syndrome (SOS).

Four dose levels (DLs) of fractionated InO (days 1, 8, and 15 of each cycle) were tested (Supplementary Table 2). Namely, 0.8 mg/m^2/cycle (0.4 + 0.2 + 0.2 mg/m^2), 1.1 mg/m^2/cycle (0.5 + 0.3 + 0.3 mg/m^2), 1.4 mg/m^2/cycle (0.6 + 0.4 + 0.4 mg/m^2) and 1.8 mg/m^2/cycle (0.8 + 0.5 + 0.5 mg/m^2). Once subjects achieved remission, they no longer received a loading dose of InO on day one in the following cycles.[19] Based on data from the phase I/II single agent arms of the trial, InO was initially administered at 1.1 mg/m^2/cycle (0.5 + 0.3 + 0.3 mg/m^2) and combined with vincristine 1.5 mg/m^2 (days 3, 10, 17 and 24), two 5-days blocks of dexamethasone 20 mg/m^2 divided in two daily doses (days 1-5 and 15-20), (later amended), and IT therapy as per the UKALL-R3 regimen without mitoxantrone and asparaginase (Supplementary Figure 1).[20] Subjects enrolled with CNS1 received IT methotrexate at day 1 and 8, while patients with CNS 2 or 3 were advised to receive intensified triple IT treatment (methotrexate, cytarabine plus steroids per local standard of care). In addition, from cycle two, patients could receive either combination therapy or InO single agent per investigator's discretion. For those continuing with single agent therapy, the dose of InO was either 1.8 mg/m^2/cycle if > 5% blasts in the BM, or 1.5 mg/m^2/cycle if

remission had been achieved, as per the single-agent RP2D established in the same trial.[13] Criteria to proceed with the next cycle included: M1 BM with absolute neutrophil count (ANC) ≥ 0.5 x 10^{9}/L and platelet count ≥ 30 x 10 9/L; or M3 BM at study entry attaining an M2 BM at the end of the cycle, irrespective of hematological parameters. A maximum of six cycles of InO was allowed for a given patient. Those not proceeding to HSCT could receive a maximum of two combination cycles, followed by a maximum of four or five cycles of single agent InO. In order to mitigate the risk of SOS, for patients who planned to proceed to HSCT, the recommended overall duration of InO treatment was two cycles, or three cycles in case the patient was not yet MRD negative after cycle two.

3.2.2 Endpoints and Statistical Design

The primary objective was to determine the RP2D of InO in combination with chemotherapy. A rolling-6 escalation design was used, assessing dose-limiting toxicities (DLTs) during cycle one (approximately 28 days).[21] The Maximum Tolerated Dose (MTD) was defined as the dose at which two or more DLTs occurred in 6 patients. The maximum escalation dose was capped at 1.8 mg/m^2/cycle, based on the RP2D for InO as single agent.

The primary end-point was the occurrence of DLTs, defined as any of the following toxicities related to InO: any grade 5 toxicity; ANC <500/μL and/or a platelet count <50 000/μL lasting > 42 days in the absence of persisting leukemia; or grade 3 to 4 non-hematologic toxicities persisting for > 48 hours (> 7 days for hepatic transaminases or bilirubin abnormalities). Secondary endpoints included safety such as frequency and severity of Adverse Events (AEs) based on the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03, occurrence of toxic death, and occurrence of SOS (diagnosed based on the modified Seattle criteria as reported in Supplementary Methods 1). Preliminary efficacy end-points included ORR and MRD negativity status (as best response after InO treatment and after cycle one), OS, EFS, duration of response (DOR), and the number of patients consolidating the treatment with HSCT and/or CAR T-Cell therapy. MRD levels were centrally determined by RQ-PCR of leukemia-specific rearranged immunoglobulin (IG) and T-cell receptor (TR) genes and by flowcytometry using 8 color staining according to EuroFlow protocols (Supplementary Methods 2).

3.2.3 Statistical Analysis

Patients were considered evaluable for the dose escalation if they either received at least one of the planned dose of InO (together with the first dose of dexamethasone) and experienced a DLT, or did not experience a DLT and received at least two out of three of the planned doses of InO during the first cycle, and at least three days of dexamethasone, one dose of vincristine and one dose of IT treatment. The full analysis set consisted of all enrolled patients who received at least one dose of study therapy and was used for the safety analysis. The response analysis set included all enrolled patients who received at least one dose of InO and completed at least one baseline and one post-baseline disease assessment. ORR was defined as the combined Complete Remission (CR; <5%

blast in BM, CNS1 and no evidence of extramedullary leukemia), CR with insufficient platelet recovery (CRp; ANC > 500/μL but platelets ≤ 50,000/μL), and CR without recovery of counts (CRi; ANC ≤ 500/μL with or without platelets ≤50,000/μL). MRD negativity was defined as either a PCR result below 10^{-4}, or a flow cytometry results below 0.01% when the QT-PCR was negative, but the QT-PCR quantitative range (QR) was > 10^{-4}.[22,23] EFS and OS probabilities were estimated using the Kaplan–Meier method. Events were defined as non-response (not achieving CR, CRi or CRp, considered as event at day 0), relapse after remission achieved as a result of InO treatment, death from any cause, or secondary malignancy. DOR was defined as the time between achieving response (CR, CRi or CRp) after starting study treatment and documented relapse or death. Data are available in consultation with the sponsor.

3.3. Results

Between 14-May-2020 and 11-Apr-2022, 37 patients were screened, 30 were treated, 29 were evaluable for the assessment of DLT, and 30 were evaluable for response (one patient received the wrong dose of InO on day 1 of cycle 1; the patient was excluded from the DLT assessment, but counted for response and overall safety as per protocol). Dataset cut-off date was 28-Feb-2023. Patient characteristics are reported in Table 1. Initially, four patients were enrolled at DL1 and received 1.1 mg/m²/cycle of InO. Two DLTs occurred; namely grade 3 hepatic transaminases elevation lasting more than 7 days, and one case of grade 3 SOS. InO was then de-escalated to 0.8 mg/m²/cycle, and seven patients completed cycle one, of which one received 0.8 mg/m² on day 1, 0.5 mg/m² on day 8, and skipped the day 15 dose (instead of 0. + 0.2 + 0.2 mg/m²) in error and therefore was not evaluable for DLT. No DLTs were recorded at this dose level. The Steering Committee decided to amend the protocol to reduce the dexamethasone dose from 20 mg/m²/day to 10 mg/m²/day. Upon approval of the amendment the dose of InO was re-escalated. The intent was twofold. First, mitigating liver toxicity which consisted of transient hepatic transaminases elevation likely caused by steroids and, secondly, allowing the use of higher doses of InO, closer to the RP2D already established for the single agent regimen (1.8 mg/m²/cycle) also given the lower response rates observed at lower doses in phase 1A (ORR 75%, MRD negativity: 66% at DL1; and ORR: 85%, MRD negativity: 100% at DL2).[13,16]

After amending the protocol, InO was first tested at 1.1 mg/m²/cycle (n=6, one DLT: grade 3 hepatic transaminases elevation > 7 days); subsequently at 1.4 mg/m²/cycle (n=3, no DLTs), and then at 1.8 mg/m²/cycle (n=7 as two patients registered contemporary; one DLT occurred: ANC below 0.5 x 10^9/L > day 42). At the same dose level, three additional patients were enrolled in an expansion cohort (not assessed for DLT), increasing the total number of patients treated at 1.8 mg/m²/cycle of InO combined with chemotherapy to 10 (Table 2). The RP2D of InO in combination with 1.5 mg/m² of vincristine (days 3, 10, 17, 24) and 10 mg/m² of dexamethasone (two 5-day blocks) was declared at 1.8 mg/m²/cycle (1.5 mg/m²/cycle once in complete remission).

Table 1. Patient Characteristics

Characteristic	N
Sex (%)	
Male	19 (63)
Female	11 (37)
Age at Enrollment (years)	
Median (range)	8.5 (1-17)
Status at Enrolment (%)	
First relapse post HSCT	2 (7)
≥ 2nd relapse	20 (67)
Refractory disease	8 (27)
Extramedullary Disease (%)	
CNS1	27 (90)
CNS2	2 (7)
CNS3	1 (3)
Testicular involvement	0 (0)
Lymph nodes enlarged	0 (0)
Other locations	1(3)
Other (range)	
Median WBC (10^9/L)	5.03 (1.27-63.60)
Median CD22 MFI[†]	1687 (359-7003)
Median CD22+ blast BM[†]	98% (66 – 100)
Selected Genetic Abnormalities (%)*	
High-hyperdiploid (51-67 chromosomes)	4 (13)
t(12;21)(p13.2;q22.1); ETV6::RUNX1	3 (10)
t(1;19)(q23;p13); TCF3::PBX1	3 (10)
t(4;11)(q21;q23); KMT2A::AFF1	1 (3)
TP53 mutation and/or deletion	1 (3)
TP53 mutation and/or deletion & t(12;21)(p13.2;q22.1); ETV6::RUNX1	2 (7)
IKZF1/7p12	1 (3)
t(9;22)(q34;q11.2); BCR::ABL1	2 (7)
Other	4 (13)
Normal	4 (13)
Not Available	5 (17)

* Known abnormalities detected either by karyotype and/or molecular methods (e.g. FISH, RT-PCR) at the local laboratory. † at screening as determined at the central laboratory on BM. WBC: White Blood Cells at screening; MFI: Mean Fluorescence Intensity; PB: Peripheral Blood; BM: Bone Marrow.

3.3.1 Safety

Sixteen patients received only one cycle of combination therapy, 10 patients one combination cycle plus one single agent cycle, three patients two combination cycles, and one patient received one combination cycle plus two single agent cycles.

All patients experienced at least one AE (Supplementary Table 3). Alanine aminotransferase increase (ALT) occurred in 23 patients (76.%) of which 15 (50%) were ≥ grade 3. Aspartate aminotransferase (AST) increase occurred in 22 patients (73.3%) of which 10 (33.3%) were ≥ grade 3. Overall, 24 (80%) patients had either AST and/or ALT elevation. Seven patients (23.3%) had bilirubin increase; of which six (20%) at grade 1-2, and one (3%) at grade 3. None met Hy's law criteria.[24] Toxicities recorded before and after amending the dexamethasone dose are provided in Table 3.

Overall, 63% of patients reported infections. Four (13%) patients had sepsis, one (3%) had grade 3 skin infection, one (3%) grade 3 urinary infection, and two (7%) other grade 3 infections. Other eleven (36.7%) patients had grade 1-2 infections. Ten patients (33.3%) had grade 3 febrile neutropenia.

Platelet count decrease was experienced by 22 patients (73%) of which 20 (67%) at grade ≥ 3. Overall, ANC decrease was observed in 19 patients (63.3%) of which 18 (60%) at grade ≥ 3. Anemia was experienced by 24 patients (80%) of which 19 (63.3%) at grade ≥ 3. The full lists of AEs, treatment-relatedness, and laboratory abnormalities are provided in Supplementary Tables 3-5. In total, five (17%) patients developed SOS. Four following HSCT (one grade 4 and three grade 3), after receiving a cumulative dose of 2.2, 2.9, 3.2 and 3.6 mg/m^2 of InO, and being transplanted 68, 38, 30 and 29 days since the last InO dose, respectively. The fifth case of SOS (grade 3) occurred on treatment after the administration of 0.8 mg/m^2 (0.5 + 0.3) of InO. Among those developing SOS post-InO, one subject had a prior transplant. Four patients with SOS recovered completely, while in one case SOS was ongoing when the patient died due to sepsis after HSTC. Overall, SOS occurred in 21% (4/19) of the patients that received a HSCT any time after InO (including patients receiving additional treatment after InO and before HSCT). The median time to onset of SOS since the last InO dose was 47.5 days (range 36 - 119). Among the transplanted patients, six received prophylaxis with defibrotide per investigators' discretion, none of which developed SOS. A 11-year-old female subject who had received chemotherapy and two prior HSCT developed posterior reversable encephalopathy syndrome while on treatment with InO at 1.8 mf/m^2/cycle and dexamethasone at 10 mg/m^2 at day 19 of the first cycle. The patient also received IT methotrexate on day 1 and 8 (15 mg) and vincristine on days 3, 10, 17. The subject recovered completely. The event was not considered related to InO but rather attributed to the background chemotherapy.[25] Four patients died while in CR after receiving HSCT. Two of them died due to infection (respiratory infection and post-SOS septic shock), one had a multiorgan failure, and the fourth death was due to thrombotic microangiopathy (without prior SOS). The

cumulative incidence of non-relapse death was 6.7% (95%CI: 1.1-19.5) at six months, and 10.2% (95% CI 2.5-24.3) at 12 months, including post-HSCT follow-up.

Table 2. Dose Escalation History

Dose of InO in cycle 1	Patients treated	DLTs	Notes	Achieved CR (%)
1.1 mg/m²	4	2 (SOS, AST ↑)	Both events resolved	3 (75)
0.8 mg/m²	7*	0		5 (71)‡
Amendment: Dexamethasone reduced to 10 mg/m² divided in 2 administrations per day (BID)				
1.1 mg/m²	6	1 (AST ↑)	AST normalized after 9 days	5 (83)
1.4 mg/m²	3	0		3 (100)
1.8 mg/m²	7**	1 (ANC ↓) > day 42)†	ANC recovered on day 45	6 (86)
1.8 mg/m²	3	NA	Expansion cohort	2 (67)

DLTs: Dose Limiting Toxicities; SOS: Sinusoidal Obstruction Syndrome of the liver; AST: Alanine Amino Transferase; ↑ Increase ≥ grade 3; ANC: Absolute Neutrophil Count; † < 500/μL. NA: Not Assessed. CR: Complete Remission. * One patient received a wrong dose of InO (1.3 mg/m²), therefore was excluded from the DLT calculation and replaced. ** Two patients were pre-registered contemporarily, therefore 7 instead of 6 were enrolled at this dose level. ‡ among the five responders one patient received 1.3 mg/m²/cycle (see specification above).

Table 3. Most Frequent Treatment Emergent Adverse Events (>20%) Divided by Grade and Before and after Dexamethasone Amended Dose

AE Term	Full Dexamethasone dose (20 mg/m²) n= 11		Reduced Dexamethasone dose (10 mg/m²) n= 19		Total (n=30)
	Grade 1-2	Grade ≥ 3	Grade 1-2	Grade ≥ 3	
Anemia	2 (18%)	7 (63%)	3 (15%)	12 (63%)	24 (80%)
AST increased	2 (18%)	5 (45%)	6 (32%)	10 (53%)	23 (77%)
ALT increased	3 (27%)	3 (27%)	9 (47%)	7 (37%)	22 (73%)
Platelet count decreased	1 (9%)	8 (72%)	1 (5%)	12 (63%)	22 (73%)
ANC decreased	1 (9%)	6 (54%)	0	12 (63%)	19 (63%)
Constipation	3 (27%)	0	12 (63%)	0	15 (50%)
Fever	6 (54%)	0	7 (37%)	0	13 (43%)
Headache	4 (36%)	1 (9%)	8 (42%)	0	13 (43%)
Febrile neutropenia	0	3 (27%)	0	7 (37%)	10 (30%)
Hypokalemia	0	2 (18%)	4 (21%)	3 (15%)	9 (30%)
Abdominal pain	3 (27%)	0	5 (17%)	0	8 (27%)
Bilirubin increased	2 (18%)	0	4 (21%)	1 (5%)	7 (23%)
GGT increased	1 (9%)	1 (9%)	2 (11%)	3 (15%)	7 (23%)

AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; ANC: Absolute Neutrophil Count; GGT: Gamma Glutamyl Transferase

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3.3.2 Efficacy

Combining all dose levels (n=30), 24 patients achieved complete response (ORR 80%; 95% CI: 61.4% to 92.3%) of which 22 (73%) after cycle 1; 20 were in CR, three in CRp and one in CRi. Response by dose level is provided in Table 2. Among responders, MRD negativity as best response was achieved by 16 (66.7%) subjects of which 13 after cycle one (Figure 1). Among those treated at 1.8 mg/m^2 in cycle 1 (n=10), 8 (80.0%) achieved response, and 6 (75.0%) also achieved MRD negativity after cycle one.

A total of 21 patients (70%) proceeded to consolidation therapy, 15 (50%) with HSCT (of which one after bridging with blinatumomab in presence of MRD positivity) and six (20%) with CAR T-cell therapy. Additionally, at the time of cut-off date, one responding patient received maintenance chemotherapy (then died due to relapse 10 months after last dose of InO) and other two responding patients did not receive consolidation treatment and relapsed a few months later (Figure 2). Other four patients received HSCT following additional therapy, of which three after relapse post InO, and one among the non-responders. Notably two of them received InO a second time and were able to proceed to HSCT (after relapse post-CAR T).

The median follow-up was 15.9 months (Interquartile Range [IQR]: 12.4 – 18.4). At 6 months, the EFS probability was 66.5% (95%CI: 51.5-85.8) and the OS probability was 76.6% (95%CI: 62.9-93.4). At 12 months, the EFS probability was 41.7% (95%CI: 27.1-64.3) and the OS probability was 62.3% (95%CI: 46.9-82.8) (Figure 3). Median DOR was 8.38 months (IQR: 2.3-11.9). In a post-hoc analysis, we did not observe statistically significant differences in EFS and OS between responders consolidated with HSCT or CAR-T cell therapy (Supplementary Figure 2-3). The cumulative incidence of relapse was 8.3% (95%CI: 1.0-27.0%) at 6 months and 13.6% (95%CI 2.9-34.0%) at 12 months. Overall, 10 patients relapsed of which five died (Figure 2), and three deaths occurred among the five non-responding subjects. Additionally, four patients died while in remission, for a total of 12 deaths.
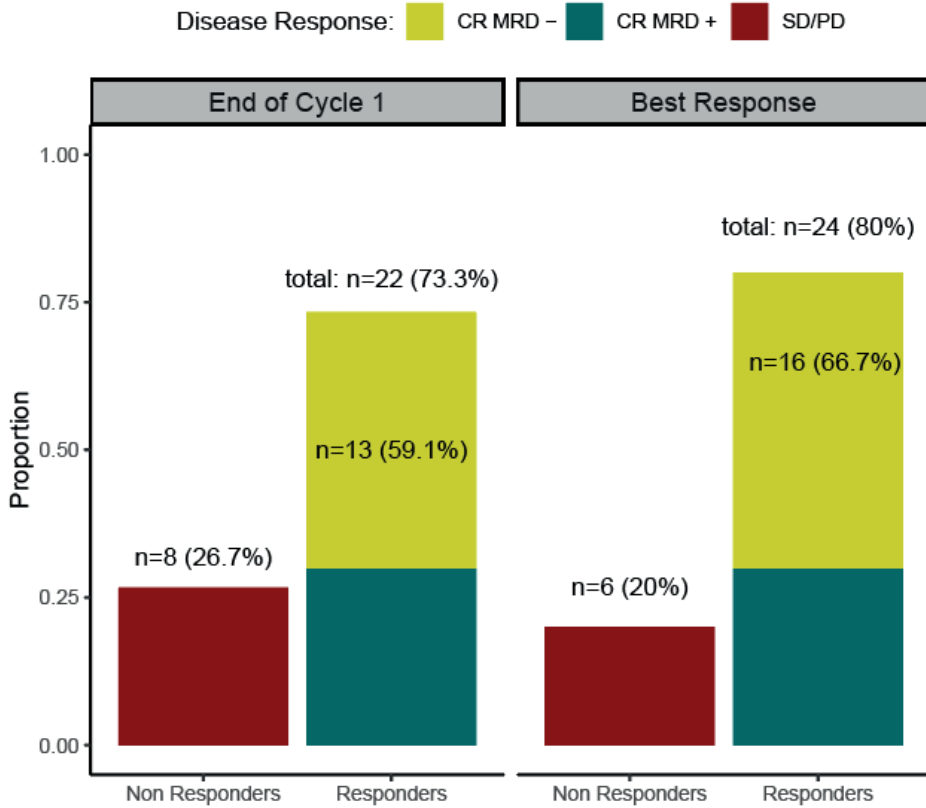
Figure 1. Proportions of Non-Responders, Responders and MRD Negativity. Responders are defined as those with <5% of bone marrow blasts regardless the recovery of the neutrophil count and platelets. MRD negativity is defined as <10⁻⁴. CR: Complete Remission; MRD: Minimal Residual Disease; SD/PD: Stable Disease/Progressive Disease
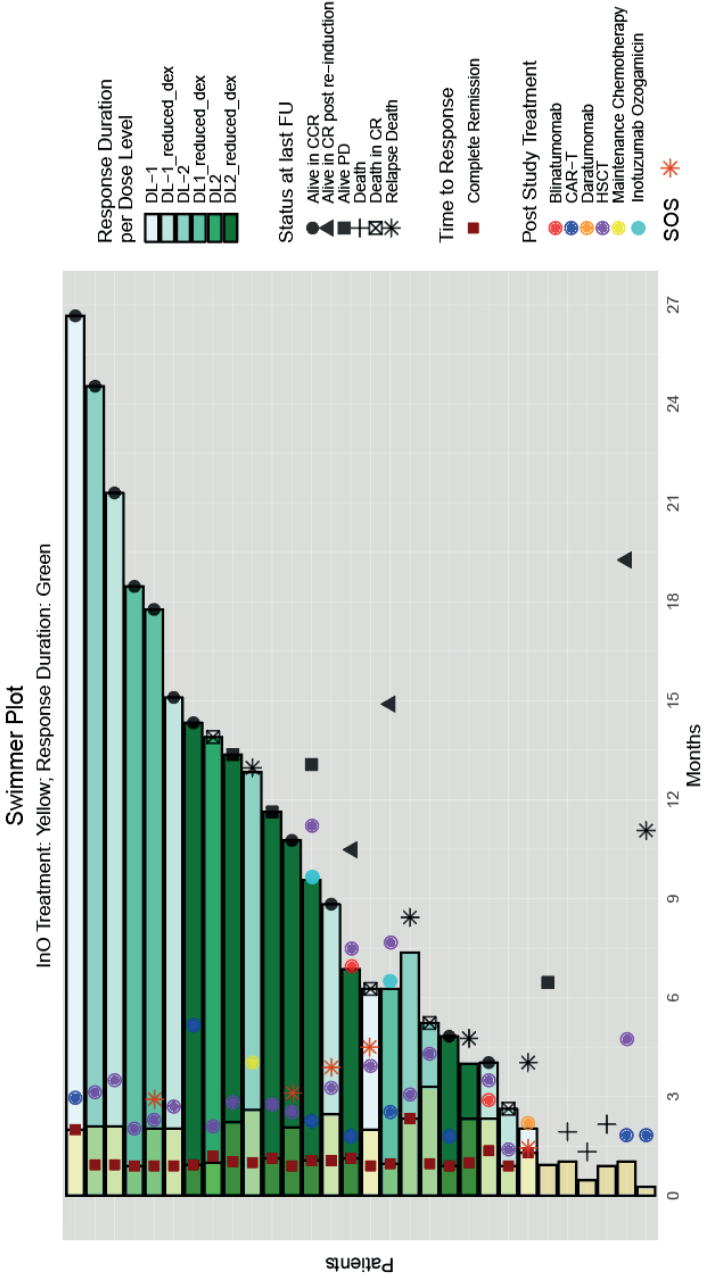
Figure 2. Swimmer Plot of Patients' Treatment and Response. Each bar starts at day 1 of cycle 1. Yellow shaded areas represent the duration of response. InO Dose Levels: DL-1: 1.1 mg/m²/cycle; DL-2: 0.8 mg/m²/cycle; DL2: 1.8 mg/m²/cycle; DL-1_amd: 1.1 mg/m²/cycle (reduced dexamethasone); DL1_amd: 1.8 mg/m²/cycle (reduced dexamethasone); DL2_amd: 1.8 mg/m²/cycle (reduced dexamethasone). CR: Complete Remission. CCR: Continuous Complete Remission achieved on InO therapy. PD: Progressive Disease/Relapse. CAR-T: Chimeric Antigen Receptor T-Cells Therapy; HSCT: Hematopoietic Stem Cell Transplantation. SOS: Sinusoidal Obstruction Syndrome. NA: Not Applicable. FU: Follow up. 16 patients received only 1 cycle (combination), 10 patients 1 combination cycle + 1 single agent cycle, 3 patients 2 combination cycles, 1 patient received 3 cycles (1 combination cycle + 2 single agent cycles).
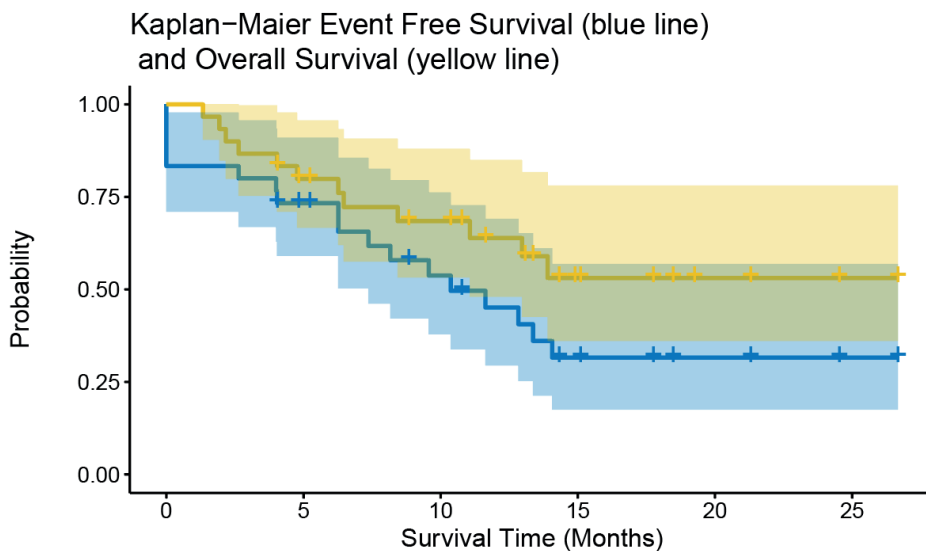
Figure 3. Overall Survival and Event-Free Survival. Probabilities were estimated using the Kaplan–Meier method. Events were defined as non-response (not achieving CR, CRi or CRp, considered as event at day 0), relapse after remission achieved as a result of InO treatment, death from any cause, or secondary malignancies. Crosses represent censored subjects. Shaded areas represent the 95% Confidence Interval.
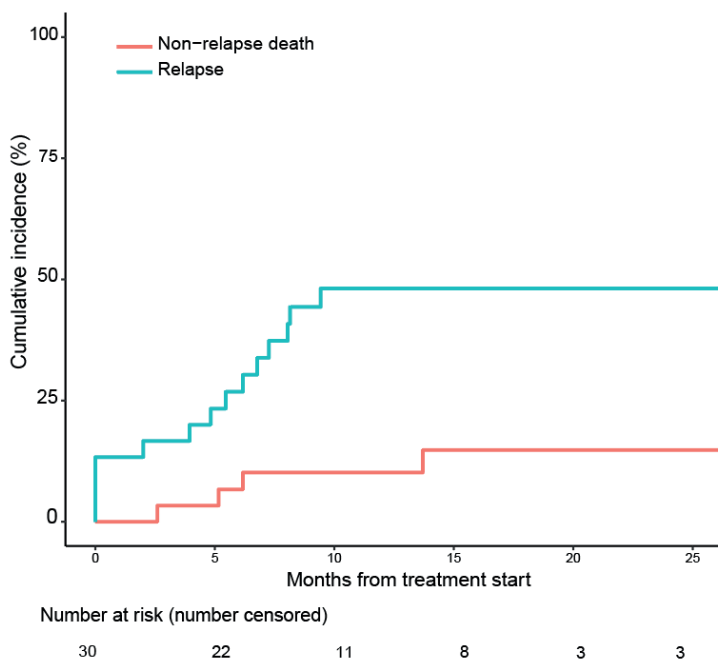
Figure 4. Cumulative Incidence of Relapses and Non-Relapse Death. Probabilities were estimated using the Kaplan–Meier method. Patients not achieving remission were counted as event at time zero for the cumulative incidence of relapse (blue line). Patients dying while in remission achieved as a result of InO treatment were counted as event in the non-relapse death curve (red line).

3.4. Discussion

This trial showed that in pediatric R/R CD22-positive BCP-ALL patients InO can be safely combined with 1.5 mg/m² of vincristine (days 3, 10, 17, 24), 10 mg/m² of dexamethasone (two 5-day blocks, BID) and IT therapy, at 1.8 mg/m²/cycle, the same RP2D as per InO single agent.[13,19]

Despite this promising safety profile, our data suggest that the combination of InO with chemotherapy might increase the risk of transaminases elevation compared to the single agent treatment. Indeed, we observed 14.3% AST elevation ≥ grade 3 and 17.9% ALT elevation ≥ grade 3 in the single agent arm of this trial, compared to 33.3% ≥ grade 3 AST elevation and 50% ≥ grade 3 ALT elevation in the combination arm reported here. It is well known that transaminases are frequently increased by chemotherapy and by dexamethasone.[26] Nevertheless, the clinical relevance of this data remains unclear as ALT/AST increase does not necessarily reflect or predict severe hepatotoxicity and, in our study, it was not associated with severe or long-lasting liver impairment, nor with clinically significant bilirubin increase, which only in one case was reported at grade 3 and none at grade 4.[26,27] By contrast, we confirm that one of the major risks associated with InO is SOS, and particularly in patients proceeding to HSCT as consolidation after InO treatment. Nevertheless, the addition of vincristine and dexamethasone to InO did not seem to further increase the incidence of SOS when compared to the single agent arms of the same trial (overall SOS incidence was 16.6% in phase IB vs 17.3% in phase IA and II combined; while among patients consolidating with HSCT after InO treatment it was 21% vs 26.1%, respectively), despite a rigorous comparison was not possible due to the non-randomized approach, the heterogeneity of the InO dose administered, SOS prophylaxis which was not uniformly performed, and the small sample size.[13,16] Non significant differences in the incidence of AEs were observed before and after the amendment of the protocol (Table 3) in this limited sample size. Nevertheless, reducing dexamethasone dose prevented the occurrence of DLTs and allowed a higher escalation of InO under the rolling-6 rules.

The data reported above are in line with other trials in older R/R patients with CD22+ BCP-ALL. In trial SWOG 1312 (NSC-772518), InO at 1.8 mg/m² was safely combined with cyclophosphamide 750 mg/m², vincristine 1.4 mg/m² and max 2 mg, prednisone 100 mg orally days 1-5 for R/R CD22+ BCP-ALL, resulting in approximately 60% response.[18] Similarly, in the EWALL-INO study (NCT03249870), InO was safely combined at 1.8 mg/m²/cycle with one triple IT injection, vincristine (1-2 mg, weekly) on day 1, 8, 15 and 22, and four 2-day blocks of dexamethasone (20 mg/day) and resulted in 87.7% response.[28]

In terms of efficacy, the ORR of InO combined with chemotherapy was comparable to the single agent arm of the trial (ORR 80% vs 81.5%).[16] In this phase 1B, though, it should be noticed that in cycle one we tested a much larger spectrum of dose levels, from 0.8 mg/m²/cycle to 1.8 mg/m²/cycle. In addition, the estimated ORR for the single agent cohort of this trial is already very high and it might be unnecessary to combine InO with toxic chemotherapy in heavily pre-treated patients to obtain a relatively small marginal improvement. Due to these considerations,

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it was decided not to proceed with the additional cohort 1B-ASP as originally planned, in which PEG-asparaginase on day 3 and 17 (1000 IU/m²) would have been simultaneously added to the combination of InO and chemotherapy.

Furthermore, it is worth noting that recent data showed that low-intensity chemotherapy schemes without asparaginase when combined with multiagent immunotherapy can deliver very high ORR in both adults and children while sparing some of the toxicities related to chemotherapy.[29,30] For example, the MD Anderson Cancer Center is developing multiagent immune/target therapy regimens that combine low-intensity chemotherapy with blinatumomab, InO and rituximab in the so-called Pedi-cRIB regimen (NCT05645718). Early results have described that the combination of mini-hyper-CVD with cRIB (InO at 1.2 mg/m²/cycle: 0.6 + 0.3 + 0.3 mg/m²) is well-tolerated also in heavily pretreated pediatric patients.[31] In adults, mini-Hyper-CVD was administered with InO at a dose of 1.3 - 1.8 mg/m² in cycle 1, which was later amended to lower dosages to mitigate the risk of liver toxicities. rituximab was added in CD20+ patients only and patients subsequently received consolidation with blinatumomab. The combination yielded a remission rate of 89%, and the 5-year progression-free survival was 44.0% (95%CI: 31.2 - 54.3), in elderly newly diagnosed patients (n=80, median age 68, IQR: 63-72); while in younger subjects (n= 31, median age 25, range: 18-57) the remission rate and 1-year OS probability were both 100%, although 3 patients (10%) had isolated CNS relapse (NCT01371630).[32–34] Such regimens, developed due to the poor tolerance of high-intensity chemotherapy in elderly patients, are now being integrated into frontline setting followed by CAR T-cell consolidation. This represents a new paradigm for front-line ALL treatment which might impact also future pediatric regimens currently still relying on conventional chemotherapy, particularly for the induction phase of the treatment.[35]

In the context of R/R pediatric patients, the trial NCT05748171 will randomize InO as single agent against the UKALL-R3 regimen in high-risk first relapse ALL patients. In newly diagnosed pediatric patients, a phase 3 randomized trial in high-risk CD22+ BCP-ALL (AALL1732) sponsored by the Children's Oncology Group, is evaluating two cycles of single agent InO at 1.2 mg/m² after standard induction and post-induction chemotherapy. Following consolidation, patients with MRD > 0.01% were randomized 1:1 (n=50) to chemotherapy (Arm A) or chemotherapy plus 2 cycles of InO (Arm B), one before the high-dose methotrexate interim maintenance and the other before proceeding to the delayed intensification blocks. From an interim analysis, no differences in grade ≥3 ALT or bilirubin elevations were recorded between arm A and B, but patients treated with InO showed a significant higher incidence of neutropenia (87.5% vs 50%) and sepsis during delayed intensification (10 cases in arm B, 1 case in arm A), as well as SOS (4 cases in arm B, 0 in arm A). The enrolment was halted and treatment was amended to mitigate toxicity during post InO chemotherapy blocks.[36] In Europe, the 'AllTogether1' group (NCT03911128) is testing InO given at 0.5 mg/m²/week for six weeks as additional consolidation block in a randomized fashion within the intermediate-high risk patient group with high MRD levels. Patients randomized to receive InO, will be given two cycles of InO during consolidation.

In conclusion, preliminary efficacy and safety data underscore the possibility to combine InO up to 1.8 mg/m^2/cycle with vincristine, dexamethasone and IT therapy in a safe manner. Nevertheless, a noticeable advantage of this combination regimen in terms of ORR when compared to the single agent arms of the same trial was not observed in these heavily pretreated patients. This study contributes to the knowledge on safety and efficacy of InO in pediatric patients, and opens the possibility to use less chemo-intensive treatments in pediatric ALL by either using InO as a single agent or in combination with immunotherapies such as blinatumumab and rituximab as already done in adults.

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Supplementary Material

Supplementary Table 1: Inclusion and Exclusion Criteria

Inclusion Criteria	
Age	≥1 and <18 years at time of enrollment The first three patients on dose level 1 must be ≥6 and <18 years Then ≥2 additional patients ≥1 year and <6 years at the same dose level
Diagnosis	First relapse of BCP-ALL post allogeneic HSCT Second or greater R/R BCP-ALL Refractory disease (newly diagnosed patients who had induction failures after ≥2 previous regimens without attainment of remission, or patients with refractory first relapse after one previous reinduction regimen without attainment of remission) **<u>AND</u>**: M2 or M3 marrow status (≥5% blasts by morphology) Malignant clone CD22 surface antigen positive (in either bone marrow or peripheral blood) by institutional standards The first six patients must have M3 marrow status (≥25% blasts by morphology)
Performance level and life expectancy	Karnofsky >60% (>16 years) or Lansky >60% (≤16 years) Life expectancy of ≥6 weeks

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	Patients must have recovered from the acute toxic effects of all prior therapy, defined as resolution of non-hematologic toxicities to ≤Grade 2 per the CTCAE 4.03 prior to entering the study
	<u>Chemotherapy</u> ≥7 days since the completion of cytotoxic therapy (exceptions: hydroxyurea, 6-mercaptopurine and steroids which are permitted up until 48 hours prior to initiating protocol therapy)
	<u>Radiotherapy</u> ≥28 days since any prior radiation therapy
	<u>Hematopoietic stem cell transplant</u> ≥90 days since previous allo-HSCT No evidence of active graft vs host disease No GVHD prophylaxis or treatment
	<u>Hematopoietic growth factors</u> ≥7 days since the completion of therapy with GCSF or other growth factors, or ≥14 days since completion of therapy with pegfilgrastim (Neulasta®)
Prior therapy	<u>Immunotherapy</u> ≥42 days after the completion of any type of immunotherapy, e.g. CART therapy. Patients may not have received prior CD22-targeted therapy (immunotoxin or CART therapy)
	f. <u>Monoclonal antibodies</u> ≥3 half-lives of the antibody must have elapsed after the last dose of a monoclonal antibody (rituximab = 66 days, epratuzumab = 69 days) Exclusion of blinatumomab: patients must have been off blinatumomab infusion for ≥14 days and all drug-related toxicity must have resolved to ≤Grade 2
	<u>Investigational drugs</u> ≥7 days or five drug half-lives (whichever is longer) since prior treatment with any experimental drug (with the exception of monoclonal antibodies) under investigation. No residual toxicities should be observed following previous treatment
	<u>Prior calicheamicin exposure</u> Patient has not received prior treatment with a calicheamicin conjugated antibody (e.g. gemtuzumab ozogamicin)
Renal and hepatic function	Serum creatinine ≤1.5 x institutional ULN according to age AST and ALT ≤2.5 x institutional ULN Total bilirubin ≤1.5 x institutional ULN unless the patient has documented Gilbert syndrome
Cardiac function	• Shortening fraction ≥30% by echocardiogram or an ejection fraction >50% by MUGA.

Reproductive function	Female patients of childbearing potential: negative urine or serum pregnancy test confirmed prior to enrollment Female patients with infants must agree not to breastfeed on study Male and female patients of child-bearing potential must agree to use a *highly effective* method of contraception (≥8 months for females and for ≥5 months for males after the last dose of InO)

Exclusion Eligibility Criteria

Isolated extramedullary relapse	Patients with isolated extramedullary disease are excluded
VOD/SOS	Any history of prior or ongoing VOD/SOS as per modified Seattle criteria, or prior liver-failure [defined as severe acute liver injury with encephalopathy and impaired synthetic function (international normalized ratio of ≥1.5)]
Infection	Systemic fungal, bacterial, viral or other infection that is exhibiting ongoing signs/ symptoms The patient may not have: A requirement for vasopressors Positive blood culture within 48 hours of study enrollment Fever above 38.2 degrees Celsius within 48 hours of study enrollment with clinical signs of infection. Fever that is determined to be due to tumor burden is allowed, with documented negative blood cultures for ≥48 hours prior to enrollment and no concurrent signs or symptoms of active infection or hemodynamic instability A positive fungal culture within 30 days of study enrollment Active fungal, viral, bacterial, or protozoal infection requiring intravenous or oral treatment. Chronic prophylaxis therapy to prevent infections is allowed
Other anti-cancer therapy	Patients will be excluded if there is a plan to administer non-protocol anti-cancer therapy during the study period
Allergic reaction	Patients with prior Grade 3/4 allergic reaction to a monoclonal antibody are excluded
Concurrent disease	Significant concurrent disease, illness, psychiatric disorder or social issue that would compromise patient safety or compliance with protocol therapy, interfere with consent, study participation, follow-up, or interpretation of study results Children with Down syndrome are excluded from participation in the dose finding parts of the study

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCP-ALL, B-cell precursor acute lymphoblastic leukemia; CART, chimeric antigen receptor T cell; GCSF, granulocyte-colony stimulating factor; GVHD, graft versus host disease; HSCT, hematopoietic stem cell transplant; InO, Inotuzumab Ozogamicin; MUGA, multiple gated acquisition scan; R/R, relapsed/refractory; SOS, sinusoidal obstruction syndrome; ULN, upper limit of normal; VOD, veno-occlusive disease.

Supplementary Methods

Supplementary Methods 1. Diagnosis of Sinusoidal Obstruction Syndrome (SOS)

Two diagnostic systems are in common use, and are shown here: the modified Seattle criteria (McDonald GB et al 1993), and the Baltimore criteria (Jones RJ et al 1987). The Baltimore criteria are more stringent, with an absolute requirement for hyperbilirubinemia. In this protocol we used the Modified Seattle Criteria to define SOS. Formally these criteria describe SOS within 20 days post-HSCT, but since SOS may also occur post-InO and/or at a later time-point, for this study we will consider all occurrences of SOS per the definition below:

Modified Seattle Criteria

Two of the following criteria must be present:
  - Total bilirubin > 34.2 μmol/l (2mg/dL)
  - Hepatomegaly or right upper quadrant pain
  - Weight gain (> 2% from pre-transplant weight)

Other factors that may point at SOS include:

* ascites
* thrombocytopenia with refractoriness to platelet transfusion
* changes in the flow of vena portae

Therefore, when evaluating liver toxicity, the radiologist should be informed of the potential for hepatic vascular disease. When SOS is in the differential diagnosis, a right upper quadrant ultrasound with color flow doppler (including indices to hepatic artery flow and evaluation of hepatic venous outflow) should be performed. In addition, the radiology report should describe common bile duct, the degree of gall bladder wall thickening in millimeters, and the volume of ascites should be estimated as closely as possible (ie, small and localized, moderate and generalized, or large and generalized).

Supplementary Methods 2. Minimal Residual Disease (MRD) Detection Methods

Molecular MRD levels were centrally determined by RQ-PCR of leukemia-specific rearranged immunoglobulin (IG) and T-cell receptor (TR) genes (van der Velden and Van Dongen, 2009). Quality control and standardized interpretation of RQ-PCR data were achieved following the guidelines of the European Study Group on MRD detection in ALL (EuroMRD) (Van der Velden et al, Leukemia 2007). For flowcytometric MRD analysis, also centrally performed, bone marrow samples were bulk-lysed and subsequently stained using 8 color stainings according to EuroFlow protocols (Theunissen et al, Blood 2017; Kalina et al, Leukemia 2012). Four million cells (if available) were acquired and MRD positivity was defined if at least 20 ALL cells could be detected. Flow MRD negativity was defined as MRD < 0,01% using an assay with a sensitivity of at least 0,01%. MRD negativity was defined as PCR below 10^{-4} or flow cytometry below 0.01% when PCR was negative but the Quantitative Range was > 10^{-4}.

Supplementary Table 2. Dose Levels of InO for Patients Enrolled in Cohort 1B in Cycle 1 and Cycles 2 to 6

Day	Cycle 1*				Cycle 2-6#*			
	1	8	15	Total Dose per Cycle	1	8	15	Total Dose per Cycle
Level -2	0.4	0.2	0.2	**0.8 mg/m²**	0.2	0.2	0.2	**0.6 mg/m²**
Level -1	0.5	0.3	0.3	**1.1 mg/m²**	0.3	0.3	0.3	**0.9 mg/m²**
Level 1 (Start)*	0.6	0.4	0.4	**1.4 mg/m²**	0.4	0.4	0.4	**1.2 mg/m²**
Level 2	0.8	0.5	0.5	**1.8 mg/m²**	0.5	0.5	0.5	**1.5 mg/m²**

Dose de-escalation will not go below Level -2.

Following Cycle 1, in patients who have achieved a CR/CRi or CRp, the day 1 dose is decreased slightly due to no loading dose requirement. In patients who have not yet achieved a CR/CRi or CRp after cycle 1, a loading dose similar to cycle 1 will be given in cycle 2, but not in subsequent cycles.

* Note that there will be no dose-capping for obese patients/patients with high BSA.

Supplementary Figure 1. Treatment Scheme

Time-table UKALL-R3 block without mitoxantrone combined with InO
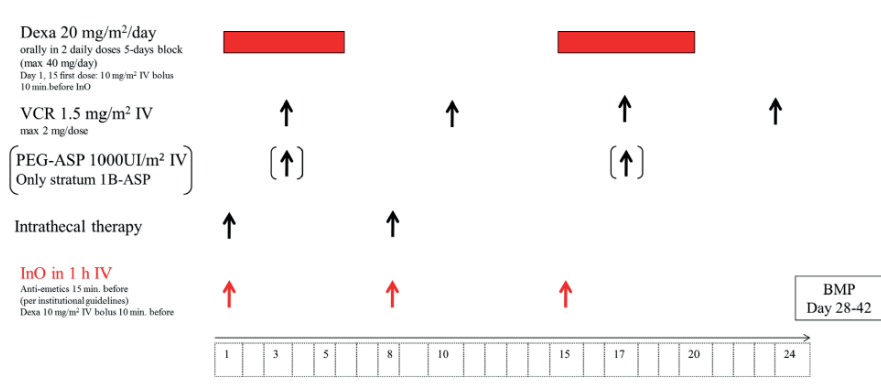
VCR: Vincristine; InO: Inotuzumab Ozogamicin; Dexa: Dexamethasone. Dexamethasone dose was then reduced to 10 mg/m²/day. IT methotrexate prophylaxis is recommended to be given intrathecally to patients with BCP-ALL who are CNS1 at study entry on day 1 and 8 of each cycle. Patients with BCP-ALL who are CNS 2 or 3 prior to enrollment may receive intensified IT therapy with triple IT agents (cytarabine plus either prednisolone or hydrocortisone) per local standard of care and based on which steroids are approved for IT use in a given country. PEG-ASP (Asparaginase) was not given in this cohort (1B) are reported in the main text.

Supplementary Table 3: List of Treatment Emergent Adverse Events (N=30)

AE term	Grade 1-2	Grade ≥ 3	Total
Anemia	5	19	24
Alanine aminotransferase increased	8	15	23
Aspartate aminotransferase increased	12	10	22
Platelet count decreased	2	20	22
White blood cell decreased	0	20	20
Neutrophil count decreased	1	18	19
Constipation	15	0	15
Fever	14	0	14
Headache	12	1	13
Febrile neutropenia	0	10	10
Hypokalemia	4	5	9
Abdominal pain	8	0	8
Blood bilirubin increased	6	1	7
GGT increased	3	4	7
Cough	5	0	5
Hypertension	5	0	5
Lymphocyte count decreased	0	5	5
Pain in extremity	5	0	5
Sinusoidal Obstruction Syndrome	0	5	5
Nausea	4	0	4
Sepsis	0	4	4
Bone pain	2	1	3
Creatinine increased	2	1	3
Diarrhea	3	0	3
Erythema multiforme	3	0	3
Generalized Edema	3	0	3
Hypertriglyceridemia	1	2	3
Hyperuricemia	3	0	3
Hypocalcemia	3	0	3
Rhinitis infective	3	0	3
Skin infection	2	1	3
Vitamin D deficiency	3	0	3
Vomiting	3	0	3
Allergic reaction	1	1	2
Anal fistula	2	0	2
Anxiety	2	0	2
Fatigue	1	1	2
Gastritis	2	0	2

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AE term	Grade 1-2	Grade ≥ 3	Total
Hyperglycemia	1	1	2
Hyperphosphatemia	2	0	2
Hypophosphatemia	2	0	2
Hypotension	2	0	2
Joint pain	2	0	2
Mucositis oral	2	0	2
Pain	2	0	2
Perianal Erythema	2	0	2
Pruritus	2	0	2
Sore throat	2	0	2
Upper respiratory infection	2	0	2
Acute kidney injury	0	1	1
Adenovirus infection	1	0	1
Allergic reaction to Ambisome	1	0	1
Allergic rhinitis	1	0	1
Anal ulcer	1	0	1
Anaphylaxis	0	1	1
Arthralgia	0	1	1
Back pain	1	0	1
Bacteremia	1	0	1
Chest wall pain	1	0	1
Depressed level of consciousness	1	0	1
Disease progression	0	1	1
Dyspnea	1	0	1
E.coli infection	1	0	1
Facial pain	1	0	1
Flank pain	1	0	1
Folliculitis	1	0	1
Gastrointestinal pain	1	0	1
Herpes simplex reactivation	1	0	1
Herpes Zoster	0	1	1
Hypoalbuminemia	1	0	1
Hypomagnesemia	1	0	1
Hyponatremia	0	1	1
INR increased	1	0	1
Lactate dehydrogenase increased	1	0	1
Laryngeal inflammation	1	0	1
Lip infection	1	0	1
Lipase increased	0	1	1

AE term	Grade 1-2	Grade ≥ 3	Total
Lung infection	1	0	1
Malaise	1	0	1
Mandible pain	1	0	1
Mandibular pain	1	0	1
Muscle weakness trunk	1	0	1
Neoplasms benign malignant*	1	0	1
Neuralgia	1	0	1
Non-cardiac chest pain	1	0	1
Omaya Catheter infection	0	1	1
Pain due to catheter removal surgery	1	0	1
Palmar erythema	1	0	1
Pancreatitis	1	0	1
Periorbital edema	1	0	1
Periorbital hyperemia	1	0	1
Peripheral motor neuropathy	1	0	1
Pharyngitis	1	0	1
Pneumonitis	1	0	1
Pyogenic granuloma	1	0	1
PRESS	0	1	1
Sars-Cov-2 Infection	1	0	1
Sinus bradycardia	1	0	1
Sinus tachycardia	1	0	1
Somnolence	1	0	1
Stomach pain	1	0	1
Toothache	1	0	1
Tumor lysis syndrome	0	1	1
Upper gastrointestinal hemorrhage	0	1	1
Urinary tract infection	0	1	1
Urinary tract pain	1	0	1

* inclusion cysts and polyps; PRESS: Reversible Posterior Leukoencephalopathy Syndrome

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Supplementary Table 4. List of Adverse Events Considered Definitely, Probably or Possibly Related to Study Treatment (N=30)

AE term	Grade 1-2	Grade ≥ 3	Total	Percentage
Platelet count decreased	2	14	16	53%
ALT increased	5	11	16	53%
Anemia	4	12	16	53%
Neutrophil count decreased	0	12	12	40%
AST increased	5	6	11	37%
White blood cell decreased	0	7	7	23%
Febrile neutropenia	0	7	7	23%
Lymphocyte count decreased	0	4	4	13%
Sinusoidal Obstruction Syndrome	0	5	5	17%
Abdominal pain	2	0	2	7%
Blood bilirubin increased	3	0	3	10%
Constipation	3	0	3	10%
Gastritis	2	0	2	7%
Headache	2	0	2	7%
Sore throat	2	0	2	7%
Fever	2	0	2	7%
E.coli infection	1	0	1	3%
Facial pain	1	0	1	3%
Flank pain	1	0	1	3%
Hyperphosphatemia	1	0	1	3%
Hypertension	3	0	3	3%
Hyperuricemia	1	0	1	3%
Hypophosphatemia	1	0	1	3%
Malaise	1	0	1	3%
Mandible pain	1	0	1	3%
Tumor lysis syndrome	0	1	1	3%
Urticaria	1	0	1	0%
Creatinine increased	1	0	1	3%
Herpes Zoster	0	1	1	3%
Hypertriglyceridemia	0	1	1	3%
Hyponatremia	0	1	1	3%
GGT Increased	1	0	1	3%
Lactate dehydrogenase increased	1	0	1	3%
Hypokalemia	1	0	1	3%
Lung Infection	1	0	1	3%
Muscle weakness trunk	1	0	1	3%

AE term	Grade 1-2	Grade ≥ 3	Total	Percentage
Nausea	1	0	1	3%
Neuralgia	1	0	1	3%
Pain in extremities	1	0	1	3%
Pancreatitis	1	0	1	3%
Pruritus	1	0	1	3%
Vomiting	1	0	1	3%

The AE relatedness to study drug was based on the treating physician's judgment (definitely, probably, possibly, unlikely, not related or unknown).
AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; ANC: Absolute Neutrophile Count; GGT: Gamma Glutamyl Transferase.

Supplementary Table 5. List of Hematologic Laboratory Abnormalities (N=30) Based on the Local Upper Limit for Normality

Grade	1	2	3	4	Total
Test Abnormality					
Anemia	1	29	0	0	30
White blood cell count decrease	1	1	7	21	30
Absolute neutrophil count decrease	0	1	6	23	30
Platelet count decrease	1	1	6	22	30
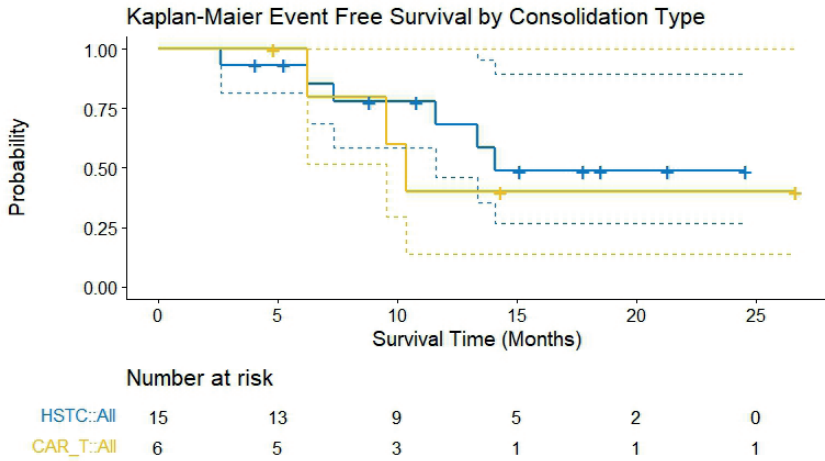
Supplementary Figure 2. Event Free Survival Among Responders (n= 21) Consolidating either by HSCT or by CAR-T Therapy (3 responders which did not consolidated after achieving remission are not reported). Event Free Survival among responders consolidating either by HSCT or by CAR-T. HSCT: Hematopoietic Stem Cell Transplant; CAR-T: Chimeric Antigen Receptors T-Cell Therapy. Other three patients achieving remission with InO received either maintenance chemotherapy or no consolidation therapy at cut-off date (not shown in the figure). Dashed lines represent the 95% confidence interval.
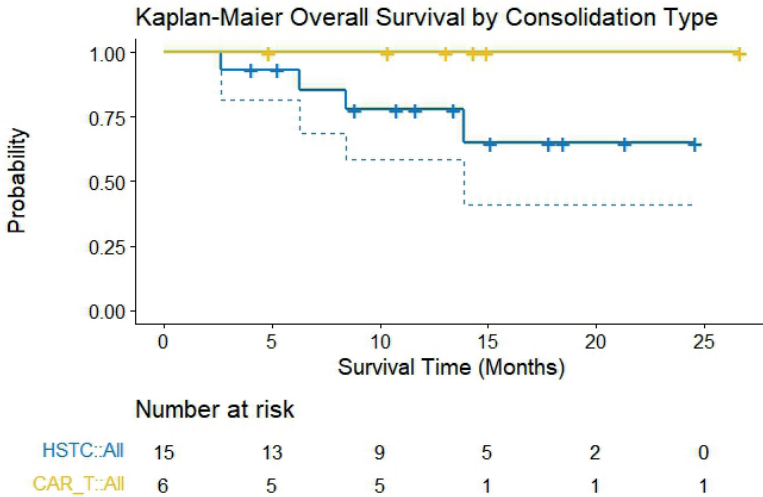
Supplementary Figure 3. Overall Survival Among Responders (n= 21) Consolidating either by HSCT or by CAR-T Therapy (3 responders which did not consolidated after achieving remission are not reported). Overall Survival among responders consolidating either by HSCT or by CAR-T. HSCT: Hematopoietic Stem Cell Transplant; CAR-T: Chimeric Antigen Receptors T-Cell Therapy. Other three patients achieving remission with InO received either maintenance chemotherapy or no consolidation therapy at cut-off date (not shown in the figure). Dashed lines represent the 95% confidence interval.

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Chapter 4

Population Pharmacokinetics of Inotuzumab Ozogamicin in Pediatric B-cell Precursors Relapsed/Refractory Acute Lymphoblastic Leukemia – Results from Study ITCC-059

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Abstract

Inotuzumab ozogamicin (InO) is an antibody-drug conjugate for treating relapsed/refractory (R/R) B-cell precursors (BCP) acute lymphoblastic leukemia (ALL) in adults. Pediatric pharmacokinetic (PK) data of InO are lacking. This study is the first to examine the population PK of InO in pediatric patients with R/R BCP-ALL. From 531 adult non-Hodgkin's lymphoma, 234 adult ALL, and 53 pediatric ALL patients, 8924 InO serum concentrations were analyzed using non-linear mixed effects modeling. A published adult InO population PK model, a two-compartment model with linear and time-dependent clearance, was adapted to describe the pediatric data. Modifications of the previous population PK model included: i) re-estimating PK parameters and covariate effects; ii) modifying covariates representation; iii) introduction of relevant pediatric covariate effects (age on the decay coefficient (k_{des}) of time-dependent clearance (CL_t) and ALL effect on initial value of CL_2). For R/R BCP-ALL patients, an increase in age was associated with a decrease in k_{des} of CL_t, reflecting that the target-mediated drug clearance declines more rapidly in children. In pediatric ALL trials, the median cumulative area under concentration-time curve was higher among responders (n = 42) versus non-responders (n = 10) at the end of the first cycle (26.1 [interquartile range (IQR) 18.9 - 35.0] vs. 10.1 [IQR 9.19 - 16.1], 10^3 ng*h/mL). From simulations performed at the recommended pediatric phase II dose, InO exposure reached similar levels as observed in responding participants in pediatric trial. Therefore, no dose adjustment is required for pediatric BCP-ALL patients despite of the impact of age.

4.1 Introduction

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy in children.[1,2] Contemporary treatment is associated with an 80-90% 5-year event-free survival (EFS) rate and a 5-year overall survival (OS) rate around 90%.[3-5] However, prognosis remains unsatisfactory in those cases refractory to first-line induction or that relapse, with a 5-year OS rate of 50-60% at the first relapse and less than 50% in patients with second or greater relapse.[6-8] Furthermore, despite the approval of immunotherapies like blinatumomab and chimeric antigen receptors T-cell (CAR-T) therapies, re-induction rates with blinatumumab approximate 40%, and 55% of those achieving remission when treated with CAR-T cell therapy relapse during extended follow-up.[9,10] Finally, it is needed to replace toxic chemotherapy agents which are still a burden for pediatric ALL patients, particularly in relapsed/refractory (R/R) settings. Therefore, novel therapeutic agents for ALL in children are needed.

Inotuzumab ozogamicin (InO) is an antibody-drug conjugate (ADC) consisting of a humanized monoclonal IgG4 antibody and a cytotoxic payload, N-acetyl-γ-calicheamicin dimethyl hydrazide, conjugated via an acid-labile linked.[11-13] The antibody of InO targets CD22, whereas calicheamicin is a DNA-binding cytotoxic agent with potent antitumor activity.[11,14] CD22 is expressed on the surface of the majority of B-lymphocyte malignancies and on healthy B-cells, but not on non-lymphoid hematopoietic cells.[11,15-17] After InO binds to CD22, it is internalized into the slightly acidic lysosomal compartment and calicheamicin is released, then CD22 is re-expressed on the cell surface. Calicheamicin, after being released from InO, binds to the minor groove of double-helical DNA, cleaves the double-strand DNA, and causes cell apoptosis.[11,18]

InO was approved by the European Medicines Agency (EMA) and the Food and Drug Administration (FDA) in 2017 for the treatment of adults with R/R B-cell precursors (BCP) acute lymphocytic leukemia (ALL). In the phase III INO-VATE trial in adults with R/R CD22+ B-cell ALL, InO as a single agent was associated with significantly superior response rate when compared to standard intensive chemotherapy and showed an acceptable toxicity profile.[19] In children with R/R ALL, the overall response rate (ORR, complete remission with or without recovery of platelets and neutrophile counts) in the single agent phase II trials conducted in Europe and the USA spans from 81.5% (95%CI: 61.9 - 93.7%) to 58.3% (90% CI: 46.5 - 69.3%).[20,21] Among responders, a 82% and 67% minimal residual disease (MRD)-negativity rate were reported from the respective trials.[20,21]

The pharmacokinetics (PK) of InO in adults has been well characterized by a two-compartments model with linear and time-dependent clearance components (representing target-mediated drug disposition) and several covariates that affect InO disposition have been identified.[22,23] In the pediatric population, PK studies are absent. The aim of this analysis was to evaluate the population PK of InO, to identify potential difference in InO disposition between pediatric and adult R/R BCP ALL patients, and to assess the PK at the pediatric recommended

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phase 2 dose (RP2D), using a dataset that included pediatric (comprising the ITCC-059 Phase IA and Phase II single agent cohorts) and adult data.[20,23-24]

4.2 Methods

4.2.1 Study Design and Patients

This population PK analysis is based on clinical data from 11 studies in adult patients with R/R BCP-ALL or R/R B-cell Non-Hodgkin Lymphoma (NHL) (treated with InO either as a single agent, or combined with rituximab or with rituximab plus chemotherapy), and one study of single-agent InO in pediatric patients with R/R BCP-ALL (Trial ITCC-059 Phase IA and Phase II). Details on the studies conducted in adults were described by Garrett et al.[23] Details on the ITCC-059 trial were published by Brivio et al. and Pennesi et al.[20,24] The data from the adult patients were provided by Pfizer Inc. ITCC-059 was sponsored by the Erasmus MC and financed by Pfizer (EudraCT Number: 2016-000227-71).

The inclusion criteria of trial ITCC-059 were, among others, age ≥ 1 and < 18 years, diagnosis of R/R CD22+ BCP-ALL, and provision of informed consent (full list in Supplementary Table 1). InO was administered as an intravenous (IV) infusion over 60 minutes in 3-week cycles in a fractionated manner on a weekly basis (days 1, 8, 15). In the phase I part of trial ITCC-059, two dose levels were tested; at dose level 1 (DL1), InO was given at 1.4 mg/m^2/cycle and 1.2 mg/m^2/cycle once remission was achieved; at DL2, InO was given at 1.8 mg/m^2/cycle and 1.5 mg/m^2/cycle after remission. The latter was selected as the recommended phase II dose (RP2D).[24] Each InO dosing regimen was fractioned in three doses/cycle on day 1, 8, and 15 (DL1: 0.6, 0.4, 0.4 mg/m^2, and after remission 0.4, 0.4, 0.4 mg/m^2; DL2: 0.8, 0.5, 0.5 mg/m^2, and after remission 0.5, 0.5, 0.5 mg/m^2). A maximum of six cycles was permitted, except for patients proceeding to transplant, for whom a maximum of two to three cycles was allowed based on MRD status. The median age was nine years (range 1- 17 years) and median white blood cells (WBC) at screening was 3.33 ·10^9 (range 0.19 - 132 ·10^9), 67.9% were male. Detailed patient characteristics were published previously.[20,24]

All studies were approved by the independent ethics committee at each participating center and were conducted in accordance with the Declaration of Helsinki and the International Conference of Harmonization Guideline for Good Clinical Practice.

4.2.2 Pharmacokinetic Sampling and Bioanalytic Methods

InO ADC and unconjugated calicheamicin pharmacokinetic samples were collected and analyzed. The serum concentration of InO and unconjugated calicheamicin from children were quantified by a validated high-performance liquid chromatography with tandem mass spectrometry (HPLC/MS/MS), with a lower limit of quantification (LLOQ) of 1.0 ng/ml and 0.05 ng/mL, for the ADC and the unconjugated calicheamicin respectively. The bioanalytical analysis method was designed for indirect measurement of N-acetyl-γ-calicheamicin dimethyl hydrazide conjugated to the antibody of InO, the same method was used for PK samples from adult patients with B-cell ALL, as described by Garrett et al.[23] A validated enzyme-linked immunosorbent assay (ELISA) method, designed to directly assess N-acetyl-γ-calicheamicin dimethyl hydrazide linked to the InO antibody, was used to measure the serum concentration of InO from adult patients with NHL.[23] Bioanalytical analysis methods were validated/revalidated by PPD (Richmond, VA, USA), and performed at laboratories designated by Pfizer Inc.

In this study, the population PK analysis refers to InO concentrations rather than unconjugated calicheamicin as all unconjugated calicheamicin serum concentrations from pediatric patients were below the LLOQ. Data in adults have shown that InO exhibits both linear- and time-dependent clearance components.[23] In adults, the steady state was achieved by the fourth cycle and the linear clearance component predominates over the time-dependent component. Therefore, in children, PK samples were taken during cycle 1, 2 and 3, to better characterize both the linear- and time-dependent clearance of InO. In total, six blood samples were collected per patient during cycle one on day 1, 8 and 15 either before InO administration or 1 hour after dose or both, and at the end of the cycle for trough samples; 5 and 4 blood samples were collected in cycle 2 and 3, respectively. The detailed sampling schedule for PK of InO is reported in Supplementary Table 2.

4.2.3 Model Development

The starting point for model development was a previously developed population PK model for adult ALL and NHL patients.[23] This model consisted of a two-compartments model with linear (CL_l, L/h) and time-dependent clearance (CL_t, L/h) (Figure 1). The two-compartments model structure aligns with the general modeling framework for the PK of monoclonal antibodies with target-mediated drug disposition.[25,26] The linear InO clearance (CL_l) is considered to represent the metabolism and elimination of endogenous IgG, mediated by the Fc receptors (mostly occurring in skin, muscles, and liver) and salvaged by neonatal Fc receptor. The time-dependent clearance, described by the formula below, relates to the target-mediated drug disposition which decreases over time as tumor burden (and thus CD22 antigen expression) reduces.

$$CL_t = CL_2 * e^{(-k_{des} * Time)}$$

The differential equations used to describe the PK data were:

$$k_{10} = CL_{total} / V_1$$

$$Q = k_{12} * V_1 = k_{21} * V_2$$

$$CL_{total} = CL_1 + CL_t$$

$$dA(1) / dt = -k_{10} * A(1) - k_{12} * A(1) + k_{21} * A(2)$$

$$dA(2) / dt = k_{12} * A(1) - k_{21} * A(2)$$

where V_i represents the volume of the ith compartment, $A(i)$ is the amount in the respective compartment, k_{10} is the elimination rate constant from V_1, and Q is the intercompartmental clearance translated into the distribution rate constant (k_{12}, k_{21}).

Covariates in the adult final model were baseline body surface area (BBSA, m²), disease type and/or analytical methods (ALL effect, NHL as the reference), and concomitant rituximab treatment (RITX, with rituximab as the reference) on CL_1; BBSA on the volume of distribution in the central compartment (V_1, L); BBSA on CL_2; ALL effect and baseline percentage of blasts in the peripheral blood (BLSTPB, %) on the decay coefficient (k_{des}) of CL_t.[23]

Interindividual variability (IIV) was modeled using the following equation:

$$P_i = P_{pop} * e^{(\eta_i)}$$

where P_i is the parameter estimate of the ith individual (empirical Bayes estimates/post hoc parameters), P_{pop} represents the fixed population parameter estimate, and depicts the IIV of the ith individual, which is assumed to follow a normal distribution with a mean 0 and a variance ω^2. Residual unexplained variability was described by two separate additive residual errors based on log-transformed data to take different bioanalytical methods used in different disease types into account. Lastly, method 3 modeling approach was applied to handle InO concentration

data that were below LLOQ.[27,28] Observations below the LLOQ were retained in the analysis and treated as censored.
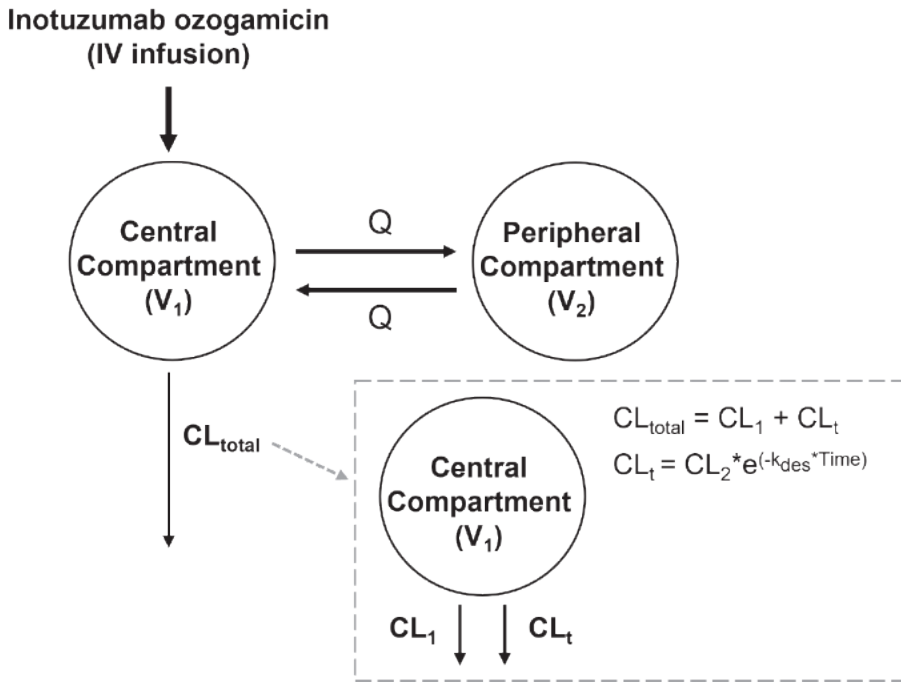
Figure 1 Inotuzumab Ozogamicin Pharmacokinetic Model Structure. Total clearance (CL_{total}) is the sum of linear clearance (CL_1) and time-dependent clearance (CL_t). V_1: volume of distribution in the central compartment; V_2: volume of distribution in the peripheral compartment; CL_2: initial value of time-dependent clearance; k_{des}: decay coefficient; Q: Intercompartment clearance

The first step in model building was to re-estimate the adult model including covariate effects on the pooled dataset and to estimate separate residual error for pediatric population to further account for variability between trials. Subsequently, specific covariates important for the pediatric population were further investigated. The included covariates relate to body size, age and disease. The covariate-parameter relationships to be examined are shown in Table 1. Baseline covariates assessed in the model include replacing certain covariates present in the adult model, namely, BBSA by body weight (BWT, kg), or lean body mass (LBM, kg), and BLSTPB by baseline absolute blast counts in peripheral blood (BLSTABL) on k_{des}.[29,30] Of note, despite some patients entered the study with low number of peripheral blasts while having bone marrow (BM) status 2 and 3, we measure InO concentration only in the central compartment (blood), therefore BLSTABL is considered a better co-variate than BM blast for describing the clearance of InO in the blood stream, particularly as far as the target mediated component of the clearance is concerned. Further testing included age (years), hepatic impairment (BHGRADE,

National Cancer Institute Organ Dysfunction Working Group criteria for hepatic impairment), albumin (BALB, g/dL), alanine aminotransferase (BALT, U/L) on CL1; age, ALL effect, age and CD22BLST on k_{des}.[31] Noteworthy, blast in peripheral blood (BLSTPB, BLSTABL) does not apply for NHL patients and, therefore, blast related covariates were not imputed and the effects were only modeled in ALL patients. In addition, age effect was also modeled solely for ALL patients, as NHL patients only consisted of adults.

Table 1. Covariates Examined in Pediatric Population Pharmacokinetics Analysis of Inotuzumab Ozogamicin

Pharmacokinetic parameter	Covariate
CL_1	BBSA/ BWT/ LBM, Age, BHGRADE, BALB, BALT
V_1	BBSA/ BWT/ LBM
CL_2	BBSA/ BWT/ LBM, Age, ALL effect, BLSTABL
k_{des}	BLSTPB/ BLSTABL, Age

CL_1: Linear clearance; V_1: Volume of distribution in the central compartment; CL_2: Initial value of time-dependent clearance; k_{des}: Decay coefficient of time-dependent clearance. BBSA: Baseline body surface area calculated by the DuBois method; LBM: Baseline lean body mass (estimated by Boer's equations for adults, and by equation established by Peters et al. for children); BHGRADE: Baseline hepatic impairment grades; BALB: Baseline albumin; BAST: Baseline aspartate aminotransferase; BALT: Baseline alanine aminotransferase; ALL effect: disease type (ALL/NHL) and/or analytical methods; BLSTPB: Baseline percentage of blasts in the peripheral blood; BLSTABL: Baseline absolute blast counts in peripheral blood.

4.2.4 Model Evaluation

Covariate selection was based on parameter precision, biological plausibility and statistical significance. For hierarchically nested models, a drop of the objective function value (dOFV) ≥ 10.83, corresponds to P < 0.001 (x^2-distribution with 1 degree of freedom (df)), was used to determine a significant improvement of the fit. Model performance was evaluated by goodness-of-fit diagnostic plots, and prediction- and variability-corrected visual predictive check (pvcVPC).[32] The PK parameter estimates precision was assessed using the sampling importance resampling (SIR) procedure.[33]

4.2.5 Model-Based Exposure Estimation

Cumulative area under concentration-time curve (AUC) for cycle 1 was estimated using Maximum a Posteriori (MAP) Bayesian estimation using the POSTHOC option of NONMEM. The final model with actual trial dosing records were used to estimate InO exposure for each pediatric patient. Difference in cumulative AUC at the end of cycle 1 was compared numerically and graphically between responders and non-responders and between positive/negative-MRD status among responders, to preliminarily evaluate the exposure-efficacy relationship. Formal testing was hampered by potential shrinkage in individual AUC estimation and subsequent violation of the independence assumption in classical statistical tests. Hematologic response was defined as patients with < 5% blasts in the bone marrow and no circulating blasts or extramedullary

disease. The relationship between pharmacodynamic paraments and the response was analyzed before and published by our group.[20]

4.2.6 Model-Based Simulations

Simulations of the final InO population PK model were performed to simulate the expected concentration-time profile in adults and pediatric ALL patients at a fixed dosing regimen (the approved dosing regimen for adult R/R ALL and the pediatric RP2D; 1.8 mg/m^2/cycle fractionated in three weekly administrations for the first cycle of 21 days, followed by 1.5 mg/m^2/cycle for up to five cycles of 28 days). The simulations were employed to assess the PK endpoints in adult and pediatric patients, such as the cumulative AUC and terminal half-life. In addition, to evaluate whether similar InO exposure can be achieved without a loading dose, the final PK model was used for simulation in adults and pediatric ALL patients at the above-mentioned fixed dosing regimen without a loading dose on day 1 in the first cycle (1.5 mg/m^2/cycle fractionated evenly in three weekly administrations for the first cycle of 21 days, followed by 1.5 mg/m^2/cycle for up to five cycles of 28 days).

4.2.7 Software

Nonlinear mixed-effects modeling was performed using NONMEM (version 7.5.0, ICON Development Solutions, Ellicott City, MD, USA) and Pearl-speaks-NONMEM (PsN, version 5.3.0) with stochastic approximation expectation maximization (SAEM) and importance sampling (IMP) expectation maximization as estimation method.[34-36] Parameter precision was obtained by SIR as implemented in PsN[33]. Pirana (version 2.9.9) was used as a graphical user interface for NONMEM.[37] R (version 4.2.1) was used for data handling and visualization.

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4.3 Results

4.3.1 Population PK Analysis Dataset and Patient Characteristics

The dataset included 8924 serum InO PK observations from 818 patients; 5609 observations were from 531 adult NHL patients, 2752 observations from 234 adult ALL patients, and 563 observations from 53 pediatric ALL patients (13 treated in DL1 and 40 in DL2). Among children, 10 patients received three cycles, 20 received two cycles, and 23 received one cycle only. A total of 3394 observations (38.03%) were below the LLOQ; whereas only two observations were below the LLOQ in pediatric ALL patients. Patient baseline characteristics and covariates are summarized in Table 2 and in Supplementary Table 3.

4.3.2 InO Population PK Model

After re-estimating the PK parameters and covariate effects of the adult InO population PK model based on observations from both adult and pediatric patients, there is a difference in the distribution of empirical Bayes estimate of IIV (η) on k_{des} between pediatric and adult patients (Figure 2a).[23] The difference in IIV distribution and the negative trend across age categories (Figure 2b) demonstrate that the model did not appropriately account for the age effect on k_{des}. Further model development steps to examine the potential pediatric population relevant differences in our analysis and the corresponding change in model fit are shown in Supplementary Table 4. The results of the model development include: i) inclusion of separate residual error on pediatric population; ii) replacing BBSA by LBM to represent body size effect and replacing percentage of blast in peripheral blood by absolute counts (BLSTPB by BLSTABL), which both did not significantly affect the model fit, yet aiding in the clinical interpretation; iii) introduction of age on k_{des} and ALL effect on CL_2 which improved the model fit associated with a P-value < 0.001. The inclusion of age effect on k_{des} reflects the difference in the decline rate of the target mediated drug clearance across age. In NHL patients, higher initial value of the target mediated drug clearance was found compared to ALL patients, indicated by the inclusion of ALL effect on CL_2. No further covariate inclusion significantly improved the model performance. InO PK parameter estimates and the final population PK model are summarized in Table 3.

Table 2. Summary of Patient Baseline Covariates

| Covariates, Median [Range]	Adult NHL patients	Adult ALL patients	Pediatric ALL patients
Number of patients	531	234	53
Age, years	65.0 [18.0, 92.0]	46.0 [20.0, 79.0]	9.00 [1.00, 17.0]
Body weight, kg	73.2 [33.5, 148]	74.0 [30.9, 154]	34.3 [12.7, 147]
Body surface area[a], m^2	1.83 [1.12, 2.56]	1.86 [1.27, 2.81]	1.18 [0.550, 2.21]
Lean body mass[b], kg	53.6 [24.4, 87.4]	54.4 [29.4, 95.9]	29.5 [10.9, 69.0]
Albumin, g/dL	4.00 [2.20, 5.20]	3.80 [1.80, 4.93]	3.60 [2.50, 4.60]
Alanine aminotransferase, U/L	20.4 [3.00, 236]	33.0 [5.00, 161]	61.0 [4.00, 187]
White blood cells in peripheral blood, *10^9 counts	NA	NA	3.33 [0.19, 132]
Blasts in peripheral blood, %	NA	4.00 [0.00, 100]	3.00 [0.00, 85.0]
Absolute blast counts in peripheral blood, *10^9 counts	NA	0.82 [0.00, 254]	0.00 [0.00, 32.0]
Blasts that are CD22+ in peripheral blood, %	NA	98.7 [11.4, 100]	9.35 [0.00, 84.2]
Baseline NCI ODWG criteria for hepatic impairment (%)			
A (normal)	444 (83.6)	167 (71.4)	31 (58.5)
B1 (mild)	75 (14.1)	58 (24.8)	20 (37.7)
B2 (mild)	9 (1.70)	8 (3.40)	2 (3.80)
C (moderate)	2 (0.40)	1 (0.40)	0
D (severe)	1 (0.20)	0	0
Unknown	0	0	0
Combination therapy with rituximab (%)			
No	147 (27.7)	234 (100)	53 (100)
Yes	384 (50.2)	0	0

NHL: Non-Hodgkin lymphoma; ALL: Acute lymphoblastic leukemia; NCI ODWG: National Cancer Institute Organ Dysfunction Working Group; NA: Non-applicable
[a]Baseline body surface area calculated by the DuBois method for adults, estimated by Mosteller formula for children
[b]Baseline lean body mass (estimated by Boer's equations for adults, and by equation established by Peters et al. for children)

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Compared to the adult ALL patients in the previous population PK analysis, similar effect magnitude of body size and blasts in peripheral blood were identified in pediatric patients using the pooled data in our analysis.[23] A larger body size was associated with a higher CL_1, V_1, and CL_2. LBM is considered to be more relevant to organs contributing to the endogenous IgG antibody clearance, therefore LBM is used to represent body size instead of BBSA.[26] Compared to patients with a median LBM (52.73kg), in individuals with a low LBM (37.53kg; 10th percentile), CL_1 decreased by 29.0%, V_1 by 29.1% and CL_2 by 21.4%, leading to higher InO exposures (e.g. cumulative AUC, given the same dose). Whereas for patients with a high LBM (69.04kg; 90th percentile), CL_1 increased by 31.3%, V_1 by 30.9%, and CL_2 by 21.0%, leading to lower exposures. An increase in peripheral blood blasts count was related to a decrease in k_{des}; hence a decrease in the decline rate of CL_t. However, considering the magnitude of the BLSTABL effect and the rapid decline in CL_t (reduce by > 50% within one week for ALL patients with typical covariates value), BLSTABL is not deemed to significantly affect InO disposition, as also described by Garrett et al.[23]

For ALL patients, besides the covariates also identified in the adult model, an additional age effect on k_{des} was observed in our analysis. Increasing age was correlated with a decrease in k_{des} (Figure 3), reflecting that the target mediated drug clearance (CL_t) declined more rapidly in children compared to adults. Relative to the time for CL_t to reduce by 50% at the age of 60 (158 h, for ALL patients with the median BLSTABL value), the corresponding time required at age 1, 12, 18, and 30 were 47.0 h, 98.2 h, 111 h, and 129 h. It is noteworthy that CL_t is one of the components contributing to the total clearance of InO, where the contribution of CL_t reduced over time. Its contribution decreased by 90% after 3.12 weeks (age 60), 0.93 weeks (age 1), 1.94 weeks (age 12) and 2.19 weeks (age 18), indicating that the linear clearance component predominates after the first few weeks of treatment.
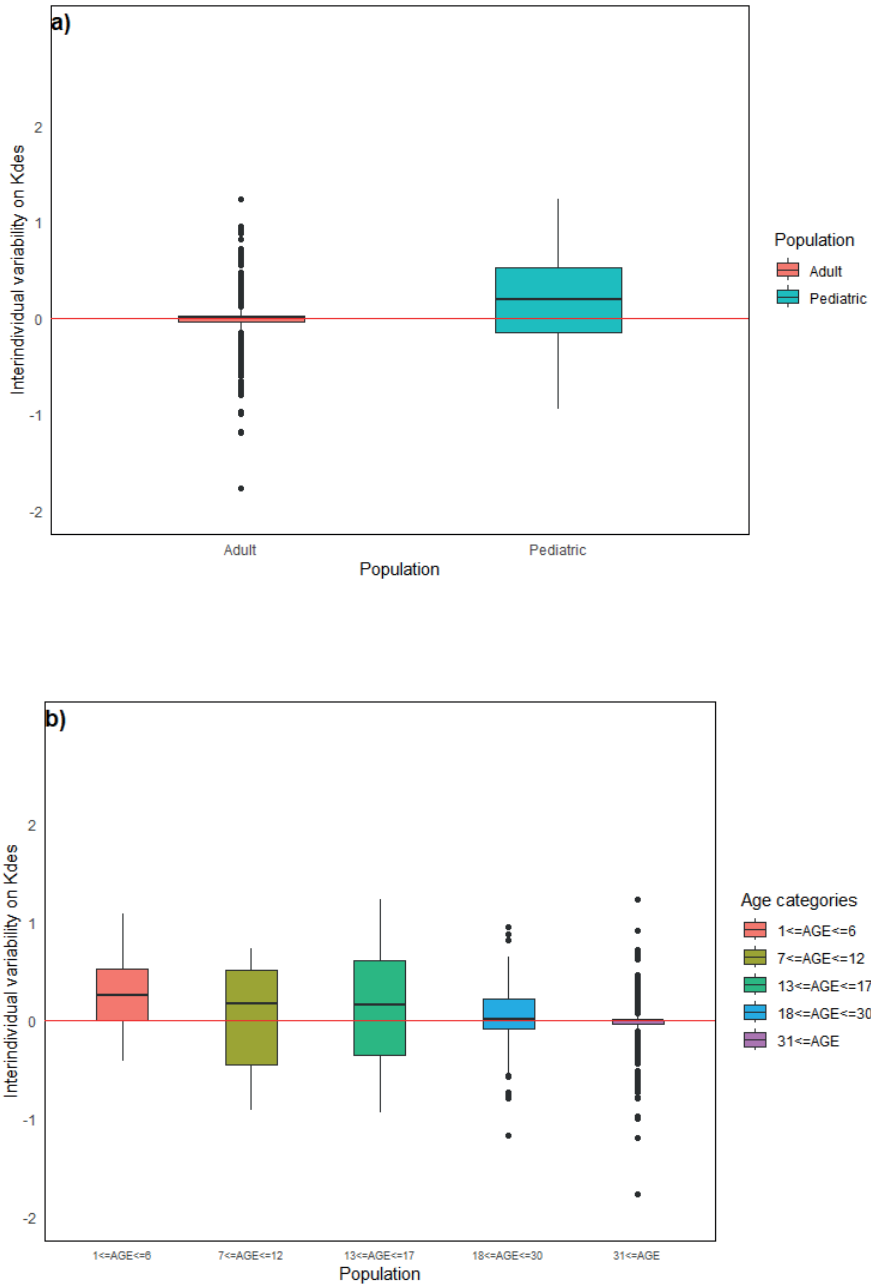
Figure 2. Distribution of Interindividual Variability on Decay Coefficient from the Previously Developed Adult Model After Re-Estimation. A) Adult and Children Population and B) Age Categories. The red solid line is the reference line (y = 0).
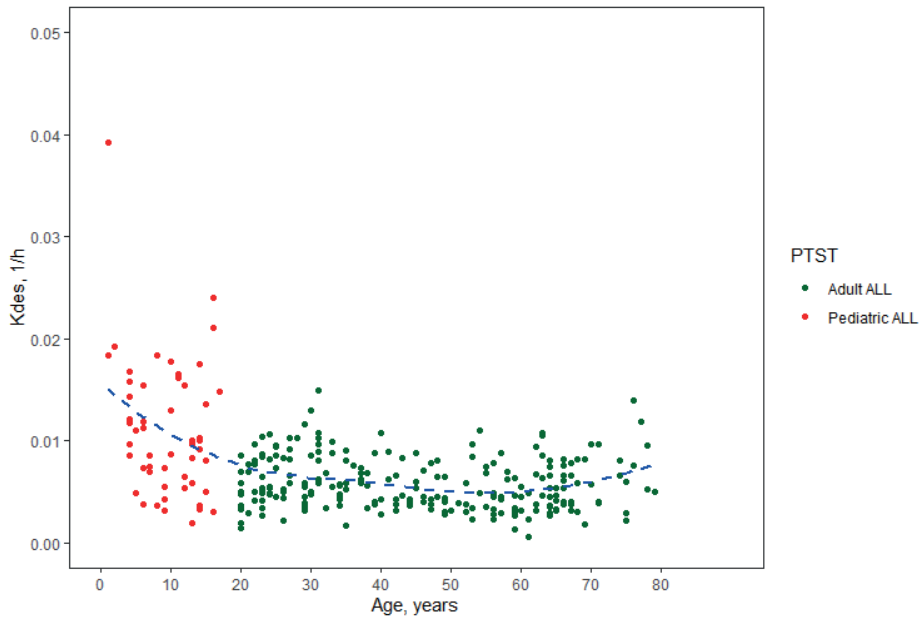
Figure 3 Distribution of Decay Coefficient of the Time-Dependent Clearance Versus Age in ALL Patients.
The blue dotted lines are the LOESS

Table 3. Result of Final Ino Population PK Model and Parameter Estimates

InO population PK model:

CL_1 : 0.130 L/h * $\left(1 - 0.767 * ALL\right)$ * $(LBM/52.73k\ g)^{1.05}$ * $\left(1 - 0.132 * RITUX\right)$

V_1 : 6.49 L * $(LBM/52.73k\ g)^{0.977}$

CL_2 : 0.569 L/h * $\left(1 - 0.362 * ALL\right)$ * $(LBM/52.73k\ g)^{0.687}$

$k_{des\ .NHL}$: 0.0577 h^{-1}

$k_{des\ .ALL}$: 0.0577 h^{-1} * $\left(1 - 0.924 * ALL\right)$ * $(BLST\ ABL/0.352)^{-0.0484}$ * $(AGE/60y)^{-0.296}$

Q : 0.0437 L/h

V_2 : 4.74 L

For all pediatric patients with R/R B-cell ALL, covariate ALL =1 and RITUX =0

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Parameters	Definition	NONMEM results				SIR results	
		Estimate	95% CI		Shrinkage%	95% CI	
			Lower	Upper		Lower	Upper
CL1, L/h	Linear clearance	0.130	0.117	0.143	-	0.120	0.141
ALL effect[a] on CL₁		-0.767	-0.793	-0.741	-	-0.793	-0.740
LBM effect on CL₁		1.05	0.891	1.21	-	0.901	1.20
Concomitant rituximab treatment effect[b] on CL₁		-0.132	-0.204	-0.0605	-	-0.202	-0.0582
V₁, L	Volume of distribution in central compartment	6.49	6.24	6.74	-	6.26	6.70
LBM effect on V₁		0.977	0.861	1.09	-	0.847	1.10
CL₂, L/h	Initial estimate of time-dependent clearance	0.569	0.380	0.758	-	0.456	0.721
ALL effect on CL₂		-0.362	-0.597	-0.127	-	-0.507	-0.173
LBM effect on CL₂		0.687	0.320	1.05	-	0.453	0.929
kdes, 1/h	Decay coefficient of the time-dependent clearance[a]	0.0577	0.0342	0.0812	-	0.0390	0.0826
ALL effect on kdes		-0.924	-0.956	-0.892	-	-0.949	-0.893
BLSTABL effect on kdes		-0.0484	-0.0686	-0.0282	-	-0.0641	-0.0336
Age effect on kdes		-0.296	-0.474	-0.118	-	-0.413	-0.189
Q, L/h	Intercompartment clearance	0.0437	0.0348	0.0526	-	0.0383	0.0493
V₂, L	Volume of distribution in peripheral compartment	4.74	2.62	6.86	-	3.97	5.49
CL₁, IIV[c] (%)		40.0%	31.6%	46.9%	20.8%	35.8%	44.9%
V₁, IIV[c] (%)		40.1%	34.9%	44.8%	16.6%	36.9%	43.0%
CL₂, IIV[c] (%)		73.7%	53.8%	89.2%	24.5%	63.8%	84.3%
kdes, IIV[c] (%)		59.7%	40.7%	73.9%	51.3%	53.3%	67.1%
CL₁ - V₁; correlations (%)		0.136; 84.7%	0.0939	0.178	-	0.111	0.166
CL₁ - CL₂; correlations (%)		0.194; 65.8%	0.125	0.263	-	0.151	0.241
V₁ - CL₂; correlations (%)		0.204; 69.0%	0.144	0.264	-	0.167	0.242
NHL, proportional residual error[d]		0.444	0.393	0.495		0.429	0.458
Adult ALL, proportional residual error[d]		0.612	0.546	0.678	18.5%	0.595	0.630
Pediatric ALL, proportional residual error[d]		0.452	0.338	0.566		0.418	0.486

InO: Inotuzumab Ozogamicin; PK: Pharmacokinetics; OFV: Objective function value; SIR: Sampling importance resampling procedure; NHL: Non-Hodgkin lymphoma; ALL: Acute lymphoblastic leukemia; LBM: Baseline lean body mass; RITUX: Rituximab combination therapy; BLSTABL: Baseline absolute blast counts in peripheral blood; IIV: Interindividual variability

[a]ALL effect accounts for disease type (NHL/ALL) and/or different bioanalytical analysis methods.

[b]Reference group of concomitant rituximab treatment was "with rituximab" in adults study. In this study the reference was "without rituximab".

[c]IIV was reported as percent coefficient of variation (%CV).

[d]Separate residual errors were included in the model to account for variability between trials. Reported as standard deviation.

4.3.3 Model Evaluation

Goodness-of-fit diagnostic plots of the final model showed no indication of model misspecification in both children (Figure 4) and adults (Supplementary Figure 1). Furthermore, the Prediction-Corrected Visual Predictive Checks indicated that the observed data were align with simulated predictions from the final model for pediatric patients (Figure 5) and adults (Supplementary Figure 2). PK parameter estimates precision was verified through the stable estimates and confidence interval from SIR procedure (Table 4). In addition, the distribution of empirical Bayes estimate of IIV (η) on k_{des} were centered around 0 against age after the inclusion of age in the model (Figure 6), suggesting that the final model appropriately describe the PK difference across ages. Lastly, IIV distribution on PK parameters against other covariates (Supplementary Figure 3) also indicates the final model properly addressed the PK variability associated with covariates.

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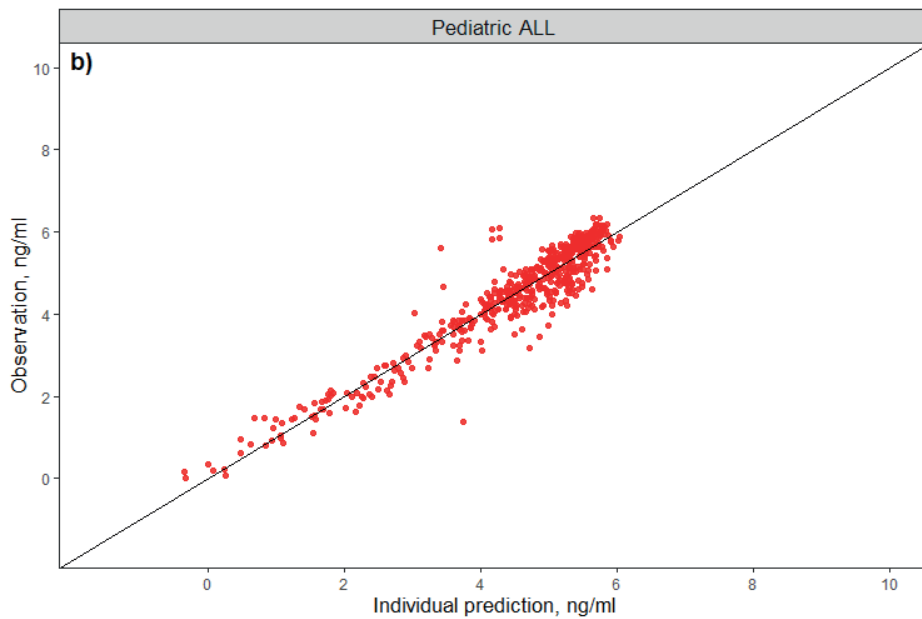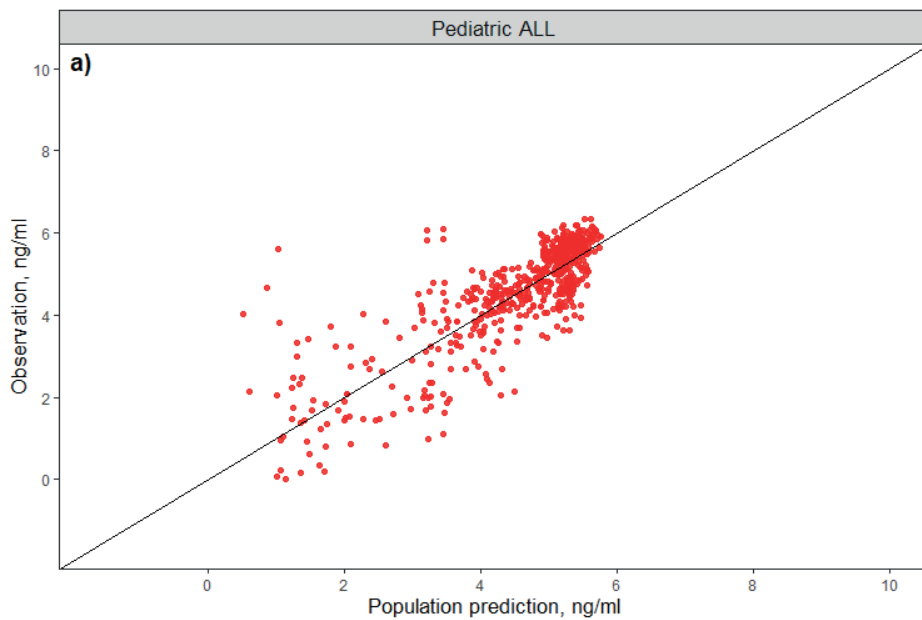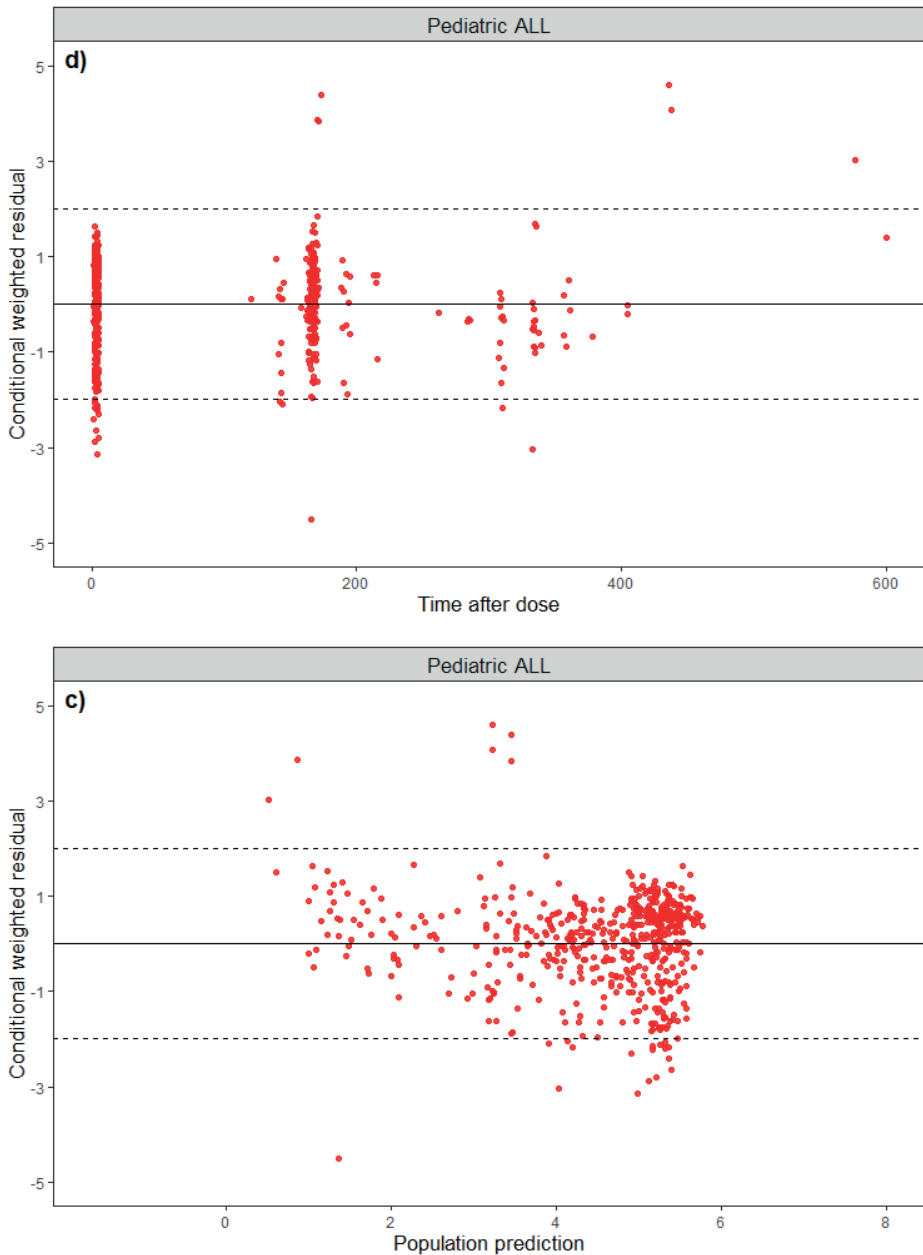
Figure 4. Goodness-of-Fit Diagnostic Plots of the Final Model for Pediatric Acute Lymphoblastic Leukemia Patients. Log observed Inotuzumab Ozogamicin concentration versus a) population prediction and b) individual prediction. The solid lines show the reference line (y = x). c) Scatter plots of conditional weighted residuals against population prediction and d) time after each dose.
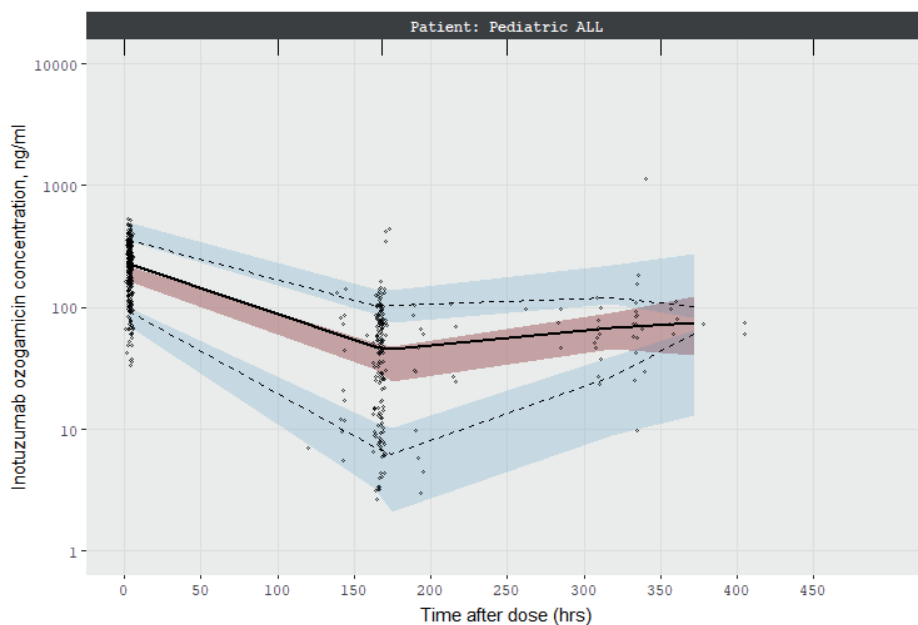
Figure 5. Prediction and Variability-Corrected Visual Predictive Check in Pediatric Acute Lymphoblastic Leukemia Population. Black circles represent the observed data. The black lines show median (solid) and the 10th and 90th percentiles (dash) of the observed data. The shaded regions show the 95% CI of the median (red) and the 10th and 90h percentiles (blue) of the simulated concentration (N = 1000).
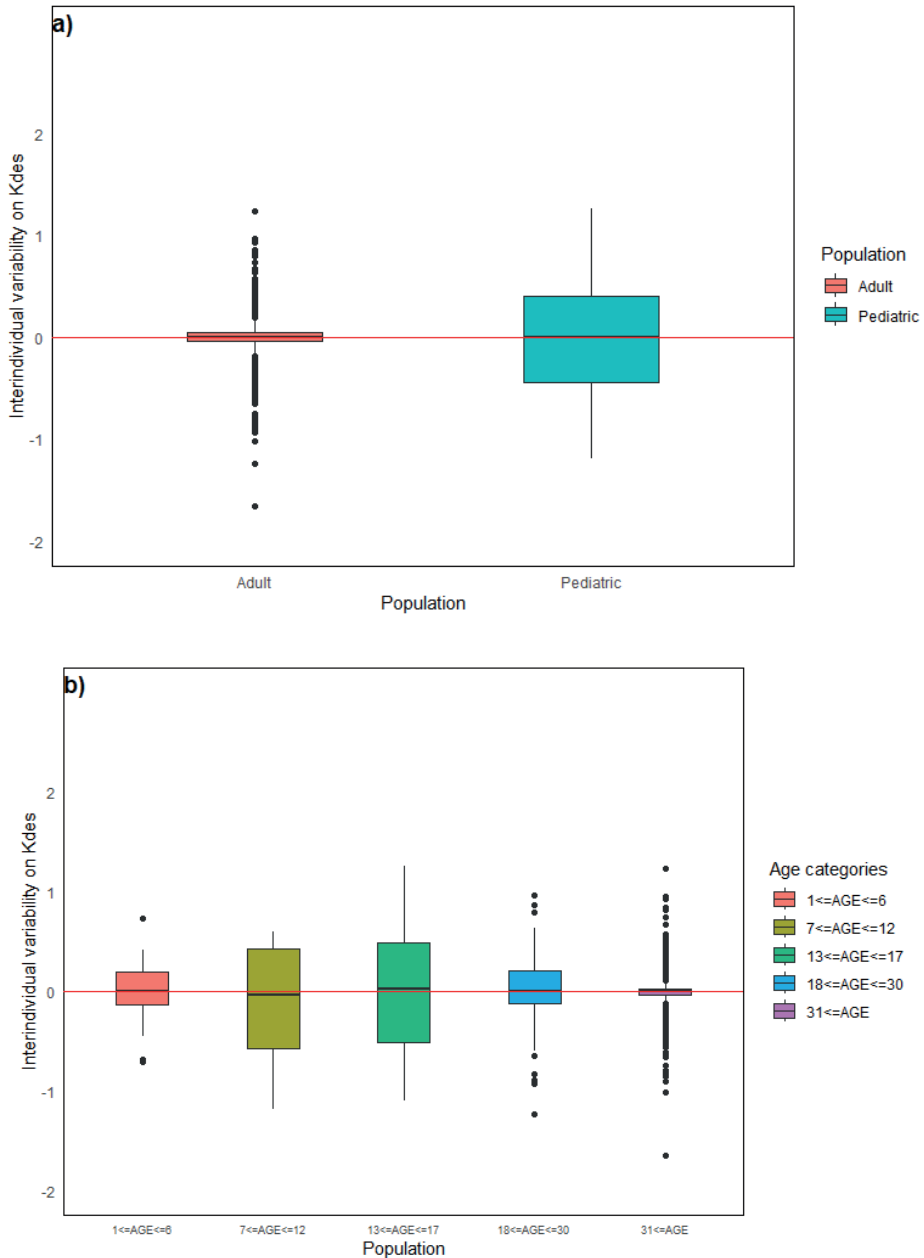
Figure 6. Distribution of Interindividual Variability on Decay Coefficient from the Final Model. A) Adult and Children Population and B) Age Categories. The Red Solid Line Is the Reference Line (Y = 0).

4.3.4 Model-Based Exposure Estimation

Estimated by the final population PK model with the clinical trial dosing records, in pediatric trial participants, the median cumulative AUC at the end of cycle 1 was higher in responders compared to non-responders (26.1· 10^3 vs 10.1 · 10^3 ng*h/mL, Figure 7 and Table 4). Among responders, the median cumulative AUC at the end of cycle one was slightly higher in MRD-negative patients when compared to MRD-positive ones (Figure 7, Table 4). Comparisons at later cycles was not considered, as receiving additional cycles might be dependent on treatment response after cycle 1 and the interpretation limited due to the small sample size. Finally, it is worth noting that responders exhibited a faster rate of decline in CL_t as compared to non-responders. This distinction is substantiated by a higher k_{des} value caused by a higher variability median (0.168 vs. -0.724; p-value < 0.001) in responders. This might explain the difference in cumulative AUC between responders and non-responders.

Table 4. Final Model-Based Inotuzumab Ozogamicin Exposure Estimation

Cumulative area under concentration-time curve (AUC) estimation (median [IQR], *10^3 ng*h/mL)				
End of Cycle1	**Responders**	**Non-responders**	**MRD-negative (responders)**	**MRD-positive (responders)**
Pediatric ALL	26.1 [18.9 - 35.0]	10.1 [9.19 - 16.1]	26.4 [20.1 - 35.1]	21.8 [10.9 - 29.4]

IQR: Interquartile range; ALL: Acute lymphoblastic leukemia; MRD: minimal residual disease
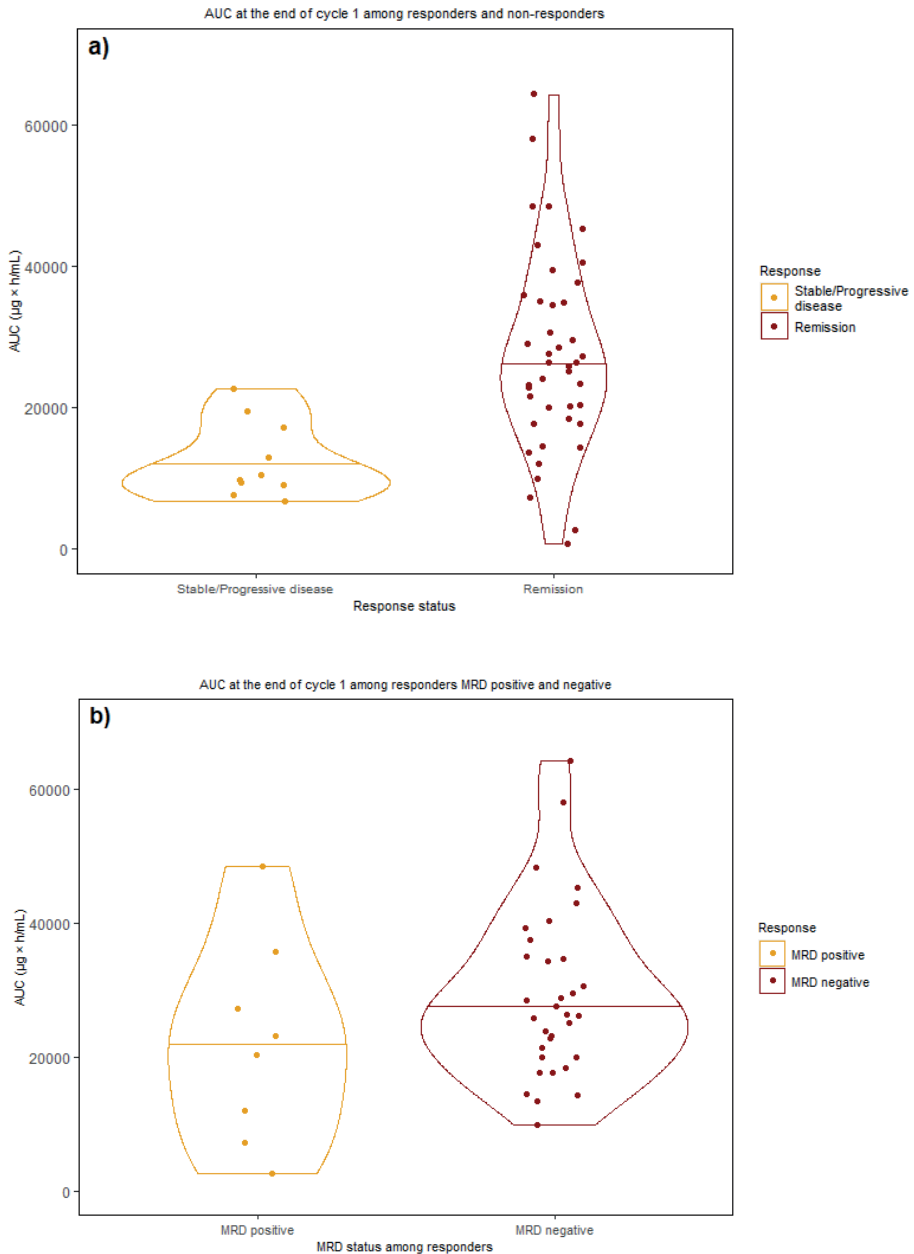
Figure 7 Estimation of Inotuzumab Ozogamicin Exposure in Pediatric Acute Lymphoblastic Leukemia Patients Using Final Model and Dosing Record in Trial. a) Cumulative area under concentration-time curve (AUC) at the end of cycle 1 for non-responders and responders. b) Cumulative (AUC) at the end of each cycle1 for MRD positive and negative patients.

4.3.5 Model-Based Simulations

A fixed dosing regimen at the RP2D was used for the final model-based simulation. For adult and pediatric ALL patients following the fixed dosing scheme, the simulated concentration time profile are generally overlapped and steady-state was fully achieved by the fourth cycle (Figure 8). The terminal beta half-life of the adult and pediatric ALL patients was 285 h (11.9 days) and 423 h (17.6 days), respectively. The predicted median cumulative AUC at the end of each cycle (Figure 7, Table 5) showed a 30 - 35% higher exposure in pediatric patients compared to adults. A 9% lower predicted median cumulative AUC was reached in patients who received the fixed dosing regimen without the loading dose on day 1 in the first cycle (Table 5). The simulation results stratified by age group are presented in Supplementary Table 5 and Supplementary Figure 4.
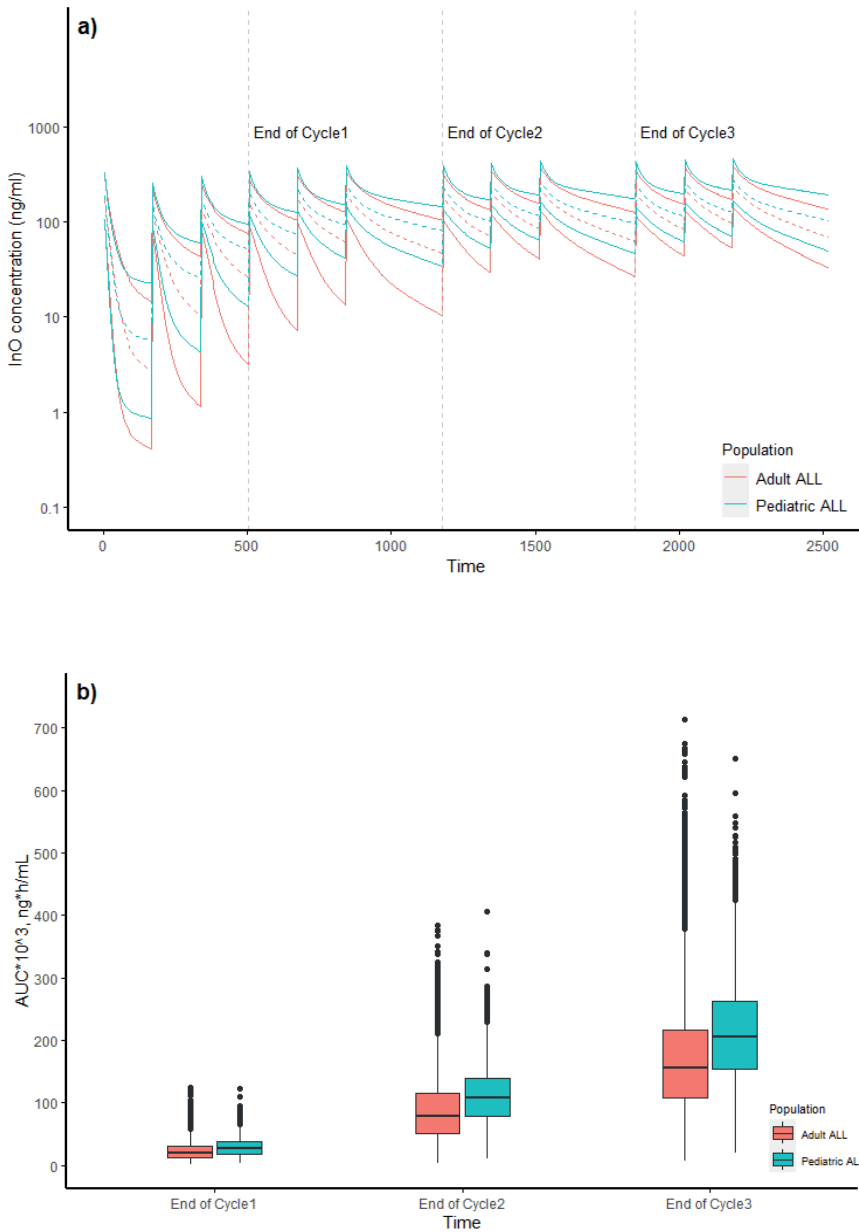
Figure 8 Simulation of Inotuzumab Ozogamicin In Adult and Pediatric Acute Lymphoblastic Leukemia Patients. a. Simulated Concentration-Time Profile For 4 Cycles. Green lines denote InO exposure in pediatric patients and red lines denote InO exposure in adults. Medians are shown with dashed lines, the 10th and 90th percentiles are shown with solid lines; b. Cumulative area under concentration-time curve (AUC) at the end of each cycle.

Table 5. Final Model-Based Inotuzumab Ozogamicin Exposure Simulation

Cumulative area under concentration-time curve (AUC) simulation (median, $*10^3$ ng*h/mL)			
	End of Cycle1	**End of Cycle2**	**End of Cycle3**
Adult ALL	19.7	79.0	155
Pediatric ALL	26.6	107	205
Difference (%, adult as reference)	+34.9	+35.9	+32.2

Cumulative AUC simulation (median, $*10^3$ ng*h/mL; without loading dose (LD) on Day1 Cycle1)		
	End of Cycle1	**Cumulative AUC difference (%; with LD and the adult/pediatric group as reference)**
Adult ALL	17.9	-9.18%
Pediatric ALL	24.4	-8.28%

ALL: Acute lymphoblastic leukemia

4.4 Discussion

This study is the first to describe the population PK of InO in a pediatric population by analyzing the pooled PK data from adult NHL and ALL, and pediatric ALL. The concentration-time profile of InO in both adult and pediatric patients were well described by the final population PK model. The structure of the model aligns with the PK analyses of similar therapeutic molecules, a two-compartment PK model was reported in most population PK analyses for monoclonal antibodies; the time-dependent/time-varying clearance component has been applied to other B-cell targeting antibodies and ADCs, e.g. rituximab and gemtuzumab ozogamicin.[25,26,38,39] The time-dependent clearance component of InO reflects the decline in the target-mediated clearance pathway, which is related to tumor burden reduction over time, supported by the fact that complete remissions were achieved by most pediatric patients by the end of the first cycle, a time when time-dependent clearance decreased by > 95% in pediatric patients.[20]

A high correlation was observed between BBSA and LBM in the dataset; therefore replacing BBSA by LBM did not significantly change the model as expected. Considering the organs contributing the most (skin, muscle, and liver) to endogenous IgG antibody clearance, LBM might be a more representative and relevant covariate to represent body size.[26] Therefore, without significantly affecting the model fit, LBM replaced BBSA in the covariate model in order to enhance the generalizability for future studies in specific subgroups. The trend and size of the LBM effect on InO disposition are consistent with the influence of BBSA reported in the adult model; hence, supporting the current BSA-based dosing strategy for pediatric R/R BCP-ALL patients.[23]

The inclusion of age effect on k_{des} implies that the target-mediated drug clearance (related to tumor burden) declines more rapidly in children compared to adults. The age effect might indicate that in pediatric ALL patients, tumor cells were depleted faster, while tumor cells were more persistent in adult ALL patients. The discrepancy in decline rate of target mediated clearance might suggest a difference in the rate of intracellular calicheamicin accumulation or in the sensitivity to this cytotoxic agent. This might be explained by a different role played by drug efflux pumps (such as P-glycoprotein and multidrug resistance-associated protein) in the unresponsiveness to InO, while in adult ALL patients drug efflux pumps have a notable role in resistance, their involvement is less likely in pediatric ALL.[16,40-42]

Estimated from pediatric ALL patients following the dosing scheme in ITCC-059 Phase IA and Phase II Trial, the cumulative AUC was higher among responders at the end of first cycle. This is in agreement with the exposure-response analysis for efficacy of InO in adults with R/R ALL patients, where InO exposure (C_{avg}, as the ratio of cumulative AUC over time) is significantly and positively correlated with achieving remission and MRD-negativity.[43] Additionally, there has been limited information regarding the relationship between InO exposure and clinical effectiveness in children. Therefore, the cumulative AUC value estimated from responding pediatric trial participants could be considered as a preliminary reference of an effective InO

exposure in pediatric ALL. From the simulation in ALL patients following the RP2D, a 30 - 35% higher difference in cumulative AUC in pediatric compared to adult ALL patients was found, reflecting the impact of age on InO exposure. However, the simulation results demonstrated that the effective InO exposure level was reached in pediatric ALL patients following the RP2D; moreover, the RP2D was well tolerated with a satisfactory response rate.[20,24] Therefore, no further dose adjustment is required for pediatric ALL patients despite of the impact of age.

A limitation of the study relates to the lack of prior knowledge on therapeutic monitoring and the causal inference of exposure and efficacy relationship of InO in children. It is commonly reported that the exposure-response relationship of monoclonal antibodies is, at least partially, confounded by general disease risk factors or underlying immune system.[44] Ergo, it was uncertain whether the InO exposure in pediatric ALL patients following a lower dosing regimen (i.e., without a loading dose) is sufficient. A reduction of InO dose could be beneficial for safety concerns as, for example, a higher risk of sinusoidal obstruction syndrome (SOS) was associated with higher InO exposure in adults.[43] At present, several trials are experimenting combinations of InO at a reduced dose with other agents such as blinatumomab and rituximab, but also standard chemotherapy.[45,46] More studies are required to unravel the exposure-response and exposure-safety correlations of InO, especially in children.

4.5 Conclusions

In conclusion, the PK profile of InO in pediatric R/R B-cell ALL patients was well described by our model using pediatric and adult data. In ALL patients, compared to the adult model, similar body size effect on InO clearance and distribution and blasts in peripheral blood effect on k_{des} were identified, whereas the additional age effect may provide further physiological insights into the difference between adult and pediatric ALL. Despite the difference in simulated InO exposure between adult and pediatric patients, children receiving the RP2D achieved a desirable cumulative AUC at the end of the first cycle; additionally, the RP2D has been reported to be well tolerated in pediatrics.[20,24] Therefore, no dose adjustment is required in pediatric R/R B-cell ALL patients for clinically reasons. Future studies may need to address issues such as reducing the cumulative dose with the intent to avoid SOS, as piloted in adult/elderly ALL patients, for examples in several studies from the MD Anderson center.

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Supplementary Material

Supplementary Table 1. Inclusion/exclusion criteria

Inclusion criteria	
Age	• Patients must be ≥ 1 and < 18 years of age at the time of enrollment
Diagnosis	Patients must have either • First relapse of BCP-ALL post allogeneic HSCT • Second or greater relapsed or refractory BCP-ALL • Refractory disease, defined as newly diagnosed patients who are induction failures after at least 2 previous regimens without attainment of remission, or patients with refractory first relapse after 1 previous reinduction regimen without attainment of remission **AND** must meet the following criteria: • M2 or M3 marrow status (≥ 5% blasts by morphology) • The malignant clone needs to be CD22 surface antigen positive (in either the bone marrow or peripheral blood) by institutional standards as determined by the local immunophenotyping laboratory • The first 6 patients must have M3 marrow status (≥ 25% blasts by morphology)
Performance level and life expectancy	• Karnofsky > 60% (>16 years of age) and Lansky > 60% (≤ 16 years of age) • A life expectancy of at least 6 weeks
Prior Therapy	Patients must have fully recovered from the acute toxic effects of all prior chemotherapy, immunotherapy, or radiotherapy defined as resolution of all such non-hematologic toxicities to ≤ Grade 2 per the CTCAE 4.03 prior to entering this study a. Chemotherapy: At least 7 days since the completion of cytotoxic therapy, with the exception of hydroxyurea, 6-mercaptopurine and steroids which are permitted up until 48 hours prior to initiating protocol therapy b. Radiotherapy: At least 28 days since any prior radiation therapy c. Hematopoietic Stem Cell Transplant: At least 90 days since previous allo-HSCT. Patient must have no evidence of active graft vs. host disease. Patient must not be receiving GVHD prophylaxis or treatment. d. Hematopoietic growth factors: At least 7 days since the completion of therapy with GCSF or other growth factors. At least 14 days since the completion of therapy with pegfilgrastim (Neulasta®). e. Immunotherapy: At least 42 days since the completion of any type of immunotherapy, e.g. CART therapy. Patients may not have received prior CD22- targeted therapy (immunotoxin or CART therapy). f. Monoclonal antibodies: At least 3 half-lives of the antibody since the last dose of a monoclonal antibody (e.g. Rituximab = 66 days, Epratuzumab = 69 days), with the exclusion of blinatumomab. Patients must have been off blinatumomab infusion for at least 14 days and all drug-related toxicity must have resolved to grade 2 or lower g. Investigational drugs: At least 7 days or 5 drug half-lives (whichever is longer) since prior treatment with any experimental drug (with the exception of monoclonal antibodies) under investigation. No residual toxicities should be observed following previous treatment. h. Prior calicheamicin exposure: Patient has not received prior treatment with a calicheamicin-conjugated antibody (e.g. gemtuzumab ozogamicin).

Renal and hepatic function	• Serum creatinine ≤ 1.5 x institutional ULN according to age. If the serum creatinine > 1.5 x institutional ULN, the patient must have a GFR ≥ 70 mL/min/1.73 m² estimated based on serum creatinine and/or cystatin C levels (e.g. Bedside Schwartz formula) • AST and ALT ≤ 2.5 x institutional ULN • Total bilirubin ≤ 1.5 x institutional ULN unless the patient has documented Gilbert syndrome
Cardiac function	• A shortening fraction ≥ 30% by echocardiogram or an ejection fraction > 50% by MUGA
Reproductive function	• Female patients of childbearing potential: must have a negative urine or serum pregnancy test prior to enrollment • Female patients with infants must agree not to breastfeed on study • Male and female patients of child-bearing potential must agree to use a highly effective method of contraception (at least 8 months for females and at least 5 months for males after the last dose of InO)
Exclusion criteria	
Isolated extramedullary relapse	• Patients with isolated extramedullary disease will be excluded
VOD/SOS	• Any history of prior or ongoing VOD/SOS as per the modified Seattle criteria will be excluded, or prior liver-failure [defined as severe acute liver injury with encephalopathy and impaired synthetic function (INR of ≥1.5)]
Infection	• Patients with a systemic fungal, bacterial, viral or other infection that is exhibiting ongoing signs/symptoms will be excluded • The patient may not have: • A requirement for vasopressors • Positive blood culture within 48 hours of study enrollment • Fever above 38.2 degrees Celsius within 48 hours of study enrollment with clinical signs of infection. Fever that is determined to be due to tumor burden is allowed if patients have documented negative blood cultures for at least 48 hours prior to enrollment and no concurrent signs or symptoms of active infection or hemodynamic instability • A positive fungal culture within 30 days of study enrollment • Active fungal, viral, bacterial, or protozoal infection requiring IV or oral treatment. Chronic prophylaxis therapy to prevent infections is allowed
Other anti-cancer therapy	• Patients will be excluded if there is a plan to administer non-protocol anti-cancer therapy during the study period. • Patients will be excluded if they have received prior treatment with anti-tumor vaccines
Allergic reaction	• Patients with prior Grade 3/4 allergic reaction to a monoclonal antibody will be excluded
Concurrent disease	• Patients with significant concurrent disease, illness, psychiatric disorder or social issue that would compromise patient safety or compliance with protocol therapy, interfere with consent, study participation, follow up, or interpretation of study results will be excluded • Children with Down syndrome will be excluded from the dose finding parts of the study

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Supplementary Table 2. Detail of Inotuzumab Ozogamicin Pharmacokinetics Sampling Schedule for Pediatric Acute Lymphoblastic Leukemia Patients

Serum concentration	Day 1		Day 8	Day 15		Day 22	Day 28
Hour (post-dose)	Pre-dose	1 hr	Pre-dose	Pre-dose	1 hr	Trough sample	Trough sample
Cycle1	x	x	x	x	x	x	
Cycle2		x	x	x	x		x
Cycle3		x	x	x			x

Supplementary Table 3. Summary of Patient Baseline Covariates

Covariates, Median [Range]	Adult NHL patients	Adult ALL patients	Pediatric ALL patients
Number of patients	531	234	53
Sex (%)			
Male	317 (59.7)	141 (60.3)	36 (67.9)
Female	214 (40.3)	93 (39.7)	17 (32.1)
Creatinine clearance[a], mL/min	81.8 [18.2, 264]	122 [29.4, 368]	223 [78.7, 636]
Total bilirubin, mg/dL	0.499 [0.100, 3.90]	0.500 [0.100, 2.16]	0.430 [0.117, 1.37]
Aspartate aminotransferase, U/L	24.0 [7.00, 163]	26.0 [5.00, 187]	40.0 [11.0, 160]
Prior radiotherapy (%)			
No	397 (74.8)	177 (75.6)	45 (84.9)
Yes	134 (25.2)	57 (24.4)	8 (15.1)
Granulocyte colony-stimulating treatment (%)			
No	434 (81.7)	148 (63.2)	48 (90.6)
Yes	97 (18.3)	86 (36.8)	5 (9.40)
Concomitant hydroxyurea (%)			
No	531 (100)	221 (94.4)	53 (100)
Yes	0	13 (5.60)	0
P-glycoprotein inhibitors (%)			
No	439 (82.7)	187 (79.9)	47 (88.7)
Yes	92 (17.3)	47 (20.1)	6 (11.3)

NHL: Non-Hodgkin lymphoma; ALL: Acute lymphoblastic leukemia; NA: Non-applicable
[a]Creatinine clearance: estimated by Cockroft & Gault formula for adults; estimated by Bedside Schwartz formula for pediatric patients

Supplementary Table 4. Model Development Examined in Population Pharmacokinetic Analysis of Inotuzumab Ozogamicin

Adult InO population PK model, after re-estimation (Model 1; model development basis) Two-compartment model with linear and time-dependent clearance Covariates: CL_1: BBSA, ALL effect[b], RITUX[c]; CL_2: BBSA; V_1: BBSA; k_{des}: ALL effect[b], BLSTPB Estimated interindividual variability (IIV) on CL_1, CL_2, V_1, k_{des}	OFV; dOFV[a] (Reference) 1449.72; 0 (Ref.)

Examined changes	OFV; dOFV (Ref.)
Estimated IIV on Q and V_2 (Model 1a)[d]	1316.14; -133.58 (Model 1)
Estimated separate residual error on pediatric population (Model 1b)	1398.04; -51.68 (Model 1)
Replaced BLSTPB by BLSTABL on k_{des} (Model 1c)	1392.24; -57.48 (Model 1)
Replaced BBSA by BWT, BWT on V_1 tested by power function (Model 2)	1425.25; +33.01 (Model 1c)
Replaced BBSA by LBM, LBM on V_1 tested by power function (Model 3)	1390.32; -1.92 (Model 1c)
Age on k_{des} (Model 4)	1371.55; -18.77 (Model 3)
Age on CL_1 (Model 5)	1385.64; -4.68 (Model 3)
Age on CL_2 (Model 6)	1392.42; +2.10 (Model 3)
BLSTABL on CL_2 (Model 7)	1389.74; -0.58 (Model 3)
ALL effect[b] on CL_2 (Model 8)	1375.81; -14.51 (Model 3)
BHGRADE on CL_1 (Model 10)	1390.20; -0.12 (Model 3)
BALB on CL_1 (Model 11)	1388.70; -1.62 (Model 3)
BALT on CL_1 (Model 12)	1393.64; +3.32 (Model 3)
Age on k_{des} and ALL effect on CL_2 (Model 13)	1356.67; -14.88 (Model 4)

Results of model development: Separate residual error on pediatric population; Replacing BLSTPB by BLSTABL on k_{des}; Replacing BBSA by LBM; Introducing Age on k_{des} and ALL effect on CL2 as an additional significant covariate
InO: Inotuzumab Ozogamicin; PK: Pharmacokinetics; OFV: Objective function value; CL_1: Linear clearance; V_1: Volume of distribution in the central compartment; CL_2: Initial value of time-dependent clearance; k_{des}: Decay coefficient of time-dependent clearance; NHL: Non-Hodgkin lymphoma; ALL: Acute lymphoblastic leukemia; BBSA: Baseline body surface area; LBM: Baseline lean body mass; RITUX: Rituximab combination therapy; BHGRADE: Baseline hepatic impairment grades; BALB: Baseline albumin; BAST: Baseline aspartate aminotransferase; BALT: Baseline alanine aminotransferase; BLSTABL: Baseline absolute blast counts in peripheral blood
[a]Difference in objective function value between the indicated model and the reference model.
[b]ALL effect accounts for disease type (NHL/ALL) and/or different bioanalytical analysis methods.
[c]Reference group of concomitant rituximab treatment in this study was "without rituximab"; whereas it was "with rituximab" in the adult study.
[d]Insufficient information to estimate IIV on peripheral PK parameters indicated by convergence issue and greatly deviated estimates on Q and V_2 observed at the end of the iterative SIR procedure.
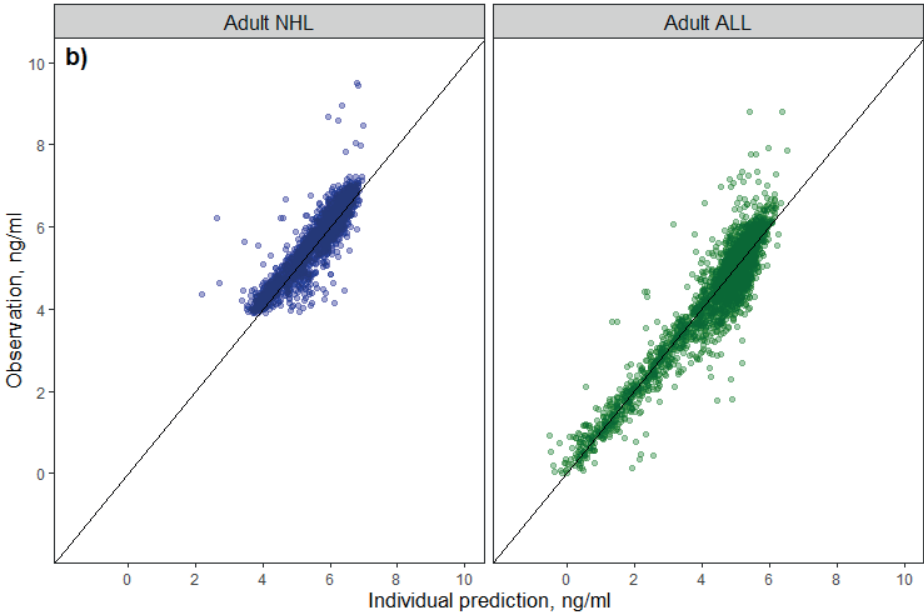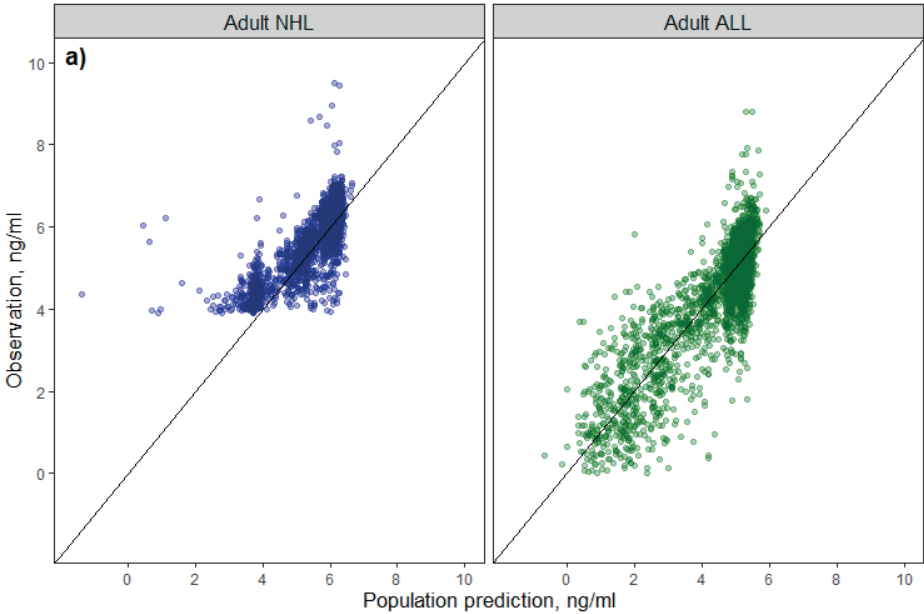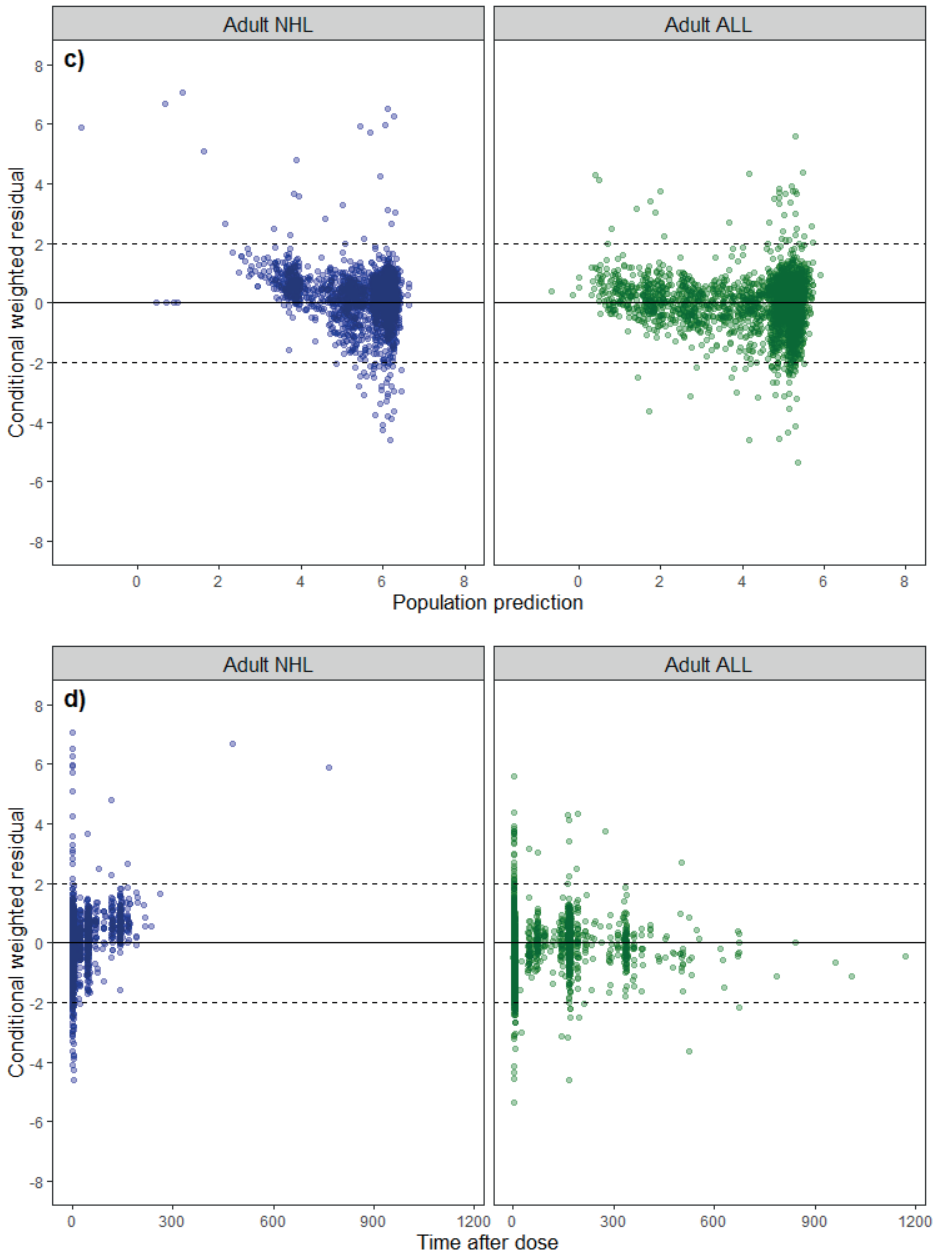
Supplementary Figure 1. Goodness-of-fit Diagnostic Plots of the Final Model for Adult non-Hodgkin Lymphoma (NHL) and Adult Acute Lymphoblastic Leukemia (ALL) Patients. Log observed Inotuzumab Ozogamicin concentration versus a) population prediction and b) individual prediction. The solid lines show the reference line (y = x). c) Scatter plots of conditional weighted residuals against population prediction and d) time after each dose.
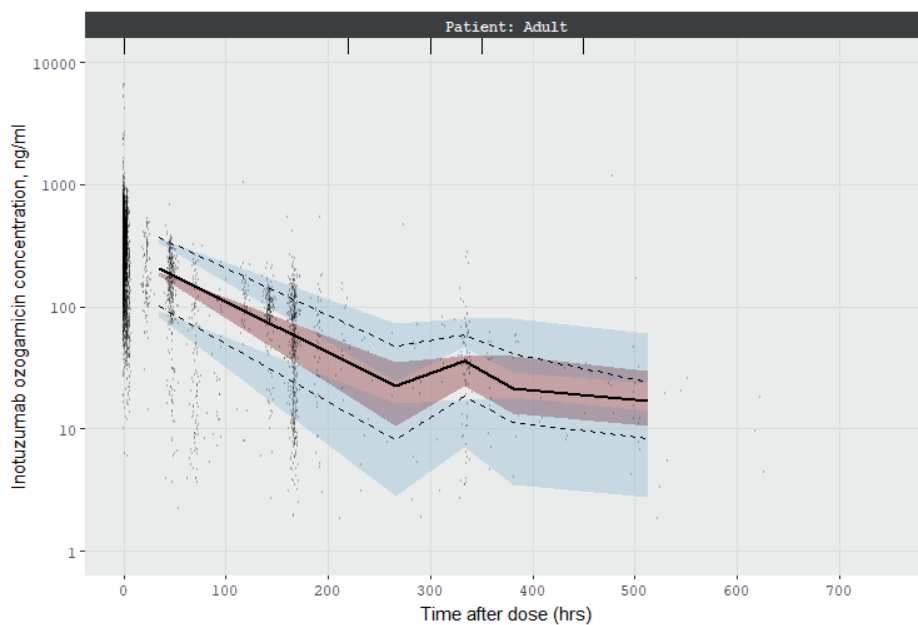
Supplementary Figure 2. Prediction- and Variability-Corrected Visual Predictive Check in Adult non-Hodgkin Lymphoma (NHL) and Adult Acute Lymphoblastic Leukemia (ALL) Patients. Black circles represent the observed data. The black lines show median (solid) and the 25th and 75th percentiles (dash) of the observed data. The shaded regions show the 95% CI of the median (red) and the 25th and 75th percentiles (blue) of the simulated concentration (N = 1000).
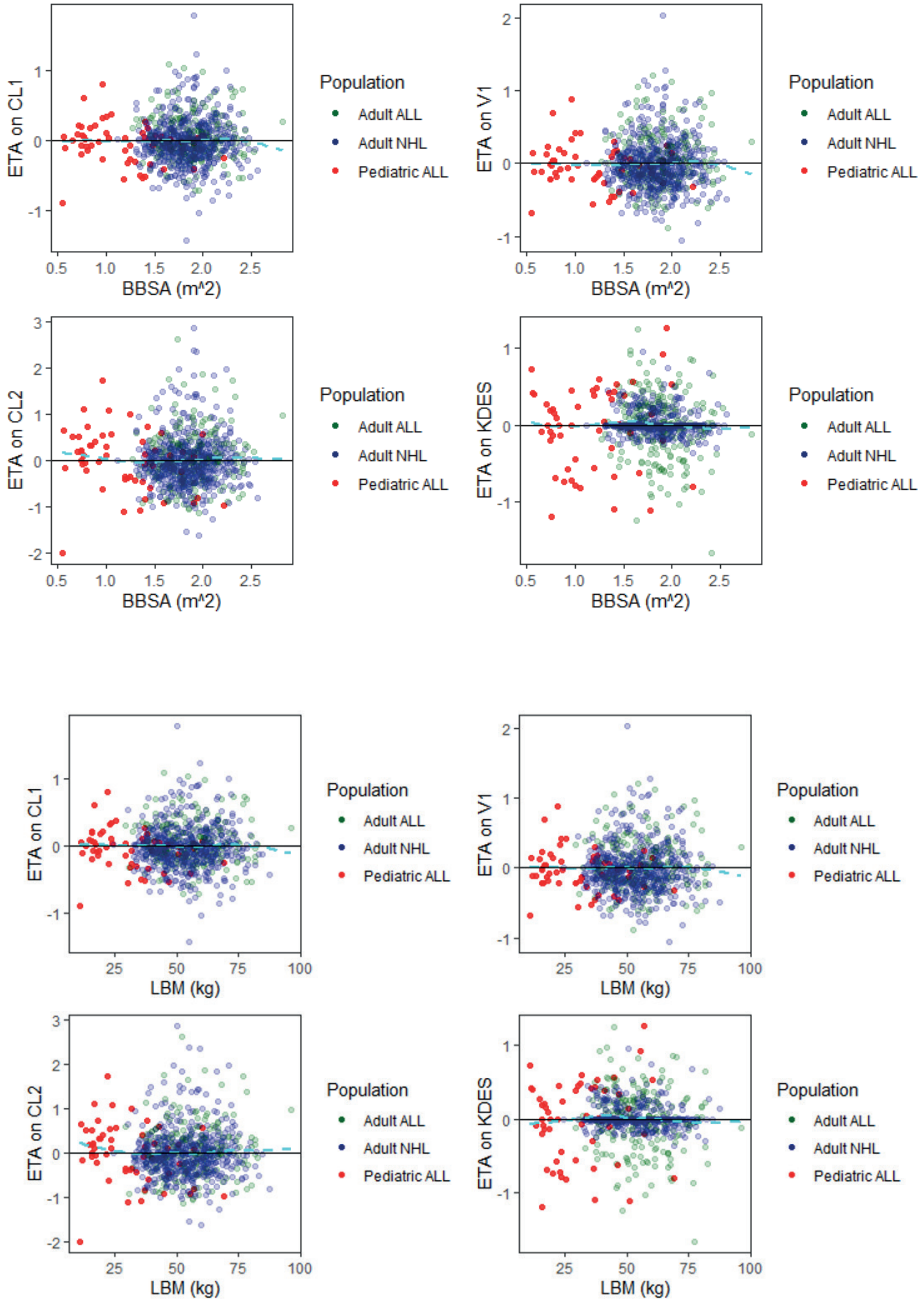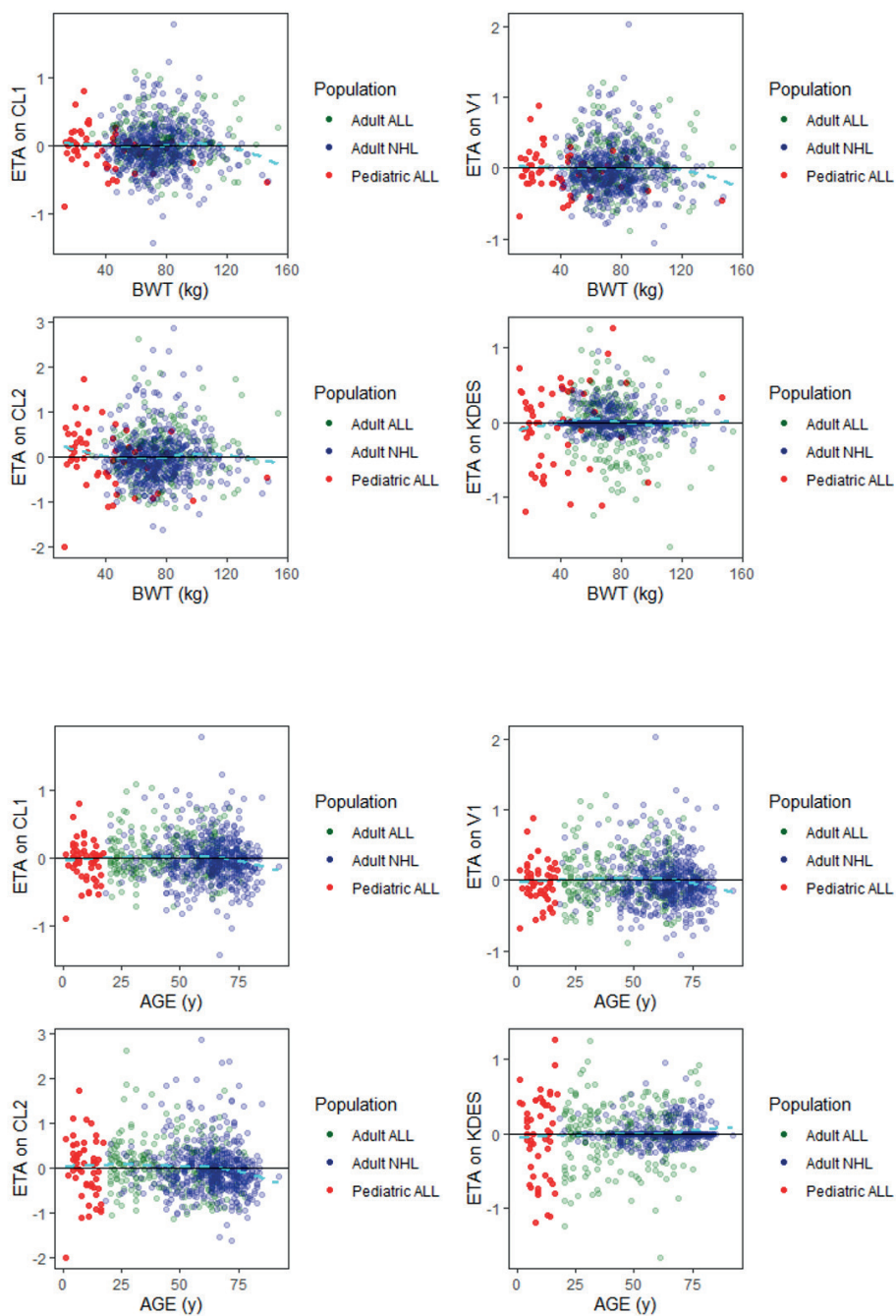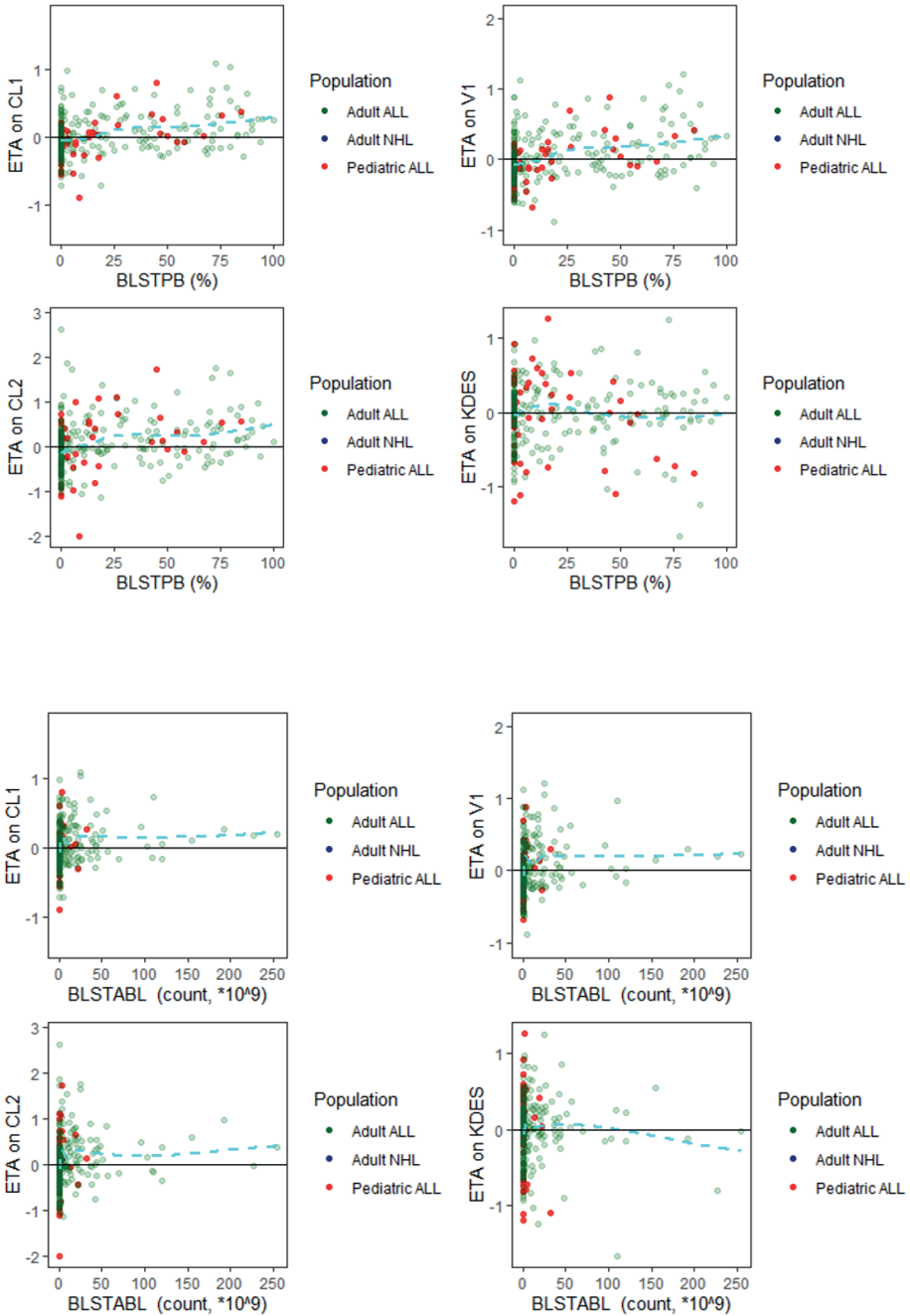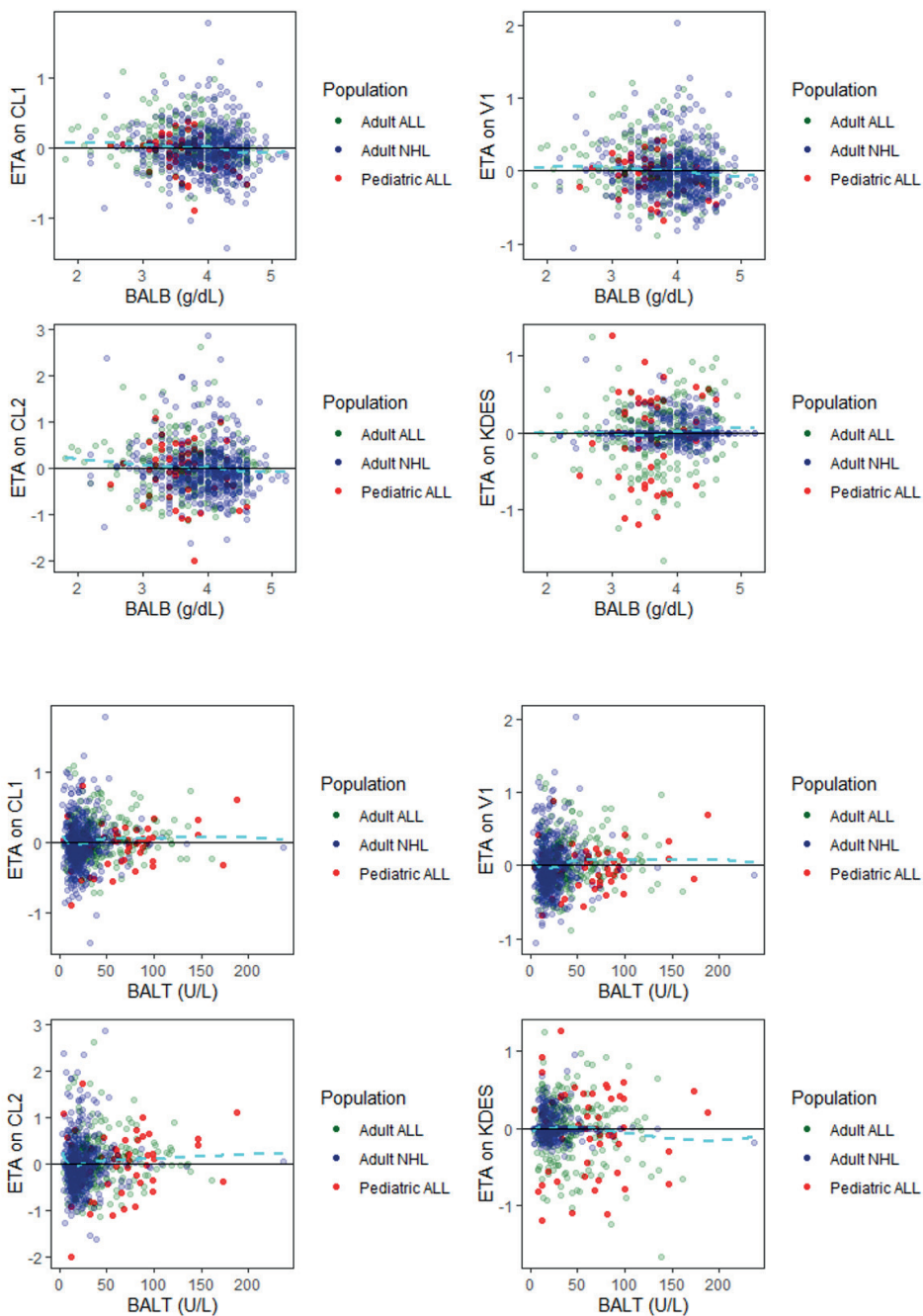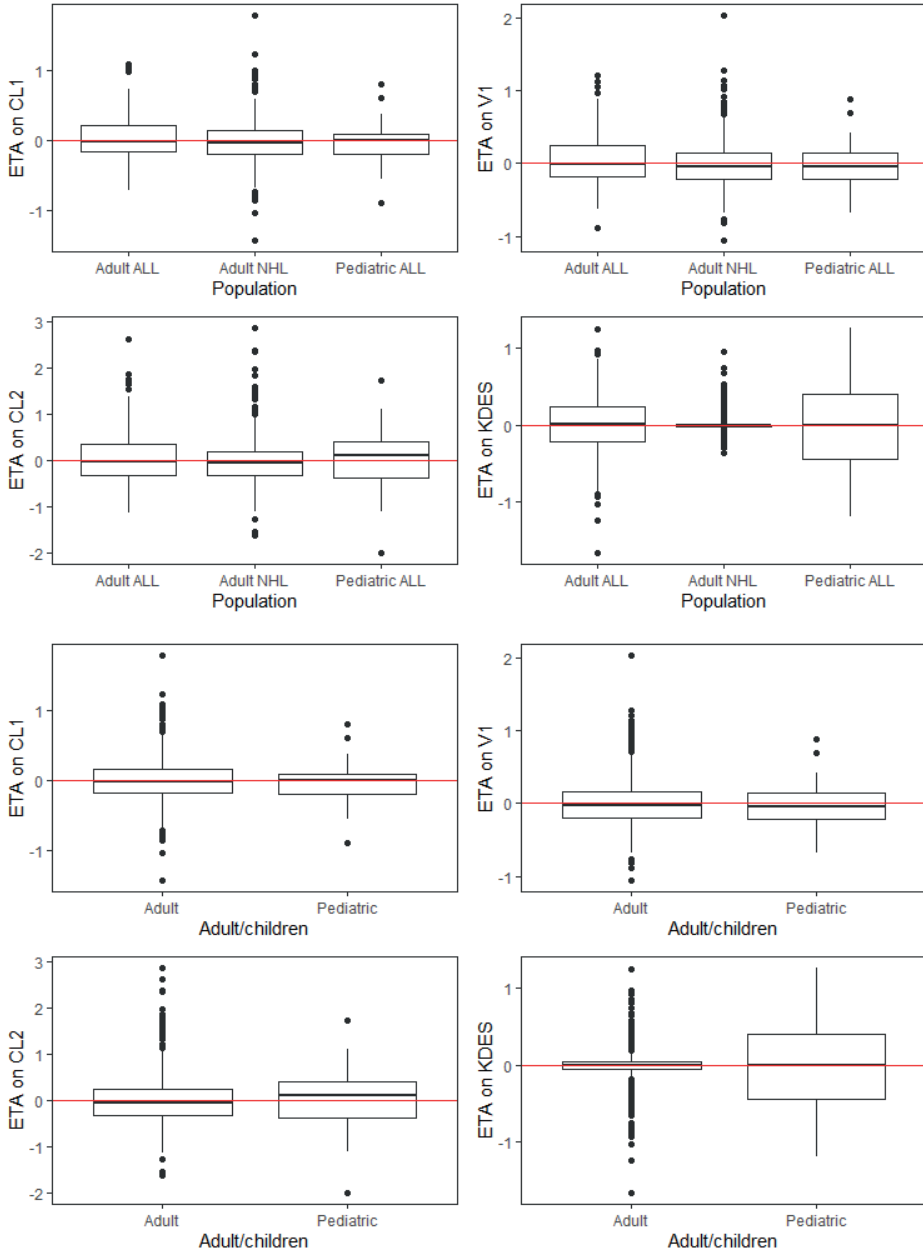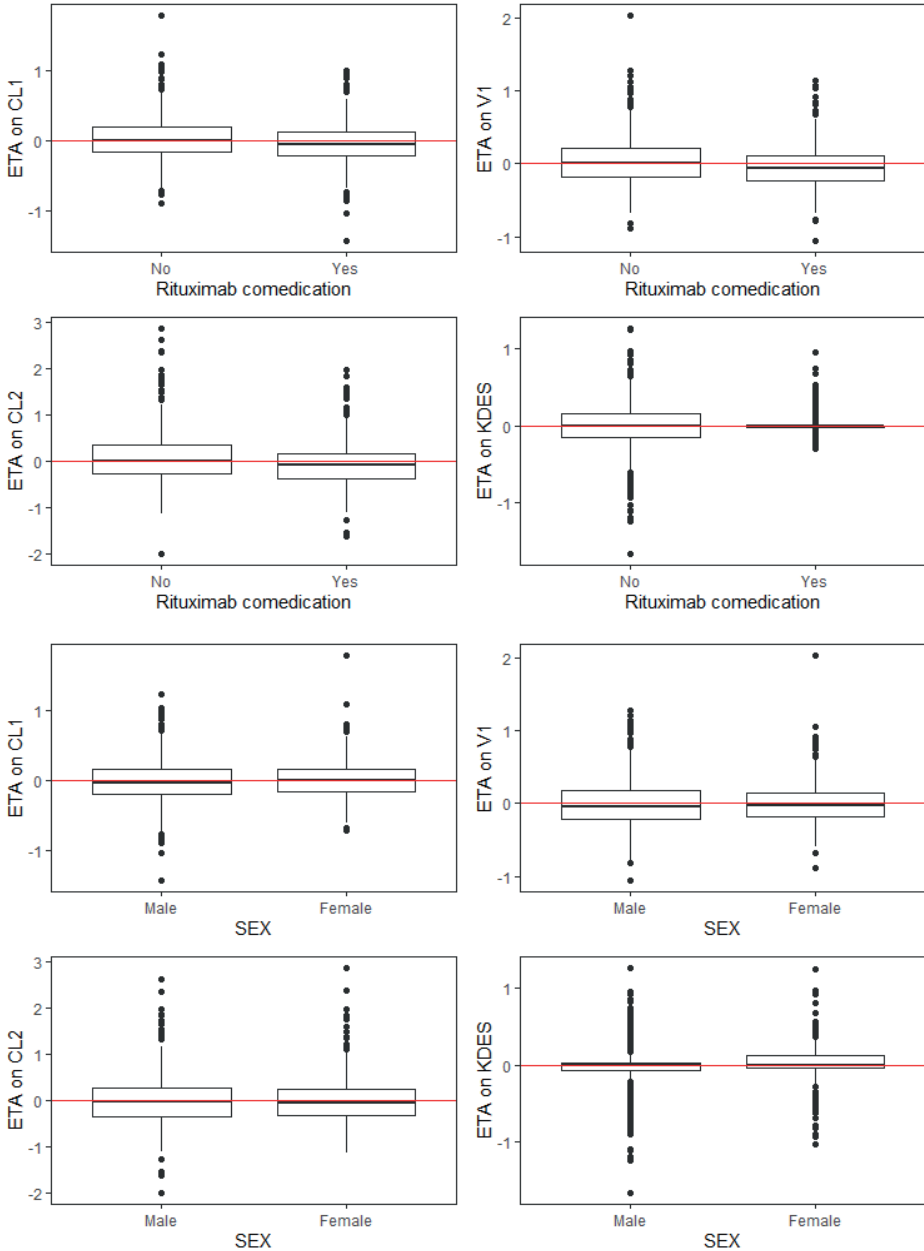
Supplementary Figure 3. Final Model ETAs on Pharmacokinetics Parameters Versus Baseline Covariates.
NHL: Non-Hodgkin lymphoma; ALL: Acute lymphoblastic leukemia; BBSA: Baseline body surface area; BWT: Baseline body weight; LBM: Baseline lean body mass; BHGRADE: Baseline hepatic impairment grades; BALB: Baseline albumin; BALT: Baseline alanine aminotransferase; BLSTPB: Baseline percentage of blasts in the peripheral blood; The cyan dotted lines are the LOESS and the red solid line is the reference line (y = 0).

Supplementary Table 5. Final Model-Based Inotuzumab Ozogamicin Exposure Simulation in ALL Patients

Simulation, cumulative AUC (median, *10³ ng*h/mL) [Difference (%)]

	End of Cycle1	End of Cycle2	End of Cycle3
Age >= 31	19.5 [-4.14]	78.1 [-4.16]	154 [-2.60]
18 <= Age <= 30	20.3 [ref.]	81.5 [ref.]	158 [ref.]
13 <= Age <= 17	24.7 [+21.4]	98.6 [+20.9]	182 [+15.1]
7 <= Age <= 12	26.9 [+32.6]	110 [+35.2]	210 [+32.7]
1 <= Age <= 6	27.8 [+37.0]	113 [+38.8]	219 [+38.4]

Simulation, cumulative AUC (median, *10³ ng*h/mL; without loading dose (LD) on Day1 Cycle1)

	End of Cycle1	Cumulative AUC difference (%; each corresponding age category with LD as reference)
Age >= 31	17.7	-9.29%
18 <= Age <= 30	18.5	-9.02%
13 <= Age <= 17	22.7	-8.07%
7 <= Age <= 12	24.9	-7.59%
1 <= Age <= 6	25.4	-8.90%

ALL: Acute lymphoblastic leukemia; AUC: area under concentration-time curve

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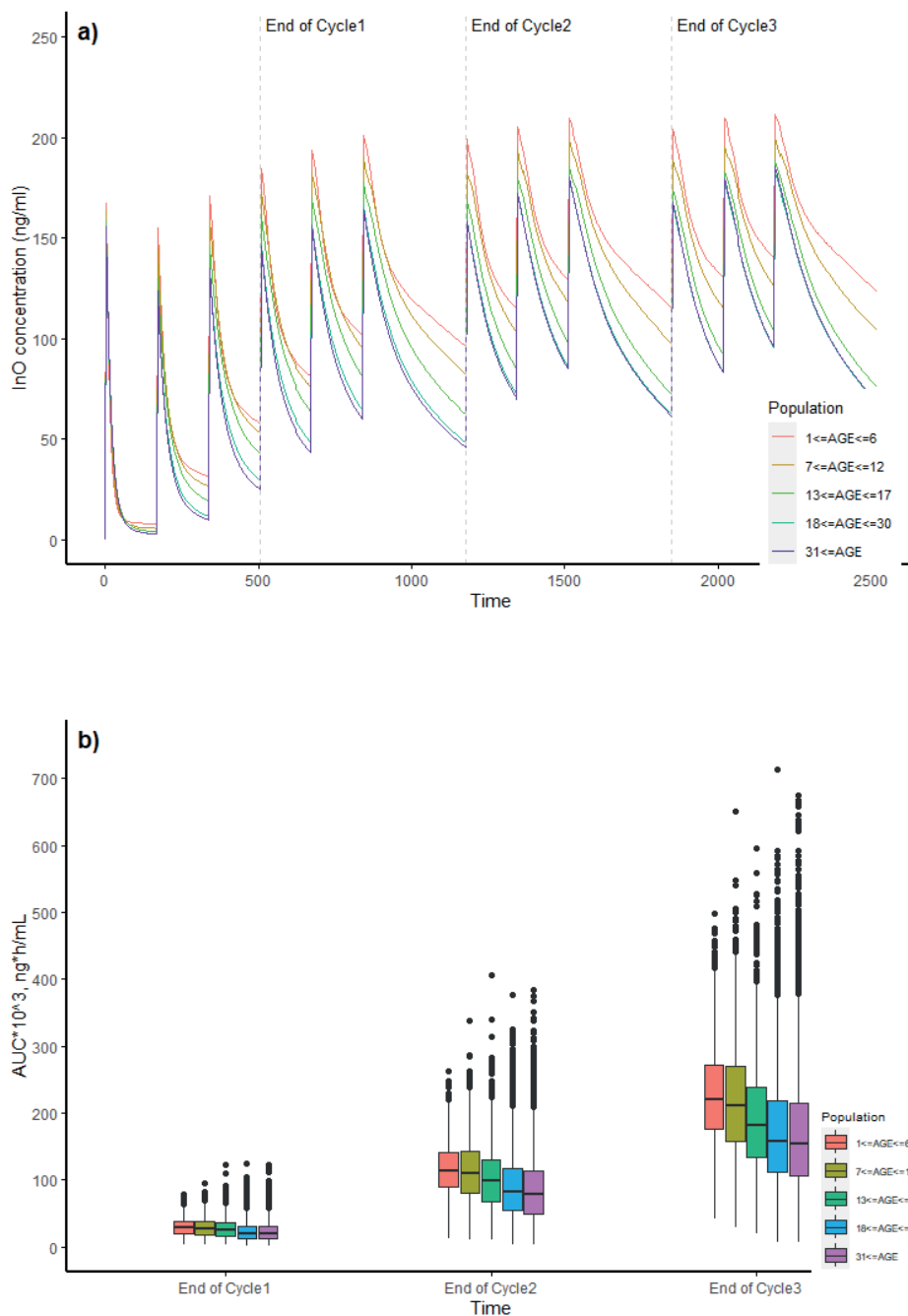
Supplementary Figure 4 Simulation of Inotuzumab Ozogamicin Stratified by Age Group. a) Simulated median concentration-time profile for 4 cycles; b) Cumulative area under concentration-time curve (AUC) at the end of each cycle.

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5

Chapter 5

Bosutinib in Resistant and Intolerant Pediatric Patients with Chronic Phase Chronic Myeloid Leukemia: Results from the Phase I of Study ITCC054/COG AAML1921

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*contributed equally

Abstract

Bosutinib is approved for adults with Chronic Myeloid Leukemia (CML); 400 mg QD in newly diagnosed (ND); 500 mg in resistant/intolerant (R/I) patients. Bosutinib has a different tolerability profile than other TKIs, and potentially less impact on growth (preclinical data). The primary objective of this *first-in-child* trial was to determine the recommended phase 2 dose (RP2D) for pediatric R/I and ND patients. In the phase I part of this international, open-label trial (NCT04258943), children aged 1-18 with R/I (per ELN 2013) Ph+ CML were enrolled using a 6+4 design, testing 300, 350 and 400 mg/m^2 QD with food. The RP2D was the dose resulting in 0/6 or 1/10 DLTs during the first cycle, and achieving adult target AUC levels for the respective indication. As ND participants were only enrolled in phase 2, the ND RP2D was selected based on data from R/I patients. Thirty patients were enrolled; 27 were evaluable for DLT: 6 at 300 mg/m^2, 11 at 350 mg/m^2 (1 DLT), and 10 at 400 mg/m^2 (1 DLT). The mean AUCs at 300 mg/m^2, 350 mg/m^2 and 400 mg/m^2 were 2.20 μg·hr/mL, 2.52 μg·hr/mL and 2.66 μg·hr/mL. The most common adverse event was diarrhea (93%; ≥ grade 3: 11%). Seven patients stopped due to intolerance, eight due to insufficient response. Complete Cytogenetic and Major Molecular Response to bosutinib appeared comparable to the other published phase I/II trials with second generation TKIs in children. Bosutinib was safe and effective. The pediatric RP2D was 400 mg/m^2 QD (max 600 mg/day) with food in R/I patients and 300 mg/m^2 QD (max 500 mg/day) with food in ND patients, which achieved targeted exposures as per adult experience.

5.1 Introduction

Chronic Myeloid Leukemia (CML) is a rare disease in children, accounting for 3% of all pediatric leukemias.[1, 2] CML is caused by the t(9;22)(q34;q11.2) translocation, resulting in the *BCR::ABL1* fusion oncogene (Ph+).

The introduction of tyrosine kinase inhibitors (TKIs) targeting the BCR::ABL1 protein, such as imatinib (approved in 2003 for children), has drastically improved the prognosis of Ph+ CML.[3] With imatinib more than 90% of children achieve Complete Hematologic Response (CHR), and around 60% achieve Complete Cytogenetic Response (CCyR) after 1 year of treatment.[4–7] However, in the long term, approximately 30% of children have an unsatisfactory response or intolerance to imatinib.[7] Dasatinib, a second-generation TKI approved for this pediatric indication, led to 82% CCyR rate in imatinib resistant/intolerant (R/I) patients with chronic phase (CP) Ph+ CML.[8–11] In addition, nilotinib was also approved for pediatric patients and led to around 80% CCyR rate in resistant subjects, but it requires twice daily administration under fasting conditions.[12, 13] Side effects of imatinib and dasatinib mostly consist of musculoskeletal pain, asthenia and skin rash.[1, 7, 10, 11] While nilotinib is more frequently associated with increased bilirubin, nausea, and vomiting.[2, 3, 12]

Bosutinib is a dual Src and Bcr-Abl inhibitor, approved for adults at the recommended dose of 400 mg (max 600 mg) orally once daily for newly diagnosed (ND) chronic phase Ph+ CML, and 500 mg (max 600 mg) once daily for patients previously treated with one or more TKIs.[14] Treatment with bosutinib in adults is mostly associated with gastrointestinal toxicities, rash, and increased transaminases (BYOND study).[15] Gastrointestinal toxicity (mainly diarrhea) may lead to dose reduction during treatment.[2, 3, 15, 16] Furthermore, animal models showed that bosutinib does not cross the blood-brain barrier, differently from dasatinib.[14]

Of particular relevance for children, there is evidence that long-term exposure to imatinib results in growth impairment.[1, 17] The mechanism of impaired growth may be related to *"off-target"* binding, such as inhibition of c-KIT and PDGF-R, and/or the development of an acquired growth hormone deficiency.[18–21] Preclinical data indicated that this toxicity may not be observed, or be less prominent, with bosutinib.[22]

We report the results of the phase I part of the ITCC-054/COG AAML1921 trial, which aimed to select the recommended phase II dose (RP2D) for R/I and ND pediatric patients with Ph+ CML.

5.2 Patients and Methods

5.2.1 Study Design

ITCC-054/COG AAML1921 (NCT04258943) is a phase I/II multicenter, single-arm, open-label study conducted in the context of a Pediatric Investigation Plan and a Pediatric Written Request. The study was conducted under the International Ethical Guidelines for Biomedical Research Involving Human Subjects, ICH Guidelines for Good Clinical Practice, the Declaration of Helsinki, and approved by the Institutional Review Board or Ethics Committee in all participating centers. The study is sponsored by the Erasmus Medical Center in Europe and the Children's Oncology Group (COG) in the United States, and funded by Pfizer Inc. It is open in 21 sites of the Innovative Therapies for Children with Cancer (ITCC) Consortium based in Europe, and 45 COG sites.

Eligible patients were aged ≥1 to <18 years at enrolment, had a diagnosis of Ph+ CML (either in chronic, acute phase (AP), or blast crisis (BC)), were resistant or intolerant to at least one prior TKI (per protocol definition according to 2013 European Leukemia Net criteria (ELN)), and did not suffer from major organ toxicities.[23] Main exclusion criteria consisted of known T315I or V299L *BCR::ABL1* mutations, and extramedullary disease only (Supplementary Table 1). Patients and/or parents, provided written informed consent, and were enrolled between November 2016 and August 2022.

A modified rule-based design (6+4), following the principles of the rolling six design, was chosen to allow a better characterization of the PK parameters defining the RP2D based on a simulation study showing that six to ten patients are needed to demonstrate that target exposure in children is in the adult range.[24,25] We defined the RP2D as the dose resulting in 0/6 or 1/10 dose limiting toxicities (DLTs, definition in Table 1; patients without DLT had to receive ≥ 75% of the planned dose in cycle 1 to be evaluable), and resulting in a geometric mean area under the concentration-time curve at steady state (AUCss) of 3.15 ng·hr/mL (±20%) for R/I patients, and 2.27 µg·hr/mL (±20%) for ND patients. Target AUCss for both ND and R/I patients were based on a population PK analysis pooling data (n=1401) from adults treated with bosutinib, and are equivalent to the adult exposure achieved at 400 and 500 mg/day respectively.[26] The PK sampling schema is provided in Supplementary Table 2. The RP2D for ND patients was extrapolated from PK and safety data obtained in R/I subjects, and target exposure was based on adult data. The protocol was amended to add a new cohort of ND patients in chronic phase in the phase 2 part of the study, after approval of bosutinib for this indication in adults (Supplementary Table 3).

Table 1. Definition of Dose-Limiting Toxicity

Non-hematologic AEs*	Hematologic AEs*
Any Grade ≥3 toxicity, despite optimal treatment	
Any Grade ≥2 toxicity requiring discontinuation/ interruption for ≥7 days	Grade 4 neutropenia or thrombocytopenia AND lasting ≥7 days (not explained by persistent leukemia)
Clinically significant laboratory abnormality Grade ≥3 AND lasting ≥7 days despite optimal treatment	

*AEs were graded based on the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 and assessed only during the first cycle of treatment (28 days)

5.2.2 Study Treatment

A treatment cycle was defined as 28 days, regardless of missed doses. Available formulations included tablets (dissolved for nasogastric administration if needed) and capsules (which could be opened and sprinkled on food), which could be used in combination. The bioequivalence of these formulations was established based on data from the trials NCT04549480, NCT05032690 and NCT04916769 provided by Pfizer Inc.

Dose levels were amended in protocol version 4 as the exposure observed in the first patients treated at 300 mg/m^2 was insufficient to yield the target AUCss for R/I patients. Consequently, we tested daily doses of 350 mg/m^2 and 400 mg/m^2 (dose schema in Supplementary Table 4). Maximum daily dose was capped at 600 mg in R/I patients as per adult label. Body Surface Area (BSA) was calculated using the Mostellar formula[27]. Moderate or strong CYP3A inducers and inhibitors and proton pump inhibitors were prohibited.

5.2.3 Endpoints and Assessment

The primary objective was to determine the RP2D of bosutinib for R/I pediatric patients with Ph+ CML. Secondary objectives included overall safety and preliminary anti-leukemic activity. The primary endpoints were the incidence of DLTs and PK parameters. Secondary endpoints included estimations of toxicity and efficacy outcomes. As a post-hoc analysis the cumulative incidence of treatment discontinuation due to unsatisfactory response and intolerance was added. Full list of endpoints and definitions of efficacy, safety assessments, and mutation analysis can be found in the Supplementary Tables 5-7.

5.2.4 Statistical analysis

Cumulative incidence of response was obtained using 1 minus the Kaplan–Meier (KM) estimate. EFS and OS were estimated using the KM method (definition in Supplementary Table 6). The cumulative incidence of treatment discontinuation was estimated using a competing risk setting (insufficient response versus intolerance). Details on the statistical methodology are given in the Supplementary Material.

5.3 Results

5.3.1. Patients

At the data cut-off of 19 September 2022, 30 patients were screened, 29 enrolled (one screen failure), 28 treated (one patient did not start the treatment due to low absolute neutrophil count; safety and efficacy analysis set); 27 were evaluable for DLT (one patient was not evaluable because of withdrawn of consent after less than 21 days of treatment in cycle 1, in absence of a DLT) (Supplementary Figure 1). Baseline demographics are summarized in Table 2. All 28 treated patients were in CP at time of enrolment.

Overall, 490 bosutinib 28-day cycles (median cycles per patient: 15; range: 1–66) were administered. Eleven patients (39%) were still on treatment at time of data cut-off: seven stopped due to intolerance, eight due to insufficient response, and two completed study treatment and transitioned to adult care (after 24 and 19 months of study treatment).

5.3.2 Safety

Six patients were enrolled at 300 mg/m^2, without DLTs. The dose was escalated to 350 mg/m^2, and 11 patients were enrolled (two patients consented simultaneously). One DLT occurred (grade 3 nausea/vomiting and diarrhea). This patient continued at 250 mg/m^2 and discontinued the treatment after seven cycles due to increased transaminase levels. The dose was further escalated to 400mg/m^2, and 11 patients were treated, as one subject was replaced (not evaluable for DLTs). One patient experienced a DLT (grade 3 transaminase increase, grade 2 bilirubin increase, and grade 3 rash with treatment interruption > 7 days) which resolved completely and continued the treatment at the reduced dose of 300mg/m^2.

The most common AEs were diarrhea (93%, n=26), abdominal pain (71%, n=20), vomiting (68%, n=19), nausea (61%, n=12), and maculo-papular rash or other skin disorders (39%, n=11; and 43%, n=12 respectively). AEs assessed as (possibly, probably or definitely) related to bosutinib are reported in Table 3.

Importantly, some patients suffered of persistent low grade gastro-intestinal toxicity, mostly diarrhea, protracted for over a year.

Among grade 3 and 4 AEs, the most common were transaminase elevation (18%, n=5), maculo-papular skin rash (11%, n=3), vomiting (11%, n=3), and diarrhea (11%, n=3). No grade 5 AEs occurred. Laboratory and hematological abnormalities are summarized in Supplementary Table 9, AEs by age class and dose level in Supplementary Table 10 A-B. No patient developed a clinically significant prolonged QTc (Supplementary Table 11). Neither cases of arrhythmia, nor abnormalities in cardiac function were registered at the echocardiograms performed every 12 months. Eleven patients had their dose level reduced due to AEs, and seven stopped the treatment

due to intolerance (protracted diarrhea, nausea/vomiting, neutropenia, rash), of which two were already intolerant to imatinib or dasatinib.

Table 2. Patient Characteristics (N=28, all patients receiving at least one dose of bosutinib)

Characteristic	Total number of patients (%)	
Sex		
Male	16 (57.1%)	
Female	12 (42.9%)	
Age at enrollment (in years)		
Median (range)	12 (1-17)	
Age category, n (%)		
>1– ≤6 years	6 (21.4%)	
>6– ≤12 years	10 (35.7%)	
>12 years	12 (42.9%)	
Reason of enrollment		
Resistant	23 (82.1%)	
Intolerant	5 (17.9%)	
Previous lines of treatment (TKIs)	**Resistant***	**Intolerant***
One	13 (46.4%)	3 (10.7%)
Two	8 (28.6%)	1 (3.6%)
Three	2 (7.1%)	1 (3.6%)
Last TKI received	**Resistant**	**Intolerant**
Imatinib	10 (35.5%)	3 (10.7%)
Dasatinib	12 (42.9%)	1 (3.6%)
Nilotinib	1 (3.6%)	1 (3.6%)
Time from diagnosis (in months)		
Median	17	
Range	3 - 82	

Characteristic	Total number of patients (%)	
**Response status at enrolment **	**Resistant**	**Intolerant**
Complete Cytogenetic Response (CCyR)	14 (50%)	2 (7.1%)
Partial CyR	3 (10.7%)	2 (7.1%)
Minor CyR	1 (3.6%)	0 (%)
Minimal CyR	2 (7.1%)	0 (%)
No CyR	1 (3.6%)	0 (%)
Not available	2 (7.1%)	1 (3.6%)
Major Molecular response (MMR/≥MR3)	1[#] (3.6%)	3 (10.7%)
MR2	9 (32.1%)	1 (3.6%)
MR1	7 (25%)	0 (%)
No MR	5 (17.8%)	0 (%)
Not available	1 (3,6%)	1 (3.6%)

* Resistance has been defined either "*Suboptimal/Warning*" or "*Failure*" response based on ELN 2013 criteria (for all) patients depending on whether they received only one or more than one line of treatment with TKIs (see appendix 3 and 4 of the protocol). Intolerance was based on the treating physician's judgment.

** results based on central lab analysis at time of screening. Molecular response was based on peripheral blood (PB) analysis, and on bone marrow when PB was not available.

one patient was included as resistant with MR2 molecular response based on local peripheral blood results. The central laboratory confirmation later showed MR3 in PB (MR2 based on bone marrow analysis), but because the patient was already enrolled treatment was continued.

Table 3. Most Frequent Adverse Events (frequency >3)

Adverse Event Term	Gr. 1-2	Gr. ≥ 3	Gr. 1-2 related to bosutinib*	Gr. ≥ 3 related to bosutinib*
Diarrhea	23 (82%)	3 (11%)	20 (71%)	2 (7%)
Abdominal pain	19 (68%)	1 (4%)	15 (54%)	1 (4%)
Vomiting	16 (57%)	3 (11%)	12 (43%)	3 (11%)
Nausea	17 (61%)	0	15 (54%)	0
Fever	11 (39%)	1 (4%)	5 (18%)	0
Skin and subcutaneous tissue disorders	11 (39%)	1 (4%)	6 (21%)	1 (4%)
Rash maculo-papular	8 (29%)	3 (11%)	4 (14%)	3 (11%)
Headache	9 (32%)	1 (4%)	5 (18%)	0
Alanine aminotransferase increased	4 (14%)	5 (18%)	4 (14%)	5 (18%)
Fatigue	7 (25%)	1 (4%)	6 (21%)	1 (4%)
Pain in extremity	7 (25%)	1 (4%)	4 (14%)	0
Constipation	7 (25%)	0	2 (7%)	0
Gastrointestinal disorders	7 (25%)	0	4 (14%)	0
Anorexia	6 (21%)	0	5 (18%)	0
Creatinine increased	6 (21%)	0	5 (18%)	0
Infections and infestations	5 (18%)	1 (3%)	0	0
Metabolism and nutrition disorders	6 (21%)	0	4 (14%)	0
Stomach pain	6 (21%)	0	5 (18%)	0
Aspartate aminotransferase increased	3 (11%)	2 (7%)	3 (11%)	2 (7%)
Rhinitis infective	5 (18%)	0	0	0
Cough	4 (14%)	0	1 (4%)	0
CPK increased	4 (14%)	0	3 (11%)	0
Flatulence	4 (14%)	0	2 (7%)	0
General disorders - Other	4 (14%)	0	2 (7%)	0
Platelet count decreased	3 (11%)	1 (4%)	2 (7%)	1 (4%)
Rash acneiform	4 (14%)	0	4 (14%)	0

* possibly, probably and definitely related based on the treating physician's judgment.

5.3.3 Pharmacokinetics

In total, 386 samples from 27 patients were available for PK analysis. The geometric mean AUCss at 300 mg/m², 350 mg/m² and 400 mg/m² were 2.20 µg·hr/mL (range 1.54 – 3.10), 2.52 µg·hr/mL (range 1.85 – 4.62) and 2.70 µg·hr/mL (range 1.47 – 3.92), respectively. The geometric mean peak plasma concentrations at 300 mg/m², 350 mg/m², and 400 mg/m² were 188.5 ng/mL, 221.2 ng/mL, and 198.1 ng/mL, respectively and it was generally reached approximately 3 hours after the administration across all dose levels. The geomean trough concentrations at 300 mg/m², 350

mg/m² and 400 mg/m² were 46.58 ng/mL, 46.33 ng/mL, and 48.88 ng/mL, respectively. In 10 patients (33%) the capped dose of 600 mg/day was administered, five of which at the highest dose level. The target steady state exposure for ND patients (2.37 ±20% µg·hr/mL) was achieved at 300 mg/m²/day, while for R/I patients the target exposure (3.15 ±20% µg·hr/mL) was achieved at 400 mg/m²/day.

5.3.4 Efficacy

The median follow-up was 23.8 months (range 1.8-61.5). At data cut-off date, the cumulative proportions of CHR, MCyR, CCyR, and MMR by end of treatment, as best response, were 100% (95% Confidence Interval (CI): 87.7-100), 96.4% (95%CI: 81.7-99.9), 92.9% (95%CI: 76.5-99.1) and 46.4% (95%CI: 27.5-66.1) respectively (Figure 1A). All patients entering the study not in CHR (n=6), achieved CHR by month 4. Considering only those patients that achieved MCyR, CCyR and/or MMR for the first time on study, the cumulative incidence of MCyR was 71.4% (95%CI: 17.9-93.6%) at 6 months (no patient at risk after months 9), while for CCyR was 83.3% (95%CI: 40.5-96.4%) at 6 months, and was maintained at 12 months. The cumulative incidence of MMR was 26.1% (95%CI: 10.3-45.2) at 6 months and increased to 39.1% (95%CI: 19.4-58.5) at 12 months (Figure 1B). In patients without baseline response (screening), the median time to respond was 3 months for MCyR and CCyR and 28 months for MMR. Among the patients achieving MMR while on study (n=10), five achieved MR4/MR4.5. All patients that achieved or entered the study in MCyR, CCyR and/or MMR maintained the response except three patients, who lost CCyR after four, 15 and 31 cycles, respectively. Baseline response for resistant versus intolerant patients is reported in Table 2. In a post-hoc analysis, no statistically significant differences in the cumulative incidence of MMR, CCyR, or CHR were observed across the dose levels, prior lines of therapy or age groups (Supplementary Figure 2-3, and Supplementary Table 12), but the study was also not powered to detect such differences. Notably MMR was reached only by one out of five children in the class age >1y ≤6y (n=6, one already in MMR at screening), with a cumulative incidence of MMR of 20% in this age group (p 0.08).

The OS was 100% (95%CI: na) at 1 and 2 years, and 85.7% (95%CI: 63.3-100%) at 3 years. One patient died due to meningitis after Hematopoietic Stem Cell Transplantation (HSCT) 15 months after the last dose of bosutinib. EFS at 1, 2, and 3 years were 96.3% (95%CI: 89.4-100%), 91.92% (95%CI: 81.7-100%), and 70.0% (95%CI: 47.0-100%), respectively (Figure 2).

At time of data cut-off, eight patients stopped the treatment due to insufficient response per investigator judgment, of which two were treated at 300 mg/m², three at 350 mg/m², and three at 400 mg/m². As a post-hoc analysis, the cumulative incidence of treatment discontinuation is shown in Figure 3. Three of the patients which did not obtain sufficient response underwent HSCT. We did not record emerging mutations of T315I or V299L in *BCR::ABL1*, nor any other mutations in exons 5 and 6 in ABL1, in patients achieving the end of treatment.

A

Cumulative Proportions for Each Response Type

5

B

No. at risk (no. censored):

CHR	6	1	0	0	0
MCyR	7	3	2	1	0
CCyR	12	4	2	2	1
MMR	24	20	17	14	12
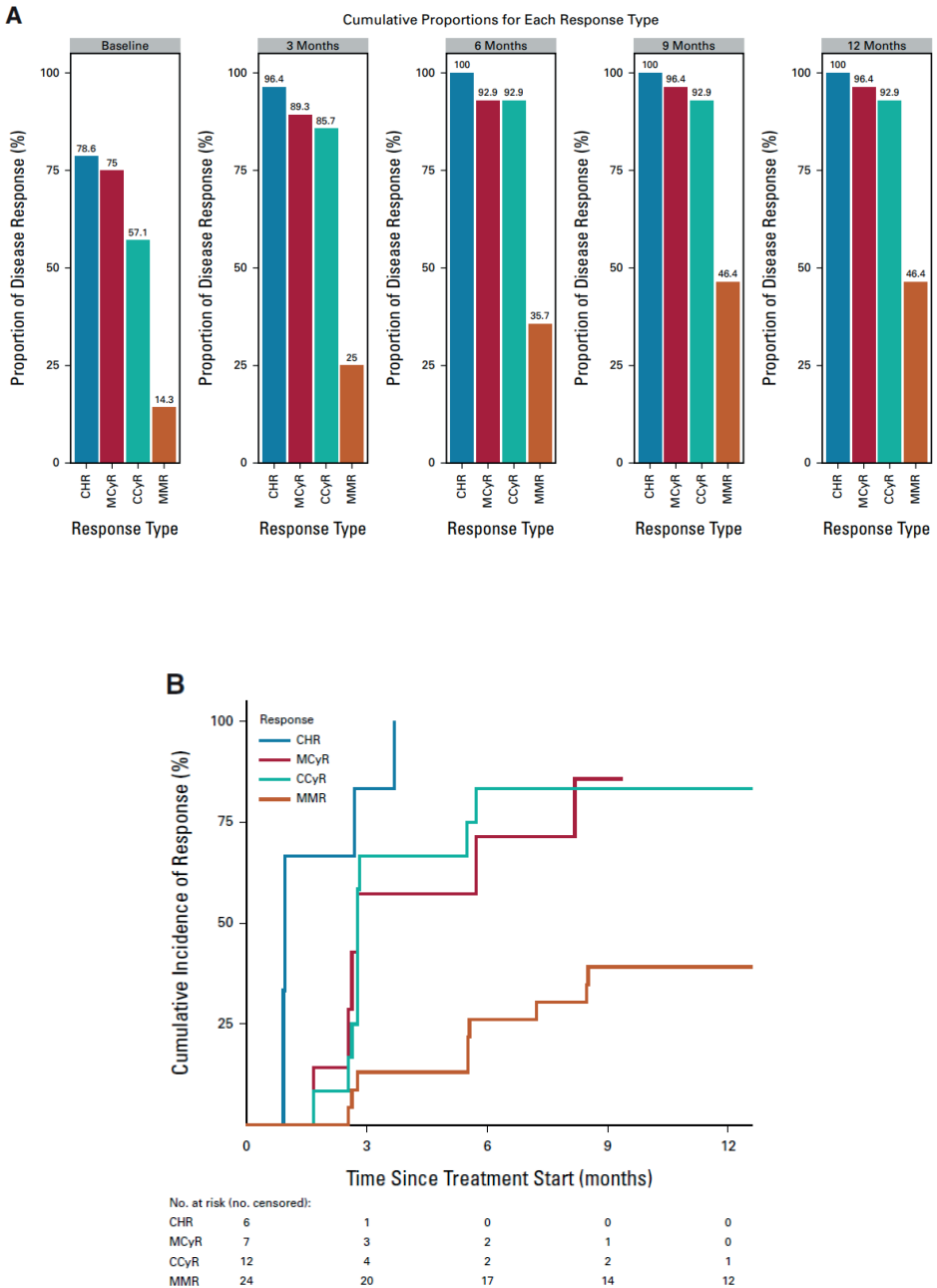
Figure 1. Proportions as best response (A) and cumulative incidence of first-time achieving response on treatment (B). A. Proportions of patients in Complete Hematologic Response (CHR), Major Cytogenetic Response (MCyR), Complete Cytogenetic Response (CCyR), and Major Molecular Response (MMR) at baseline (screening), 3, 6, 9 and 12 months. **B.** Cumulative incidence of first-time achieving CHR, MCyR, CCyR and MMR on treatment at 3, 6, 9 and 12 months (patients with response at screening excluded).
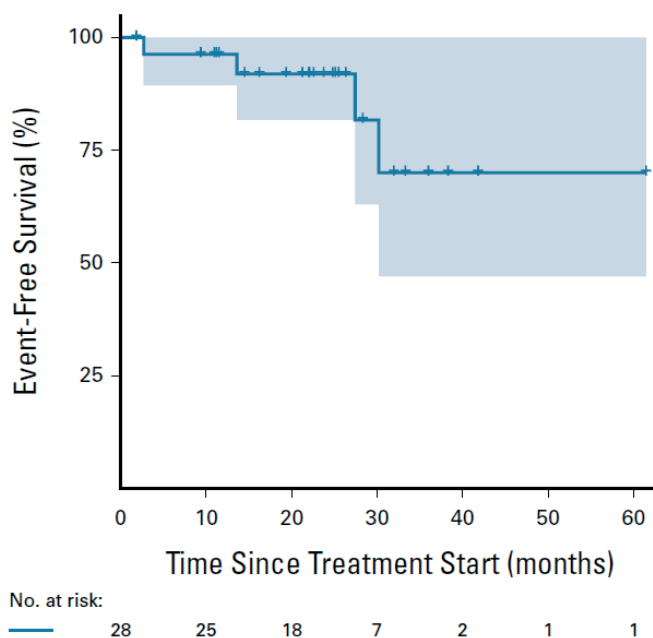
Figure 2. Event Free Survival (Kaplan-Meier method). Events were defined as either: 1. Death due to any cause; 2. Transformation to accelerated phase or blastic crisis at any time; 3. Loss of Complete Hematologic Response (CHR, as defined in Supplementary); 4. Loss of Complete Cytogenetic Response (CCyR, as defined in Supplementary); 5. Loss of Major Molecular Response (MMR, as defined in Supplementary); 6. For patients not achieving a CHR: doubling of WBC at least 1 month apart with the second value > 20 x 10⁹/L and maintained in subsequent assessments for at least 2 weeks. Only one patient died, two lost CCyR, and one lost both CCyR and MMR (counted as one event at the time of loss of CCyR). Crosses represent censored patients.
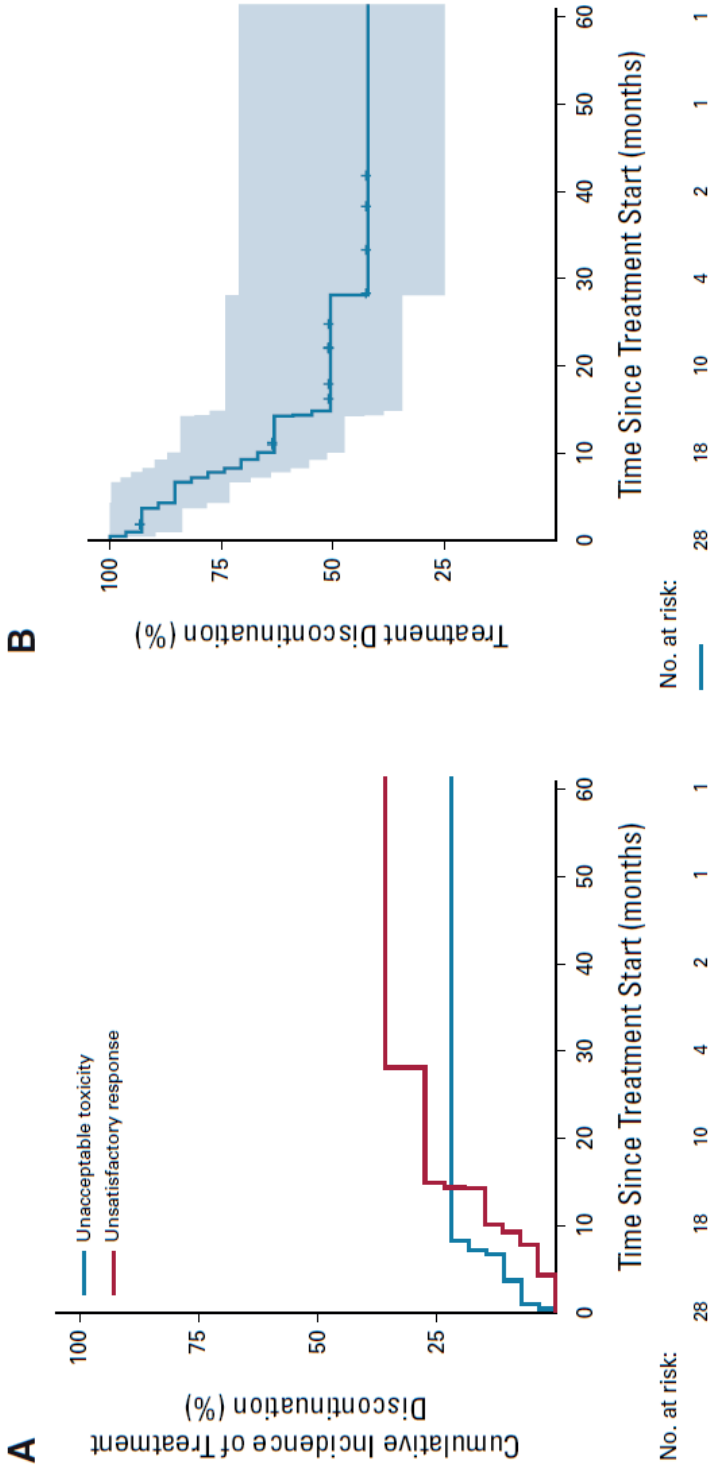
Figure 3. Cumulative incidence of treatment discontinuation. Panel A: Cumulative incidence of treatment discontinuation as per investigator judgment in a competing risk setting. Discontinuation due to insufficient response is displayed in blue and included loss of Complete Cytogenetic Response (CCyR) and/or Major Molecular Response (MMR) or failure to achieve CCyR and/or MMR. Discontinuation due to toxicities is displayed in red. Patients having a dose limiting toxicity discontinued the treatment within the first month (black line). **Panel B:** Cumulative incidence of treatment discontinuation due to all causes. Crosses represent censored patients

5.4 Discussion

In this *first-in-child* dose-finding study, bosutinib showed a tolerability profile consistent with data known from adults.[15] Only two DLTs occurred (at 350 and 400 mg/m²). The RP2D for R/I patients was established at 400 mg/m² (max 600 mg/day). The RP2D for ND patients was extrapolated from safety and exposure data in R/I pediatric patients based on the AUC of the recommended dose for ND adult patients, and was established at 300 mg/m² (max 500 mg/day).

The most common AEs were gastrointestinal toxicities, with almost all patients experiencing at least mild (grade 1-2) events; while grade 3 or higher AEs occurred in approximately 10% of the patients. This frequency is higher when compared to imatinib and dasatinib (2-5% grade 3-4 gastrointestinal toxicities).[28,29] A small proportion of patients had persistent gastrointestinal complaints, mainly diarrhea, which may affect compliance and quality of life. A '*real world*' strategy to prevent early discontinuation in adult patients consists of starting with a lower dose (200-300 mg) of bosutinib followed by gradually increasing the dose if needed, but this was not tested in this dose-finding study.[16] While musculoskeletal pain is commonly reported in patients treated with imatinib (40-50%), our study confirms that these events were less common with bosutinib (~10-15%).[28–30] Another frequent AE was skin rash, which occurred in 40% (n=11) of the subjects, similarly to published data for other TKIs used in children.[7, 11, 12] As observed in adult patients, the impact of bosutinib on cardiac function was negligible, however, longer follow-up may be needed to better assess cardiac side effects.[30]

It remains to be established whether bosutinib might show a less toxic profile on longitudinal growth as demonstrated in murine models.[20, 22] All TKIs approved in children for Ph+ CML show a negative impact on height, especially when started prior to puberty.[18, 21, 31] The potential benefit of bosutinib will be better evaluable in our phase II cohort in ND patients, as the enrolment of pretreated subjects precludes a firm assessment in the R/I cohort.

In terms of PK, the AUC increased almost linearly with each dose level, even if 50% of the patients treated at 400 mg/m² received the maximum dose of 600 mg/day. This might suggest that the solubility and saturation in the gastro-intestinal tract were not saturated in the investigated dose range. Such phenomena were observed in adults receiving 600 mg per day (selected as maximum daily dose in our R/I cohort).[30, 32] A higher BSA-adjusted dose was necessary in younger children to achieve the target exposure as defined in adult studies, whereas in older children the dose was capped as in adults if the BSA-adjusted dose was higher than 600 mg. These differences in pharmacokinetics might be influenced by a different absorption of the drug in younger children, who generally have an higher gastric pH compared to adults and less water in the gastro-intestinal tract.[33] In addition, although bosutinib was instructed to be administered following a meal, food intake was not standardized. Bosutinib is likely classifiable as a Biopharmaceutics Classification System (BCS) class IV drug, characterized by low permeability and low solubility, the latter being pH-dependent and increased by food intake, especially when rich in fat.[14]

In adults, higher bosutinib concentrations have been associated with higher probability of response, likely reaching the plateau of exposure-efficacy at recommended doses in adults.[14] In adults resistant to imatinib, it was suggested that bosutinib doses ≥ 350 mg/day were associated with an increased rate of MCyR.[25, 30] In our study, we did not identify a clear dose-efficacy relationship, which might be due to the limited sample size or that participants are at or near the exposure-efficacy plateau.

In terms of preliminary efficacy, the cumulative incidence of CCyR and MMR appears comparable to the other published pediatric phase I/II trials with 2nd generation TKIs.[11, 12] The main reasons to discontinue treatment in this study were equally attributable to intolerance and loss of response/insufficient response.

Currently, a dose-finding trial of asciminib (targeting the ABL Myristoyl Pocket STAMP) in children is ongoing (NCT04925479).[34] In adults, it showed a higher MMR rate and a lower treatment discontinuation rate due to toxicities compared to bosutinib.[35] Ponatinib is the other TKI under investigation in children (NCT03934372); available data are mostly based on case reports.[36, 37] In adults, it proved effective, particularly in patients with *T315I* mutated CML, but at the expense of more frequent cardiovascular events.[38, 39]

Since Ph+ CML is a very rare disease in children, one of the main limitations of this trial was the slow enrolment rate. Six years were needed to complete the phase I part, despite adding additional centers in the US since 2019, and finally recruiting in over 60 centers globally. The number of TKIs now approved for children further reduces the number of eligible patients for dose-finding trials. To resolve this problem, it might be crucial to limit the number of dose-levels tested, and use PK-modelling to define the starting dose, and implement extrapolation from adult data where feasible.[40]

In conclusion, the phase I portion of this study indicate that bosutinib is a safe and effective in the R/I pediatric population. The phase II part of the trial, enrolling ND and R/I patients, is ongoing.

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Supplementary Material

Supplementary Table 1. Inclusion/Exclusion Criteria Phase 1 (R/I patients only)

Inclusion Criterion	Specifications
Cytogenetic and molecular diagnosis of Philadelphia chromosome-positive Chronic Myelogenous Leukemia at either time of initial CML diagnosis or at time of study screening	Cytogenetics must be performed by chromosome banding analysis (CBA) of bone marrow cell metaphases, and requires at least 20 metaphases.
	Only if dividing marrow cells cannot be obtained, or if there is an insufficient number of metaphases, CBA can be substituted by interphase fluorescence in situ hybridization (I-FISH) of bone marrow or peripheral blood cells, using dual color dual fusion probes, that allow the detection of BCR-ABL+ nuclei; at least 200 nuclei should be counted.
	Qualitative RT-PCR should be performed on RNA extracted from freshly collected bone marrow or peripheral blood cells. It identifies the transcript type, either e14a2 or 13a2 (also known as b3a2 and b2a2), or much more rarely e19a2, or e1a2, indicating the BCR-ABL protein weight (P210, rarely P230 or P190).
Resistance (suboptimal response or failure, as defined by 2013 European Leukemia Net guidelines) or intolerance (with or without suboptimal response or failure) to at least one prior tyrosine kinase inhibitor (TKI)	The 2013 European LeukemiaNet guidelines will be used to define suboptimal response and failure to prior TKI therapy.
	Intolerance to prior TKI therapy will be determined by the treating investigator, but generally applies to patients who are unable to receive standard or reduced doses of a TKI due to significant drug-related toxicity and/or when the drug-related toxicity is not responding to appropriate medical management. Patients who enrol as a result of intolerance to prior TKI therapy may have any level of response to their prior therapy and still be eligible.
Age ≥1 and <18 years at day of attaining the informed consent.	
Lansky performance status ≥50% for patients ≤16 years of age, or Karnofsky scale ≥50% for patients >16 years of age (appendix 5).	

Inclusion Criterion	Specifications
Adequate bone marrow function:	For second-line and third-line CP CML patients: Absolute neutrophil count >1000/mm3 (>1.0 x109/L); Platelets ≥75,000/mm3 (≥75 x109/L) without any platelet transfusions during the preceding 7 days. For fourth-line CP and all for all AP/BP CML patients: Absolute neutrophil count >500/mm3 (>0.5 x109/L); Platelets ≥50,000/mm3 (≥50 x109/L) without any platelet transfusions during the preceding 7 days.
Adequate Renal Function	Subjects must have a calculated creatinine clearance (CrCl) ≥ 60 mL/min/1.73 m2, using the Schwartz formula to estimate GFR
Adequate liver function	AST/ALT ≤2.5 x upper limit normal (ULN) or ≤5 x ULN if attributable to disease involvement of the liver; Total bilirubin ≤1.5 x ULN unless the patient has documented Gilbert syndrome.
Recovered to Grade 0-1, or to baseline, from any acute toxicities of prior chemotherapy, immunotherapy, radiotherapy, differentiation therapy, or biologic therapy, with the exception of alopecia.	
Able to reliably swallow whole capsules, whole tablets; or drug added to a suitable foodstuff (from capsule contents, added to either apple sauce or yoghurt); or tablets and/or capsules dissolved in water as an oral syringe drinking solution, or tablets dissolved and administered by NG tube when needed.	
Serum/urine pregnancy test (for all girls ≥ age of menarche) negative at screening.	
Male and female patients of childbearing potential and at risk for pregnancy must agree to use a highly effective method of contraception throughout the study and for at least 30 days after the last dose of assigned treatment.	A patient is of childbearing potential if, in the opinion of the Investigator, he/she is biologically capable of having children and is sexually active.
Written informed consent of parent(s)/legal guardian(s) and/or patients (when applicable depending on age and local law and regulations)	
Patients (including legally acceptable representative for minors where applicable) who are willing and able to comply with scheduled visits, treatment plan, laboratory tests, and other study	
Exclusion Criterion	**Specifications**
Diagnosis of primary Ph+ acute lymphoblastic leukemia.	as defined by 2013 European Leukemia Net guidelines

Inclusion Criterion	Specifications
In patients with AP/BP CML leptomeningeal leukemia, This assessment is not required for inclusion of CP CML patients.	defined as positive cytology on lumbar puncture (including both CNS2 and CNS3 status), or clinical symptoms or signs present.
Extramedullary disease only.	
Documented prior history of T315I or V299L BCR-ABL1 mutations (Note: BCR-ABL1 mutation testing will be performed at screening for a baseline assessment, but results are not used to determine eligibility.	This exclusion criterion is based on whether there is a known history of these mutations at the time of study entry. If these mutations become evident during the study the patient will go off study).
Any prior treatment with a TKI within 7 days prior to starting bosutinib treatment, or other anti-tumor or anti-leukemia treatment (with the exception of hydroxyurea and/or anagrelide) within 14 days prior to start of bosutinib treatment.	
Prior growth factors or biologic agents within 7 days prior to bosutinib treatment.	
Use of strong or moderate CYP3A4 inhibitors and inducers (see Appendix 8) within 7 days prior and/or concomitant to bosutinib treatment	
Use of proton pump inhibitors (Ph-modifying agents) within 7 days prior and/or concomitant to bosutinib treatment.	
Prior radiotherapy within 3 months prior to bosutinib treatment.	
Allogeneic stem cell transplantation within 3 months prior to bosutinib treatment.	
Donor lymphocyte infusion (DLI) within 1 month prior to bosutinib treatment.	
Hereditary bone marrow failure disorder.	
Graft-versus-host disease (GVHD) within 60 days prior to bosutinib treatment.	
Major surgery within 14 days prior to bosutinib treatment (recovery from any previous surgery should be complete before day 1.	
History of clinically significant or uncontrolled cardiac disease	History of or active congestive heart failure; Clinically significant ventricular arrhythmia (such as ventricular tachycardia, ventricular fibrillation, or Torsades de pointes); Diagnosed or suspected congenital or acquired prolonged QT syndrome; History of prolonged QTc.
Prolonged QTc	>450 msec, average of triplicate ECGs
Need for medications known to prolong the QT interval.	
Pregnant and/or nursing women	

Inclusion Criterion	Specifications
Uncorrected hypomagnesemia or hypokalemia due to potential effects on the QT interval.	
Left ventricular ejection fraction <50% or shortening fraction <28%.	
Recent or ongoing clinically significant gastrointestinal disorder that may interfere with the intake or absorption of the drug.	
Evidence of serious active or uncontrolled bacterial, fungal or viral infection.	
Known history of hepatitis B (HBV), hepatitis C (HCV), or human immunodeficiency virus (HIV) infection or acquired immunodeficiency syndrome (AIDS)-related illness.	
Other severe acute or chronic medical or psychiatric condition or laboratory abnormality that may increase the risk associated with study participation or investigational product administration or may interfere with the interpretation of study results and, in the judgment of the Investigator, would make the patient inappropriate for entry into this study.	

Supplementary Table 2. Pharmacokinetics Blood Collection Schedule

PK samples were collected on five timepoints, which were selected with a clinical trial simulation based on the adult pop PK model[1].

Study Visit	Screening	Cycle 1 Day 14						Cycle 1 Day 15	Cycle 2 Day 1	Cycle 3 Day 1	Cycle 4 Day 1	Dose escalation	Drug related SAE	End of Treatment
Hour (post-dose)		Pre-Dose	1	3	6	8		24	Pre-Dose	Pre-Dose	Pre-Dose	Pre-Dose		
Phase 1														
Whole blood collection for Pharmacokinetics[1]			x	x	x	x		x	x	x	x	x	x	

1. Janssen JM, Zwaan CM, Schellens JHM, et al: Clinical trial simulations in paediatric oncology: A feasibility study from the Innovative Therapies for Children with Cancer Consortium. Eur J Cancer. 2017;85:78–85.

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Supplementary Table 3: Amendment History

Protocol version number	Date of amendment	Type of amendment	Summary of amendment
Version 2.0	14-April-2016	Substantial	Adding HbsAg screening before treatment
Version 3.0	12-July-2018	Substantial amendment	- The definition of hematologic, and molecular response has been modified (Appendix 2) to add definitions of CHR for AP/BP CML and loss of CHR - Clarification on wash out period of TKIs before starting treatment - Clarification on screening of laboratory items allowing repeated sampling in case of abnormalities (especially for LFTs) - Addition of dosing instructions for younger children - Adding background pharmaceutical data about administration of alternative formulations for patients who are not able to swallow whole tablets or capsules - Updating approval status of bosutinib for newly diagnosed CML in adult patients and background section - Dexa scans eliminated for younger children < 4 years due to lack of normal values - Addition of clarification on DLT and SUSAR reporting requirements - Some lay-out changes to the schedule of events to better explain the timing of procedures - Administrative changes in e-mail addresses, adding a new statistician - Replacing Dr. Suttorp in the steering committee with Dr. Metzler - Revising the duration of treatment for phase I patients to 2 years from LPFV, and clarifying the follow-up period for patients after treatment discontinuation (detailed in table 4 and 5) - Revising the total trial duration and clarification on end of trial definition - Clarification on test results reporting as adverse events - Addition of exclusion criteria on pregnant and nursing women - Clarification on contraceptive methods - Addition of a palatability questionnaire

Protocol version number	Date of amendment	Type of amendment	Summary of amendment
Version 4.0	10-Oct-2019	Substantial amendment	- New approval for newly diagnosed adult CML patients was added - A cohort of newly diagnosed (ND) pediatric patients with CML in chronic phase was added in Phase 2 using the 300 mg/m2 once daily dose and the title was changed to refeclect this - DL2 was changed to 400 mg/m2 (DL2B) instead of 350 mg/m2 (DL2A) based on PK results obtained in the DL1 cohort - The objectives of the Phase 1 and 2 were modified according to the new design of the study; i.e. safety and PK are the primary objectives in all cohorts, and response is considered a secondary objective - Inclusion/exclusion criteria for ND patients for phase 2 added - Based on the simulations from an updated population PK analysis using data from adult patients with CML or solid tumors and healthy volunteers, the target adult exposure at steady-state (AUCss) are now updated to 3150 ng•hr/mL for the dose of 500 mg/day, and 2270 ng•hr/mL for the dose of 400 mg/day. - Sample size has been updated to 60 patients in total - Updated guidelines for CYP3A inducers/inhibitors and PPI concomitant medication at inclusion and during treatment - COG study number changed from AAML1621 to AAML1921 according to COG standard procedures, as the study will be active from 2019 in USA - Addition of the Bayesian analysis for futility and early stopping rules for toxicity for ND patients

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Supplementary Table 4. Body Surface to Dose Scheme

Actual Daily Dose in mg

Body Surface Area (m²)	200mg/m²	250mg/m²	300mg/m²	350mg/m²	400mg/m²	450mg/m²	500mg/m²
0.3	50	75	100	100	125	125	150
0.4	75	100	125	150	150	175	200
0.5	100	125	150	175	200	225	250
0.6	125	150	175	200	250	275	300
0.7	150	175	200	250	275	325	350
0.8	150	200	250	275	325	350	400
0.9	175	225	275	325	350	400	450
1.0	200	250	300	350	400	450	500
1.1	225	275	325	375	450	500	550
1.2	250	300	350	425	475	550	600#
1.3	250	325	400	450	525	575	600#
1.4	275	350	425	500	550	600#	600#
1.5	300#	375	450	525	600#	600#	600#
1.6	300#	400#	475	550	600#	600#	600#
1.7	300#	400#	500#	600#	600#	600#	600#
1.8	300#	400#	500#	600#	600#	600#	600#
1.9	300#	400#	500#	600#	600#	600#	600#
2.0	300#	400#	500#	600#	600#	600#	600#
2.1	300#	400#	500#	600#	600#	600#	600#
2.2	300#	400#	500#	600#	600#	600#	600#
2.3	300#	400#	500#	600#	600#	600#	600#
2.4	300#	400#	500#	600#	600#	600#	600#

Note: dosages were rounded off to allow dosing with the available formulations. Please take into account that only tablets can be dissolved for administration through a NG tube, and that only capsules can be opened for sprinkling on food stuff, or a combination of tablets and capsules can be used for oral administration with a syringe. # Dosages do not exceed the maximum adult equivalent.

Supplementary Table 5. Endpoints

Endpoint	Outcome
Primary endpoints	Incidence and severity of Dose-Limiting Toxicities (DLTs) assessed during the first 28 days of treatment.
	PK parameters of bosutinib: Maximum observed plasma concentration (Cmax), time to Cmax (Tmax), area under the plasma concentration versus time curve from time zero to the dosing interval (AUCτ), pre-dose concentration (Ctrough) and apparent clearance (CL/F).
Secondary endpoints	AEs, as characterized by type, frequency, severity (as graded using CTCAE version, v4.03), timing, seriousness, and relation to study therapy;
	Laboratory abnormalities as characterized by type, frequency, severity and timing;
	ECG and performance status abnormalities;
	Overall cumulative disease response: complete hematologic response (CHR), major cytogenetic response (MCyR, defined as complete cytogenetic response [CCyR] plus partial cytogenetic response [PCyR]), CCyR, major molecular response (MMR) and deep molecular response (definitions in table 3 below).
Exploratory endpoints	Parameters of bone metabolism and growth, including linear growth, bone age, bone mineral density of lumbar spine, physical signs of pubertal maturation (Tanner stage and testicular volume of boys), and hormones associated with growth and pubertal development (IGF-1, LH, FSH, estradiol for girls, and testosterone for boys) and a marker of bone formation and bone resorption (bone alkaline phosphatase and CTX). Patient and/or caregiver-reported assessments of gastrointestinal symptoms, as measured by selected domains from the PedsQL Gastrointestinal Symptom Scale.
	Patient and/or caregiver-reported assessment of the taste and ability to swallow the medicine, as measured by the Palatability Questionnaire for Bosutinib in patients aged 4-18 years of age.

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CHR, complete hematologic response; CCyR, complete cytogenetic response, PCyR, partial cytogenetic response; Ph+, Philadelphia Chromosome positive; MCyR, major cytogenetic response; minCyR, minor cytogenetic response; noCyR, no cytogenetic response; MR, molecular response; MMR, major molecular response; IS, international scale; EFS, event free survival.

*If bone marrow metaphases cannot be obtained or evaluated by chromosome banding analysis, the definition of CCyR (defined as <1% for FISH) may be based on interphase FISH of blood cells, provided that it is performed with BCR-ABL1 extrasignal, dual color, dual fusion, or in situ hybridization probes and at least 200 nuclei are scored. In this study, PCyR and CCyR are counted together and reported as MCyR.

**Molecular response is best assessed according to the International Scale (IS) as the ratio of BCR-ABL1 transcripts to ABL1 transcripts, or other internationally recognized control transcripts, and it is expressed and reported as BCR-ABL1% on a log scale, where 10%, 1%, 0.1%, 0.01%, 0.0032%, and 0.001% correspond to a decrease of 1, 2, 3, 4, 4.5, and 5 logs, respectively.

***If an on-treatment CBC is scheduled within 1 week from the last platelet transfusion or last dose of G-CSF, it should be considered "Not Evaluable" for hematological response assessment, and a CBC should be repeated at least 1 week from the last platelet transfusion or last dose of G-CSF received.

****CHR for AP/BP <5% BM blasts is only required when a BM differential is available.

Supplementary Methods

1. Statistical analysis

The sample size of phase I was dependent on the number of dose levels tested, using a modified rule based design (6+4), as cohorts of 6-10 patients were needed to properly assess PK. The safety analysis set included all patients who received ≥1 dose of study drug. The per protocol analysis set (evaluable for RP2D) included patients who received ≥75% (≥21 doses) of the planned 28 doses of bosutinib in cycle 1 (unless fewer dosages were given due to a DLT) and in which the AUC for bosutinib was estimated. The PK concentration set included all enrolled patients who had at least one reportable bosutinib plasma concentration. The efficacy analyses was based on the safety analysis set.

2. Mutational analysis

DNA isolation (Qiagen, 51306) was performed on white blood cells isolated from bone marrow or peripheral blood at study screening and end of treatment. PCR followed by Sanger sequencing was performed to detect mutations in ABL1 (NM_005157) exon 5, containing the hotspot V299L, and exon 6, containing the hotspot T315I. This region encodes amino acids 275 to 361, representing approximately 35% of the protein tyrosine kinase domain of ABL1. RPS20 was used as a positive control for DNA quality and PCR. For primers see below.

Supplementary Table 7. Primers Used for ABL1 Mutation Screening

Primer	Region	Location primer binding	Primer sequence 5' -> 3
957	*ABL1* exon 5 whole	intron 4	AACCTGTCTGCAGCAATGT
958		intron 5	CAACGAGGTTTTGTGCAGT
5202	*ABL1* exon 5	spanning intron 4 - exon 5 boundary	CTTCTGCAGGAGGACACCAT
5203		intron 5	ACGTCGGCAGAGCACAAATA
959	*ABL1* exon 6 whole	intron 5	GGAGCCACGTGTTGA
960		intron 6	TGCCAGCACTGAGGT
2521	*RPS20* part of exon 1 and 2	exon 1	AAGGGCTGAGGATTTTTG
2522		exon 2 amino acids 24-28	CGTTGCGGCTTGTTAG

Supplementary Table 8 List of Adverse Events (any grade)

Adverse Event	N	(%)
Diarrhea	26	92.86
Abdominal pain	20	71.43
Vomiting	19	67.86
Nausea	17	60.71
Fever	12	42.86
Skin and subcutaneous tissue disorders - Other	12	42.86
Rash maculo-papular	11	39.29
Headache	10	35.71
Alanine aminotransferase increased	9	32.14
Fatigue	8	28.57
Pain in extremity	8	28.57
Constipation	7	25.00
Gastrointestinal disorders - Other	7	25.00
Anorexia	6	21.43
Creatinine increased	6	21.43
Infections and infestations - Other	6	21.43
Metabolism and nutrition disorders - Other	6	21.43
Stomach pain	6	21.43
Aspartate aminotransferase increased	5	17.86
Rhinitis infective	5	17.86
Cough	4	14.29
CPK increased	4	14.29
Flatulence	4	14.29
General disorders - Other	4	14.29
Platelet count decreased	4	14.29
Rash acneiform	4	14.29
Anemia	3	10.71
Blood and lymphatic system disorders - Other	3	10.71
Blood bilirubin increased	3	10.71
Cardiac disorders - Other	3	10.71
Eye disorders - Other	3	10.71
Hypophosphatemia	3	10.71
Myalgia	3	10.71
Periorbital edema	3	10.71
Respiratory thoracic disorders - Other	3	10.71
Serum amylase increased	3	10.71
Upper respiratory infection	3	10.71
Urticaria	3	10.71
Alkaline phosphatase increased	2	7.14
Alopecia	2	7.14
Dental caries	2	7.14
Dry eye	2	7.14
Dry skin	2	7.14
Ear pain	2	7.14
Hypocalcemia	2	7.14

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Adverse Event	N	(%)
Injury poisoning and procedural complications	2	7.14
Lipase increased	2	7.14
Musculoskeletal disorder - Other	2	7.14
Neck pain	2	7.14
Oral pain	2	7.14
Otitis externa	2	7.14
Otitis media	2	7.14
Pain	2	7.14
Palpitations	2	7.14
Pruritus	2	7.14
Reproductive system and breast disorders	2	7.14
Urinary frequency	2	7.14
Vertigo	2	7.14
Weight loss	2	7.14
White blood cell decreased	2	7.14
Agitation	2	7.14
Allergic rhinitis	2	7.14
Anal ulcer	2	7.14
Arthralgia	1	3.57
Avascular necrosis	1	3.57
Back pain	1	3.57
Blurred vision	1	3.57
Bone pain	1	3.57
Bruising	1	3.57
Catheter related infection	1	3.57
Chest pain - cardiac	1	3.57
Chills	1	3.57
Conjunctivitis	1	3.57
Cystitis noninfective	1	3.57
Dehydration	1	3.57
Delirium	1	3.57
Depression	1	3.57
Dizziness	1	3.57
Dysphagia	1	3.57
Edema face	1	3.57
Edema limbs	1	3.57
Epistaxis	1	3.57
Eye infection	1	3.57
Hematuria	1	3.57
Hepatobiliary disorders - Other	1	3.57
Hypercalcemia	1	3.57
Hyperkalemia	1	3.57
Hypoalbuminemia	1	3.57
Hypoglycemia	1	3.57
Hypokalemia	1	3.57
Hypomagnesemia	1	3.57
Hyponatremia	1	3.57

Adverse Event	N	(%)
Hypothyroidism	1	3.57
Infusion site extravasation	1	3.57
Insomnia	1	3.57
Investigations - Other	1	3.57
Lethargy	1	3.57
Lung infection	1	3.57
Lymphocyte count decreased	1	3.57
Lymphocyte count increased	1	3.57
Malaise	1	3.57
Nasal congestion	1	3.57
Neutrophil count decreased	1	3.57
Non-cardiac chest pain	1	3.57
Oral dysesthesia	1	3.57
Proteinuria	1	3.57
Pulmonary hypertension	1	3.57
Rectal hemorrhage	1	3.57
Renal and urinary disorders - Other	1	3.57
Restlessness	1	3.57
Sinus tachycardia	1	3.57
Skin hyperpigmentation	1	3.57
Skin ulceration	1	3.57
Sneezing	1	3.57
Sore throat	1	3.57
Syncope	1	3.57
Tremor	1	3.57
Urinary incontinence	1	3.57

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Supplementary Table 9. List of Chemistry and Hematologic Laboratory Tests Abnormalities

9a. Chemistry Tests

Grade	0	1	2	3	4	Total
Albumin	26	4	0	0	0	30
Alkaline phosphatase	28	2	0	0	0	30
ALT	13	11	1	4	1	30
Amylase	18	7	3	1	0	29
AST	9	17	1	3	0	30
Bilirubin (Total)	24	4	2	0	0	30
Creatinine	21	9	0	0	0	30
Glucose	10	20	0	0	0	30
Lipase	25	2	0	2	0	29
Magnesium	22	7	0	0	0	29
Phosphorous	19	8	2	0	0	29
Potassium	23	6	1	0	0	30
Sodium	24	6	0	0	0	30
total calcium	29	1	0	0	0	30
Uric acid	22	5	0	0	1	28

9b. Hematologic Tests

Grade	0	1	2	3	4	Total
Hemoglobin	12	14	4	0	0	30
Platelet count	19	8	1	2	0	30
White blood cell count	15	10	4	1	0	30

Supplementary Table 10. Most Frequent AEs (frequency >3, all grades) by Dose Level and Age

10a. Most Frequent Adverse Events (frequency >3, all grades) by Dose Level

Adverse Event Term	Allocated dose level 300 mg/m² N=6	Allocated dose level 350 mg/m² N=11	Allocated dose level 400 mg/m² N=11
Diarrhea	6 (100%)	10 (90%)	10 (90%)
Abdominal pain	1 (17%)	11 (100%)	8 (73%)
Vomiting	5 (83%)	8 (73%)	6 (54%)
Nausea	6 (100%)	6 (54%)	5 (83%)
Fever	4 (67%)	5 (45%)	3 (27%)
Skin and subcutaneous tissue disorders - other	6 (100%)	3 (27%)	3 (27%)
Rash maculo-papular	0	4 (36%)	7 (64%)
Headache	3 (50%)	3 (27%)	4 (36%)
Alanine aminotransferase increased	1 (17%)	4 (36%)	4 (36%)
Fatigue	4 (67%)	2 (18%)	2 (18%)
Pain in extremity	3 (50%)	2 (18%)	3 (27%)
Constipation	3 (50%)	1 (9%)	3 (27%)
Gastrointestinal disorders - other	2 (33%)	2 (18%)	3 (27%)
Anorexia	3 (50%)	0	3 (27%)
Creatinine increased	2 (33%)	2 (18%)	2 (18%)
Infections and infestations - other	1 (17%)	3 (27%)	2 (18%)
Metabolism and nutrition disorders - other	2 (33%)	2 (18%)	2 (18%)
Stomach pain	3 (50%)	1 (9%)	2 (18%)
Aspartate aminotransferase increased	0	3 (27%)	2 (18%)
Rhinitis infective	4 (67%)	0	1 (9%)
Cough	2 (33%)	5 (45%)	0
CPK increased	0	2 (18%)	2 (18%)
Flatulence	2 (33%)	0	2 (18%)
General disorders - Other	1 (17%)	1(9%)	2 (18%)
Platelet count decreased	0	1 (9%)	3 (27%)
Rash acneiform	2 (33%)	0	2 (18%)

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10b. Most Frequent Adverse Events (frequency >3, all grades) by Age

Adverse Event Term	>1 y ≤6 y N=6	>6 y ≤12 y N=10	>12 y N=12
Diarrhea	6 (100%)	9 (90%)	11 (90%)
Abdominal pain	4 (67%)	7 (70%)	9 (75%)
Vomiting	5 (83%)	7 (70%)	7 (58%)
Nausea	3 (50%)	7 (70%)	7 (58%)
Fever	5 (83%)	4 (40%)	3 (25%)
Skin and subcutaneous tissue disorders - other	3 (50%)	4 (40%)	5 (42%)
Rash maculo-papular	2 (33%)	4 (40%)	5 (42%)
Headache	2 (33%)	3 (30%)	5 (42%)
Alanine aminotransferase increased	0	5 (50%)	4 (33%)
Fatigue	3 (50%)	2 (20%)	3 (25%)
Pain in extremity	4 (67%)	2 (20%)	2 (17%)
Constipation	1 (17%)	4 (40%)	2 (17%)
Gastrointestinal disorders - other	2 (33%)	2 (20%)	3 (25%)
Anorexia	1 (17%)	3 (30%)	2 (17%)
Creatinine increased	0	3 (30%)	3 (25%)
Infections and infestations - other	2 (33%)	1 (10%)	3 (25%)
Metabolism and nutrition disorders - other	1 (17%)	4 (40%)	1 (8%)
Stomach pain	1 (17%)	3 (30%)	2 (17%)
Aspartate aminotransferase increased	0	3 (30%)	2 (17%)
Rhinitis infective	2 (33%)	2 (20%)	1 (8%)
Cough	3 (50%)	1 (10%)	0
CPK increased	0	1 (10%)	3 (25%)
Flatulence	0	2 (20%)	2 (17%)
General disorders - Other	2 (33%)	0	2 (17%)
Platelet count decreased	0	1 (10%)	3 (25%)
Rash acneiform	2 (33%)	0	2 (17%)

Y= years

Supplementary Table 11. QTc Measurements per Patient

Patient	Sex	Age*	Time Point	QTc (ms)
1	Female	17	SCR	394.33
1	Female	17	C1D14	392.67
1	Female	17	C1D14 timepoint2	417.33
1	Female	17	C1D15	386.00
1	Female	17	C2	402.00
1	Female	17	C3	390.67
1	Female	17	C4	395.33
1	Female	17	EOT	399.67
2	Male	9	SCR	404.00
2	Male	9	C1D14	390.00
2	Male	9	C1D14 timepoint2	387.00
2	Male	9	C1D15	387.00
2	Male	9	C2	394.67
2	Male	9	C3	382.00
2	Male	9	C4	388.67
2	Male	9	EOT	405.00
3	Male	4	SCR	343.00
3	Male	4	C1D14	361.00
3	Male	4	C1D14 timepoint2	337.67
3	Male	4	C1D15	372.00
3	Male	4	C2	337.00
3	Male	4	C3	342.33
3	Male	4	C4	363.00
3	Male	4	EOT	352.33
4	Male	11	SCR	421.33
4	Male	11	C1D14	401.33
4	Male	11	C1D14 timepoint2	409.33
4	Male	11	C1D15	391.67
4	Male	11	C2	391.67
4	Male	11	C3	391.67
4	Male	11	C4	397.00
4	Male	11	EOT	414.67
5	Male	1	SCR	337.00
5	Male	1	C1D14	355.33
5	Male	1	C1D14 timepoint2	339.67
5	Male	1	C1D15	360.67
5	Male	1	C2	351.33
5	Male	1	C3	357.00
5	Male	1	C4	360.00
5	Male	1	EOT	365.00
6	Male	8	SCR	385.00
6	Male	8	C1D14	410.67
6	Male	8	C1D14 timepoint2	411.00
6	Male	8	C1D15	401.67
6	Male	8	C2	410.00
6	Male	8	C3	414.33
6	Male	8	C4	407.00

Patient	Sex	Age*	Time Point	QTc (ms)
7	Female	5	SCR	407.67
8	Female	6	SCR	389.00
8	Female	6	C1D14	389.33
8	Female	6	C1D14 timepoint2	389.00
8	Female	6	C1D15	400.67
8	Female	6	C2	393.00
8	Female	6	C3	416.00
8	Female	6	C4	384.00
9	Female	11	SCR	365.00
9	Female	11	C1D14	403.00
9	Female	11	C1D14 timepoint2	406.00
9	Female	11	C1D15	401.67
9	Female	11	C2	405.67
9	Female	11	C3	413.00
9	Female	11	C4	413.00
9	Female	11	EOT	409.67
10	Female	11	SCR	403.67
10	Female	11	C1D14	396.00
10	Female	11	C1D14 timepoint2	408.00
10	Female	11	C1D15	404.00
10	Female	11	C2	392.67
10	Female	11	C3	397.00
10	Female	11	C4	395.67
10	Female	11	EOT	394.33
11	Female	12	SCR	398.00
11	Female	12	C1D14	381.33
11	Female	12	C1D15	390.67
11	Female	12	C2	388.00
11	Female	12	C3	394.67
11	Female	12	C4	400.33
12	Male	12	SCR	423.00
12	Male	12	C1D14	386.33
12	Male	12	C1D14 timepoint2	395.00
12	Male	12	C1D15	399.67
12	Male	12	C2	398.00
12	Male	12	C3	408.67
12	Male	12	EOT	400.33
13	Male	7	SCR	367.00
13	Male	7	C1D14	357.67
13	Male	7	C1D14 timepoint2	362.33
13	Male	7	C1D15	372.67
13	Male	7	C2	381.33
13	Male	7	C3	379.33
13	Male	7	C4	366.00
13	Male	7	EOT	385.67
14	Female	16	SCR	391.67
14	Female	16	C1D14	382.33
14	Female	16	C1D14 timepoint2	387.67
14	Female	16	C2	382.00

Patient	Sex	Age*	Time Point	QTc (ms)
14	Female	16	Unscheduled Event	381.00
14	Female	16	Unscheduled Event	387.00
14	Female	16	C3	384.33
14	Female	16	C4	379.67
15	Male	17	SCR	393.67
15	Male	17	C1D14	378.67
15	Male	17	C1D14 timepoint2	394.00
15	Male	17	C2	375.33
15	Male	17	C3	397.00
15	Male	17	C4	385.67
15	Male	17	EOT	384.00
16	Female	4	SCR	395.00
16	Female	4	C1D14	383.67
16	Female	4	C1D14 timepoint2	365.00
16	Female	4	Unscheduled Event	378.33
16	Female	4	Unscheduled Event	365.00
16	Female	4	C1D15	353.00
16	Female	4	Unscheduled Event	353.00
16	Female	4	C2	350.67
16	Female	4	C3	377.33
16	Female	4	C4	383.67
17	Male	14	SCR	376.00
17	Male	14	C1D14	367.33
17	Male	14	C1D14 timepoint2	369.67
17	Male	14	C2	386.67
17	Male	14	C3	378.67
17	Male	14	C4	389.33
17	Male	14	EOT	267.00
18	Male	13	SCR	402.33
18	Male	13	C1D14	385.67
18	Male	13	C1D14 timepoint2	395.00
18	Male	13	C2	382.33
18	Male	13	C3	390.00
18	Male	13	C4	387.33
19	Female	17	SCR	425.00
19	Female	17	C1D14	412.33
19	Female	17	C2	404.00
19	Female	17	C3	400.00
19	Female	17	C4	401.67
19	Female	17	C1D14 timepoint2	404.00
20	Male	8	SCR	400.67
20	Male	8	Unscheduled Event	358.00
20	Male	8	Unscheduled Event	373.00
20	Male	8	C1D14	400.00
20	Male	8	C1D14 timepoint2	401.00
20	Male	8	C2	391.00
20	Male	8	C3	391.00
20	Male	8	C4	393.00
20	Male	8	EOT	388.33

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| Patient	Sex	Age*	Time Point	QTc (ms)
21	Female	17	SCR	391.00
21	Female	17	C1D14	393.33
21	Female	17	C1D14 timepoint2	386.33
21	Female	17	C2	396.00
21	Female	17	C3	372.67
21	Female	17	C4	377.33
21	Female	17	EOT	269.33
22	Female	15	SCR	408.33
22	Female	15	C2	402.67
22	Female	15	C3	399.00
22	Female	15	C3 timepoint2	417.00
22	Female	15	Unscheduled Event	417.00
22	Female	15	C4	389.00
23	Male	13	SCR	386.67
23	Male	13	C1D14	398.00
23	Male	13	C1D14 timepoint2	418.00
23	Male	13	C2	397.67
23	Male	13	C3	408.67
23	Male	13	C4	396.67
23	Male	13	EOT	379.00
24	Male	16	SCR	407.00
24	Male	16	C1D14	396.67
24	Male	16	C1D14 timepoint2	419.33
24	Male	16	C1D15	390.33
24	Male	16	C2	388.67
24	Male	16	C3	392.33
24	Male	16	C4	400.00
25	Female	16	C1D14	411.00
26	Male	15	EOT	390.00
27	Male	10	SCR	385.33
27	Male	10	C1D14	383.00
27	Male	10	C1D14 timepoint2	385.67
27	Male	10	C2	371.00
27	Male	10	C3	385.33
27	Male	10	C4	383.33
28	Male	6	SCR	398.00
28	Male	6	C1D14	399.67
28	Male	6	C1D14 timepoint2	407.00
28	Male	6	C1D15	392.33
28	Male	6	C2	391.00
28	Male	6	C3	408.33

C: cycle, D: day, EOT: End of Treatment

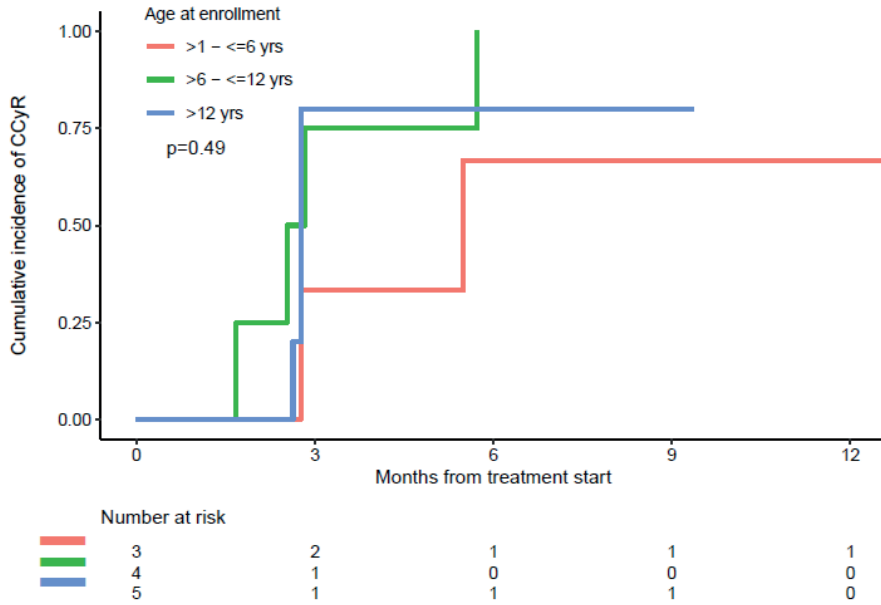*Age is reported at time of enrolment
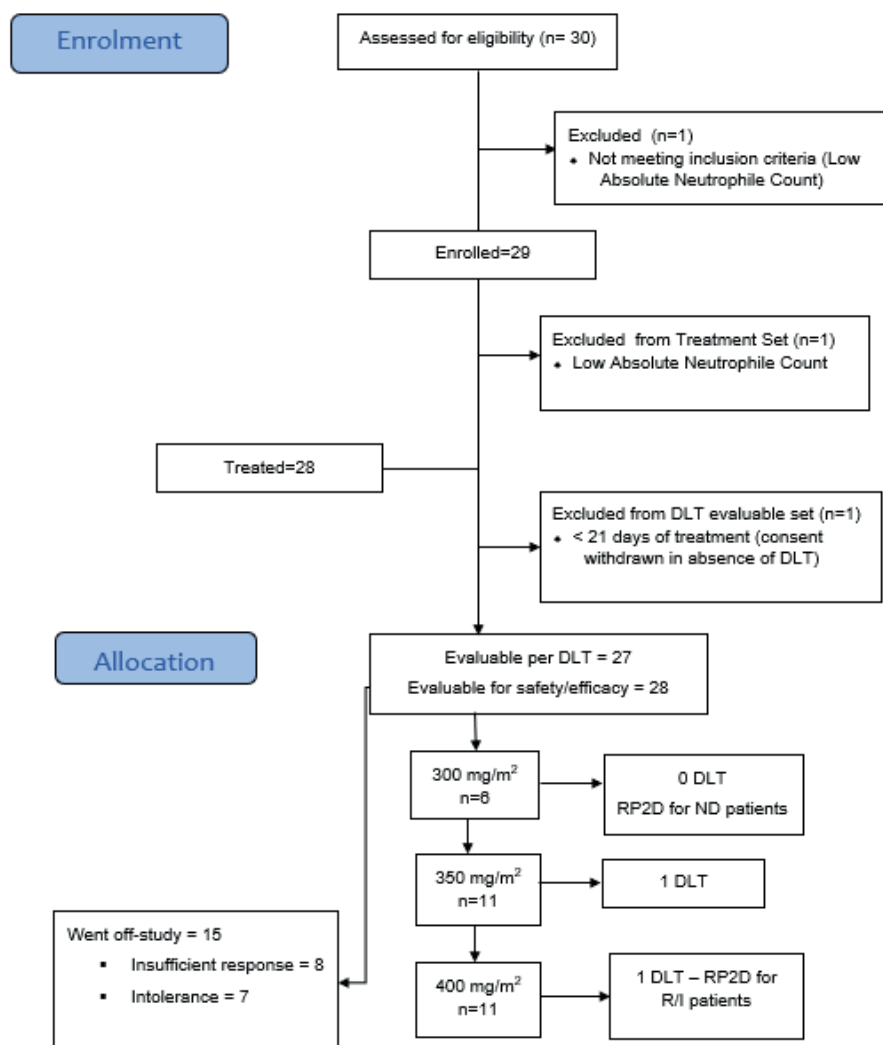
Supplementary Figure 1. Consort Diagram

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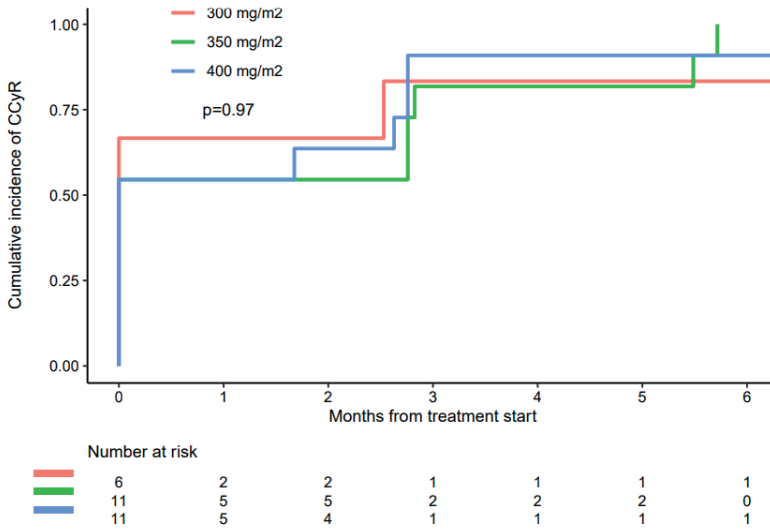
**2A. Cumulative Incidence of Complete Cytogenetic Response (CCyR) per Dose Level**
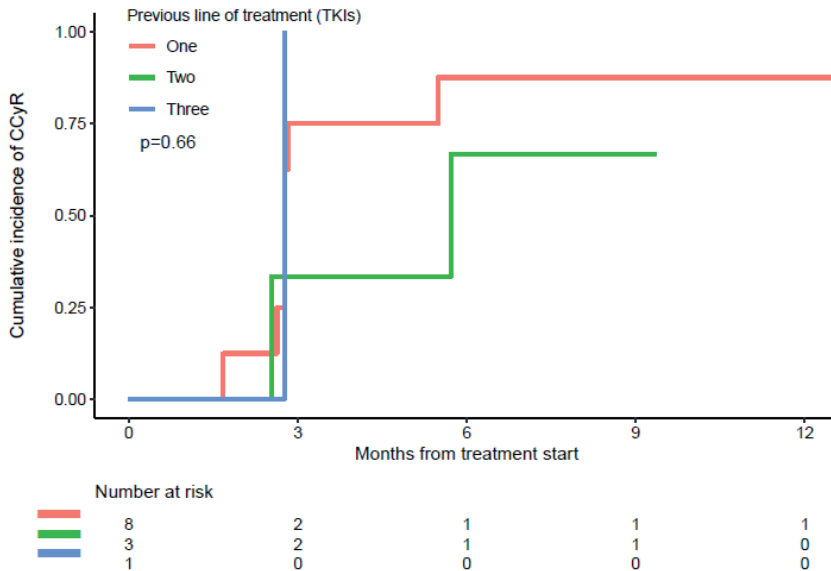
2B. Cumulative Incidence of Complete Cytogenetic Response (CCyR) per Previous Line of Treatment

2C. Cumulative Incidence of Complete Cytogenetic Response (CCyR) per Age Class

Supplementary Figure 2. Cumulative Incidence of Complete Cytogenetic Response (CCyR) per Dose Level (2A), per Previous Line of Treatment (2B), per Age Class (2C). Subjects entering the study already in CCyR are displayed as event at time zero, respectively 2, 3 and 4 patients at 300, 350 and 400 mg/m². No difference in response among the three dose levels was shown.
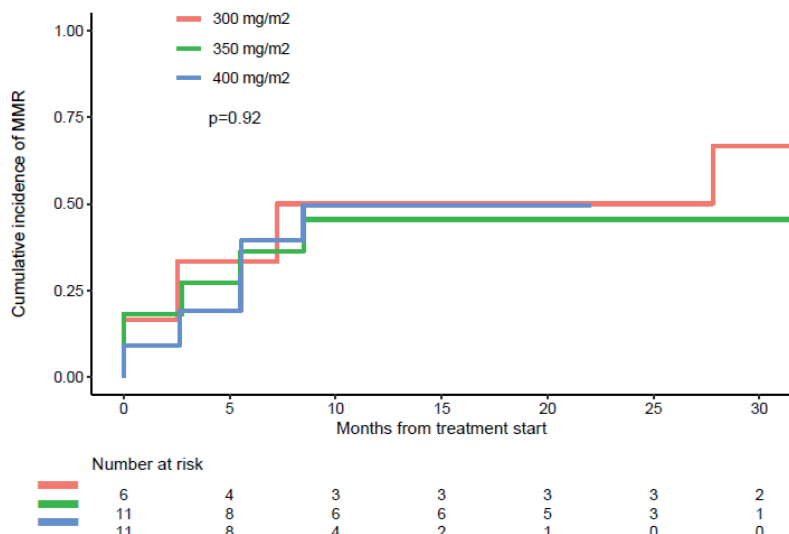
3A. Cumulative Incidence of Major Molecular Response (MMR) per Dose Level
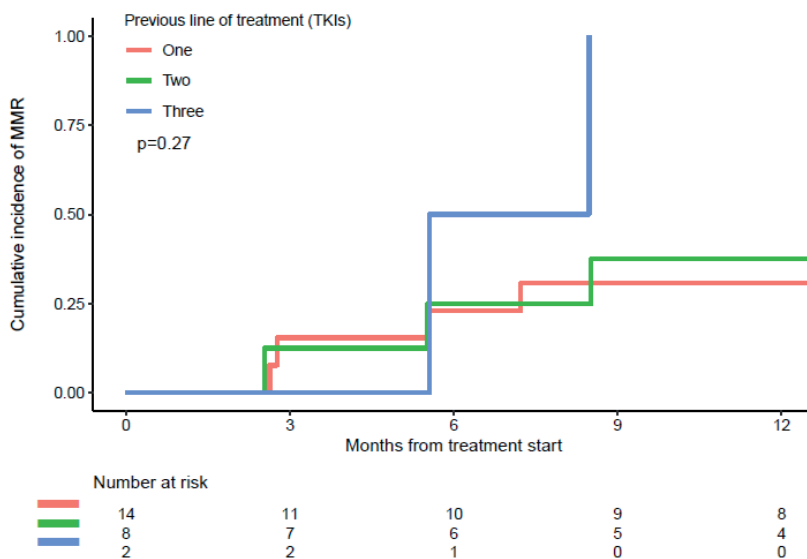
3B. Cumulative Incidence of Major Molecular Response (MMR) per Previous Line of Treatment

3C. Cumulative Incidence of Major Molecular Response (MMR) per Age Class

Supplementary Figure 3. Cumulative Incidence of Major Molecular Response (MMR) per Dose Level (3A), per Previous Line of Treatment (3B), per age class (3C). Subjects entering the study already in MMR are displayed as event at time zero, respectively 2, 3 and 4 patients at 300, 350 and 400 mg/m². No difference in response among the three dose levels was shown.
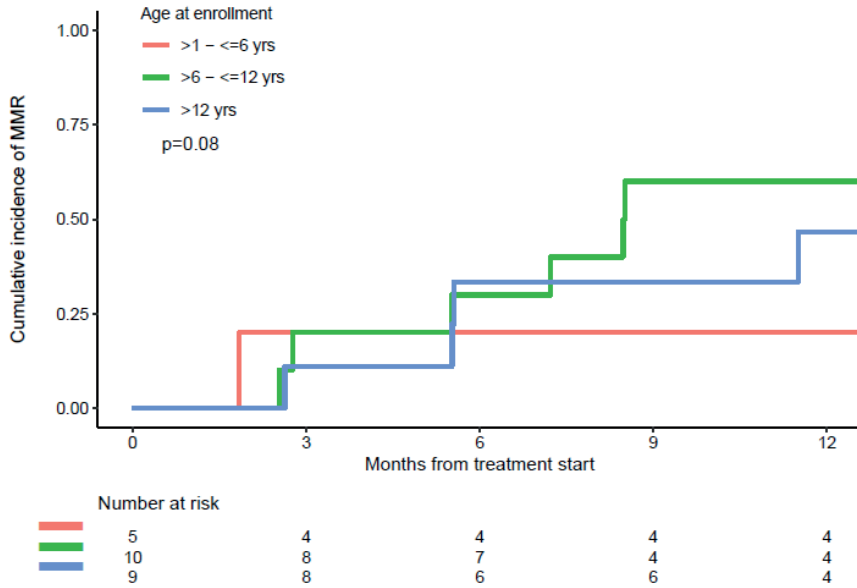
Supplementary Table 12. Overall Cumulative Confirmed Disease Response of CHR, MCyR, CCyR and MMR per Dose Level

Dose level	Hematologic Response
300 mg/m²	All 6 (100%) participants in the Phase 1 300 mg/m² cohort had a CHR (95% CI: 54.1%, 100.0%) The median time to CHR was 1.86 months (range: 1.64, 4.76 months) among responders.
350 mg/m²	10 (90.9%) participants in the Phase 1 350 mg/m² cohort had a CHR (95% CI: 58.7%, 99.8%). The median time to CHR was 1.87 months (range: 0.95, 2.79 months) among responders.
400 mg/m²	8 (72.7%) participants in the Phase 1 400 mg/m² cohort had a CHR (95% CI: 39.0%, 94.0%). The median time to CHR was 2.27 months (range: 0.99, 2.79 months) among responders.

Dose level	Cytogenetic Response
300 mg/m²	100% (95% CI: 54.1%, 100.0%) and 83.3% (95% CI: 35.9%, 99.6%) of the participants attained or maintained MCyR and CCyR, respectively, while receiving bosutinib. The median time to MCyR and CCyR was 2.76 months (range: 2.56, 8.21 months) and 2.76 months (range: 2.56, 2.79 months), respectively, among responders.
350 mg/m²	90.9% (95% CI: 58.7%, 99.8%) of the participants attained or maintained MCyR/CCyR while receiving bosutinib. The median time to both MCyR and CCyR was 2.79 months (range: 2.76, 5.75 months) among responders.
400 mg/m²	63.6% (95% CI: 30.8%, 89.1%) of the participants attained or maintained MCyR/CCyR while receiving bosutinib. The median time to both MCyR and CCyR was 2.79 months (range: 1.71, 3.71 months) among responders.

Dose level	Molecular Response
300 mg/m²	66.7% (95% CI: 22.3%, 95.7%), 33.3% (95% CI: 4.3%, 77.7%), and 33.3% (95% CI: 4.3%, 77.7%) of the participants attained or maintained MMR, MR4, and MR4.5, respectively. Of participants without the respective response at baseline 60.0% (95% CI: 14.7%, 94.7%) achieved MMR, 20.0% (95% CI: 0.5%, 71.6%) achieved MR4 and/or MR4.5. The median time to MMR was 5.01 months (range: 2.56, 27.83 months) among MMR responders
350 mg/m²	45.5% (95% CI: 16.7%, 76.6%), 36.4% (95% CI: 10.9%, 69.2%), and 27.3% (95% CI: 6.0%, 61.0%) of the participants attained or maintained MMR, MR4, and MR4.5, respectively. 1 (9.1%) participant was still on-treatment without MMR attained at the time of cut-off. Of participants without the respective response at baseline, 25.0% (95% CI: 3.2%, 65.1%) achieved MMR, 30.0% (95% CI: 6.7%, 65.2%) achieved MR4 and/or MR4.5. The median time to MMR was 5.55 months (range 2.79, 8.54 months) among MMR responders.

Dose level	Hematologic Response
400 mg/m²	45.5% (95% CI: 16.7%, 76.6%), 0% (95% CI: 0.0%, 28.5%), and 0% (95% CI: 0.0%, 30.8%) of the participants attained or maintained MMR, MR4, and MR4.5, respectively. 2 (18.2%) participants were still on-treatment without MMR attained at the time of cut-off.
	Of participants without the respective response at baseline, 44.4% (95% CI: 13.7%, 78.8%) achieved MMR, 0% (95% CI: 0.0%, 28.5%) achieved MR4 and/or MR4.5.
	The median time to MMR was 5.59 months (range: 2.66, 8.51 months) among MMR responders.

Response is unconfirmed for cytogenetic and confirmed for hematologic with 2 consecutive responses at least 28 days apart. To be considered a responder, a patient with a baseline response must have maintenance of response for ≥5 weeks from baseline for hematologic response or ≥4 weeks from baseline for cytogenetic response. Molecular response is unconfirmed and is based on the definitions provided in the Supplementary Table 5 above. See main paper for the cumulative proportion and cumulative incidence of first-time reaching CHR, MCyR, CCyR and MMR.

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Chapter 6

Conclusions and Discussion

Developing drugs for pediatric patients confronts the researcher with peculiar challenges. The rarity of these pathologies, the difficulties in recruiting patients, the heterogeneity of the pediatric population in terms of organ development and metabolic functions, as well as the longer life expectancy compared to adults are just some examples. The following paragraphs first provide considerations on how regulatory incentives can promote pediatric drug development together with an overview on possible improvements in terms of experimental design; then a concise outlook on future directions for pediatric ALL and CML research is presented.

6.1 Considerations on Regulations and Experimental Designs in Dose Finding Trials

6.1.1 Regulatory Incentives: The Introduction of Pediatric Investigation Plans, Class Waivers and the Need for Multi-Stakeholders Collaboration

The development of pediatric anti-cancer drugs is still slow-moving and mostly adult driven. For example, InO was approved for adults with ALL in 2017, after the completion of the INO-VATE ALL trial.[1] The results of the first pediatric registrational trial (ITCC-059) presented in chapter 2 and 3 where transferred to FDA and EMA six years later in 2023. Similarly, the phase I of the trial ITCC-054/COG AAML1921, testing bosutinib in R/I CML pediatric patients, opened in 2016, and finally enrolled approximately 60 patients in 2022, despite more than 50 centers in continental Europe and the US were active (however it should be underlined that CML is a very rare form of tumor in children). This finally led to approval of bosutinib in September 2023 by the FDA, awaiting EMA submission in early 2024. Meantime, in adults, a phase III trial testing the third generation TKI asciminib against bosutinib is already completed.[2] This is not an issue regarding leukemic diseases only, it affects pediatric oncology in its totality, and is potentially worse for example in solid tumors where many pediatric cancers do not occur or are extremely rare in adults (for example neuroblastoma and Wilms tumor). Between 1995 and 2021, EMA has approved over 169 anti-cancer drugs for use in adults.[3] Of these, only 16 received marketing authorizations in pediatrics (it should be noticed though that not all drugs developed for adults can have an application in pediatrics as some diseases just do not occur in this population).[3] Efforts to speed up pediatric drug development converged towards the creation of dedicated platforms such as *ACCELERATE* (a multi-stakeholder organization aiming at devising strategies to develop pediatric anti-cancer drugs faster); or international consortia to coordinate multiple research groups such as the *"Innovative Therapies for Children with Cancer"* (ITCC) consortium in Europe.[4,5]

Also at the regulatory level significant steps forward were made. In the context of the FDA *Pediatric Research Equity Act* [21U.S. Code 355B] in 2003, then amended as *Research to Accelerate Cures and Equity* (RACE) for children act in 2017, companies developing anti-cancer drugs for adults against molecular targets listed as relevant also for the pediatric population

are now required to additionally submit a development plan for children (Pediatric Study Plan or PSP).[6] Similar initiatives were taken in Europe by EMA with the introduction of the pediatric regulation and *Pediatric Investigation Plans* (PIP) in 2007, which requests Marketing Authorization Applicants (MAAs) to present an agreed PIP when filing for adult approval (European Paediatric Regulation, EC No 1901/2006).[7] The PIP is assessed by the European Medicines Agency's Paediatric Committee (PDCO) and the successful completion of the PIP rewards the MAA with a six month extension of their supplementary protection certificate.[8] A report examining data collected during the first 10 years since the PIP was introduced showed that indeed the development of pediatric drugs has improved in Europe and it is now integrated in the development cycle of new compounds at the company level.[8,9] Since 2007, 16 new molecular entities (not only in oncology), developed in the PIP framework, were approved of which nine obtained first joint approval for adult and children, while seven were approved first in adults and then in children, with an average delay of approximately six years.[10]

Nevertheless, drug development remains mostly adult driven and the increase in the number of drugs registered for pediatric use also reflects a general increase in the total number of drugs developed.[10] Among the possible caveats of the current "*PIP environment*" there is the fact that the EU regulation allows PIP waivers for pediatric studies in those cases in which the drug is preliminary assessed by the EMA PDCO to have low probability of being effective, or is considered unsafe in children; or when the disease is exclusively occurring in adults (e.g. ALK and MET inhibitors for non-small cell lung cancer); or it may not represent a significant therapeutic benefit over existing treatments. The waiver can be granted either at the class level (for example all compounds intended for a specific disease) or at the product-specific level (for example for a specific age sub-group). In the former case, the justification is mostly based on the fact the indication of the drug under development does not affect children (e.g. prostate cancer) and a review of the list of granted waivers showed that the majority of class waivers were indeed granted in oncology.[11] The main issue with this system is that class waivers, even when granted in compliance with the European regulation, might not reflect updated scientific evidence, particularly for targeted therapies.[11–13] For example, crizotinib was granted a class waiver in 2010 as its initial target indication (ALK positive non-small cell lung cancer) does not occur in children. Nevertheless, ALK and MET inhibitors are known to be relevant in pediatric cancers such as neuroblastoma, and Anaplastic Large Cell Lymphoma (ALCL). Indeed, crizotinib was later developed for pediatric ALK positive ALCL and approved by FDA in 2021, and recently also by EMA.[14] A published review of class waivers, agreed by EMA between 2012 and 2015, showed that among the 89 anticancer drugs which were granted the waivers, 48 might have had a potential application in pediatrics.[11] It is worth nothing though, that examples of drugs developed in the context of voluntary PIP exists (e.g. dabrafenib for advanced V600 BRAF pediatric solid tumors), and that also pediatric drug development projects outside the PIP regulation are pursued by companies in some cases.

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Nonetheless, it has been advocated to revoke the current EMA class waiver list (already updated by EMA in 2015) and to redact a list of pediatric relevant molecular targets, based on an harmonized database that collects data on oncological molecular profiles (as already in place in the US), to be used as a basis for grating the waivers.[12] An example of this strategy is the collaboration between the Cancer European Network and the ITCC Biology Committee which aims at collecting clinical and pre-clinical data on cancer biology, biomarkers and molecular targets based on which assessing the relevance of new drugs for pediatric oncology.[15,16] Similarly, in the US, the National Cancer Institute, the FDA and the pediatric oncology community already developed the so-called *Relevant Molecular Target List* (RMTL) which reports more than 200 molecular targets considered relevant to pediatric oncology and based on which companies are requested to submit an *initial Pediatric Study Plan* (iPSP) when developing new drugs for adults.[17]

A third aspect which is relevant in this context is the concept of a *"flexible"* PIP to incorporate new information acquired during the life-cycle of the PIP itself. Indeed, the amendment of the original agreement between the MAA and the regulatory authority should be facilitated and even supported when new data are acquired after the original submission of the PIP. As the PIP is submitted at an early development stage, typically no later than when pharmacokinetic studies in adults are completed, it is reasonable to expect new knowledge to be generated after its submission. In this sense, as reported by Karres D. et al., the PIP can be considered a "*living' document*".[18] Among the new regulations, published by EMA in April 2023, there is the introduction of a pilot project named "*stepwise PIP*" (sPIP) which simplifies the initial submission, but conditionally on submitting a full PIP as soon as the crucial information is made available, together with a stricter set of commitments expected in the full PIP, such as the obligation to submit a detailed report in case the MAA drops the PIP, the possibility for EMA to request a compulsory PIP in case the drug is intended for a disease absent in children but which targets a biological mechanism "*responsible for a different disease or condition in the same therapeutic area in children*", and the need to "*describe any measures to adapt the pharmaceutical form, the strength, the route of administration and the eventual administration device*" appropriate for pediatric use.[19] The pilot project will initiate with eight sPIPs "*to inform decision making on its use in the future*".[19]

Finally it is important to stress the role of collaboration at multiple levels. The first level is collaboration among regulatory authorities, for example EMA and FDA.[20] The second level is collaboration between academia and the industry.[21] A third level of collaboration is the one among research institutions globally.

With the introduction of "*Common Commentary on Paediatric Oncology Drug Development*", MAAs which submit simultaneously their PIP/iPSP to EMA and FDA can receive guidance on how to address institutional requirements respectively in Europe and the US, and therefore "*adequately addressing these issues upfront will permit focused discussions during cluster calls, allowing for coordination of global development plans*".[22] Discussions take place on a monthly

basis during the pediatric cluster calls to which also the Pharmaceuticals and Medical Devices Agency Japan (PMDA), the Therapeutic Goods Administration Australia (TGA) and the Health Canada (HC) representatives participate. This initiative is meant to foster a global harmonization of the regulatory landscape for pediatric drug development and to shorten the process that leads to the final approval or rejection of the MAA submission in multiple countries at once.

In regard to academia-industry collaboration, it is important for the former to develop knowledge and systems capable of generating sufficient documentation and data to produce a *"ready-to-file"* package for the respective regulatory body. Academic institutions might need to strengthen their data management and monitoring plans beyond what is needed for publication purposes, and already foresee the requirements for filing the clinical study report to the competent authority. This means the establishment of a comprehensive adverse event reporting system, detailed reports of laboratory investigations (particularly for complex laboratory procedures such as PCR and Flow Cytometry), development of data management plans in compliance with the ICH E6(R2) directives, among other challenges.[21] Academia sponsored trials with *intent-to-file* are still not common, but as witnessed by the results presented in this thesis, they can be successful and might represent a new and effective approach to develop pediatric drugs.

The third level of collaboration is the one among research groups, and in particular on the two sides of the Atlantic ocean. An example has already been presented in chapter 5 about the partnership between ITCC and COG to test bosutinib in children. Another example is the *The Leukemia & Lymphoma Society* (LLS) *Pediatric Acute Leukemia* (PedAL) program, coordinated in Europe by the *European Pediatric Acute Leukemia* consortium. In this context, the concept of Master Protocol finds its *proof-to-concept* application as an example of global Master Clinical Trial for pediatric oncology.

In general, the concept of a complex trial entails the idea of having an overarching protocol (Master Protocol) which contains several sub-protocols (or strata) and which allows either simultaneous testing of multiple interventions for a common disease, or investigating sub-populations or multiple diseases sharing a similar biology. This boils down to one of three categories: *Platform trial*, *Basket trial* or *Umbrella trial*. Under a Platform Trial framework, multiple interventions are tested against a common control with the possibility of including additional drugs as they are developed and drop the ones not promising. Among the advantages there is the reduction of the number of patients required for the control group compared to the number of participants required for numerous separate randomized controlled trials. In cases in which multiple tumors share the same genetic drive, Basket Trial designs allow for testing the same drug against multiple diseases (e.g. anti-VEGF drugs, and, in adults, checkpoint inhibitors like pembrolizumab). This might be advantageous when the researcher deals with rare diseases and, therefore, combing disease with the same biology might increase the recruitment speed. Another example might be the Drug Rediscovery protocol (DRUP), a multi-drug and pan-cancer trial, which amins at expanding the spectrum of indication of existing anti-cancer compounds (trial

NCT02925234 sponsored by The Netherlands Cancer Institute). Patients with advanced forms of cancer, and that extinguished all therapeutic options available, are screened for actionable genetic or molecular variants and can be assigned to a commercially available treatment based on a whole-genome sequencing and a national centralized database containing molecular targets.

On the contrary, when the same disease can be stratified into molecular or risk subcategories, multiple agents/combinations could be tested in parallel under Umbrella Trials (Trial ITCC-059 resembles this concept and might be considered an example of complex clinical trial as well). Trial ITCC-101/APAL2020D is an instance of complex clinical trial stratified based on molecular profiling of known/suspected relapsed and refractory Acute Myeloid Leukemia (AML) pediatric patients.[23] The first sub-trial opened is a randomized phase III trial (NCT05183035) investigating the combination of fludarabine, high-dose cytarabine, and gemtuzumab ozogamicin with and without the addition of venetoclax (a Bcl-2 inhibitor).[24] Other sub-trials, testing e-selectin ligand inhibitors, menin inhibitors, CD47 inhibitors, CD123-targeted immunotherapies, and other targeted immunotherapies, are planned to open soon.[23,25] Other examples of Master Protocol frameworks applied to pediatric oncology are the Phase II COG sponsored Pediatric MATCH (Molecular Analysis for Therapy Choice) trial (NCT03155620), and AcSe´-ESMART trial (NCT02813135) in Europe (see details below).

6.1.2 Statistical Considerations on Early Phase Trials

If we look at the predominant designs of phase I trials, the 3+3 in adults and the rolling-6 in pediatrics are the most prevalent. A literature review showed that 98.4% of dose-finding trials implement variations of standard rule-based dose escalation designs.[26] These designs though present some limitations, and alternatives are now available.[27] The first two issues are the trial duration and the risk of under- and over-treatment. For example, despite the rolling-6 design was developed as a method to accelerate phase I pediatric trials (because it allows for concomitant accrual of two to six patients at the same dose level), it is only a marginal improvement compared to the 3+3 design.[28,29] The gain in terms of duration of the trial under the rolling-6 design, and compared to the 3+3, is dependent on several variables such as the length of the DLT evaluation period and the accrual speed. The slower the accrual is, the smaller the advantage (compared to the 3+3) is, with the latter almost disappearing when the enrollment time from one patient to another is equal or above three cycles (assuming one cycle of 21 days).[29] Perhaps the only advantage of the rolling-6 is that the trial does not need to be on hold after three patients are recruited, while in the 3+3 the accrual is suspended until the DLT evaluation period expires for all three patients in a cohort at a given dose level. As practical applications demonstrate, when the treated population is rare (e.g. CML), escalation trials following a rule-based design can indeed last for many years before the RP2D is selected and often undertreat patients enrolled at the beginning of the trial (particularly in adults when the starting dose level is well below the MTD).[7] For example, in the Phase IB of the Trial ITCC-059 presented here, 20 of the 30 patients enrolled were treated below the RP2D, and seven were treated at three dose levels (0.8 mg/m²) below the RP2D (1.8 mg/m²). In this regard it is worth noting that usually the starting dose level for pediatric studies is approximately 80% or even 100% of the MTD in adults, and given the high correlation between pediatric and adult MTD (r=97%), this makes unlikely that children will be treated at a completely ineffective dose, even if lower than the final RP2D.[28] Another limitation of the 3+3 (and its variations) is that the goal is always to find the *maximum tolerated dose*, assuming a "*naïve*" linear positive relationship between the dose and probability of toxicities and between the dose and probability of response. This is probably an heritage from a past in which most drugs tested were non-specific chemotherapeutic agents. But the paradigm "*the more the better*" makes less sense in the era of targeted therapy and precision medicine (despite might still have relevance for some ADCs containing cytotoxic agents like InO). A more applicable goal may be to find the *optimal biological dose* (the lowest dose providing the highest rate of efficacy while being safely administered) and not the *maximum tolerated dose*, as advocated for example by Paoletti et al.[30,31] Finally, another aspect to consider is that, at least from a theoretical viewpoint, in traditional dose escalation designs, the decision to escalate to the next dose level is based mostly on the current cohort, what happened before is only partially accounted for. For example, escalating to dose level 3 having observed one DLT at dose level 1 and another DLT at dose level 2, is treated exactly the same way as a scenario in which no DLTs were observed in any of the previous dose levels. Moreover, the decision to escalate to higher dose levels is based on a small sample size which

makes the uncertainty regarding this decision rather high. For example, it is possible to escalate to the next dose level if zero DLTs in three patients occurred.[29] In this latter case though, the 95% confidence interval for the DLT rate is 0% - 71%. In other words, we do not really know if the drug is tolerated or not. In practice however, clinical trials usually have a Data and Safety Monitoring Board and/or a Streeting Committee which regularly monitor safety data and can overrule escalation/de-escalation design algorithms, for example in cases when severe toxicities are observed beyond the DLT evaluation period, or multiple AEs occurs in the first cycles despite not fulfilling completely the DLT definition.

So what are the alternatives? Among the relatively well known early phase designs, Continual Reassessment Method (CRM) designs are probably those applied for longer as they were developed by O'Quigley et al. in the 90's.[32] The idea is simple, instead of assigning the next patient to a certain dose level based only on the proportion of patients with DLTs at the current dose level, the researcher specifies a dose-toxicity function that best describes the relationship between the dose and the probability of toxicities and then selects a target toxicity rate considered "*acceptable*". The function is allowed to be non-linear but is till restricted to be monotonic and positive in most cases. Then the parameter(s) of the function are estimated using the *maximum likelihood method*.[32] Information is borrowed across dose levels as more and more patients are enrolled, and the model is reassessed as new information (patients) comes in. Once the required level of precision in the estimate of the MTD probability is achieved, or safety constraints are met, the accrual is completed and the MTD is declared.[32] This approach is more flexible compared to rule-based models and therefore deals better with the uncertainty that investigators face at the beginning of the trial.[32,33] In addition, the information coming from all patients is integrated in one estimate with a gain in terms of precision and confidence interval when compared to rolling-6 (caveats with the application of this approach in pediatrics are presented later in this section).

Since its first proposal over 30 years ago, CRM has been readapted, modified and refined and other model-based (or "model-assisted") designs were proposed. Over the years, as new drugs became less and less toxic, the need to incorporate some measurement of efficacy in the decision making process of phase I trials became clear. This might be the case of CML, a chronic disease for which three or four TKIs are already available in children and all have different toxicities profiles but relatively high levels of efficacy and good tollerability.[34]

An interesting design proposed by the MD Anderson Cancer Center is the Bayesian Optimal Interval (BOIN) design and its variants.[35] The idea is to reproduce the simplicity of a rolling-6 while retaining the operational characteristics of CRM designs. The BOIN designs family also allows to incorporate in the selection of the RP2D a measurement of clinical utility which is based on clinical judgment and to select the *optimal biologic dose* based on a toxicity-efficacy trade-off which can be tailored to the disease under study.[36,37] This last feature appears particularly useful for at least two reasons. First, it allows to introduce expert opinion into the model in a formal way. And in a discipline with limited data like pediatric oncology this might

be the most practical solution for very rare diseases. Secondly, it allows to tailor the design to the disease. The probability of treatment response might indeed assume a different relevance whether the disease is life-threatening or chronic. Consequently, the utility-based approach, in which clinicians weight costs and benefits of each of four possible clinical outcomes (efficacy with/without toxicities and inefficacy with/without toxicities), adds an additional level to the model for the selection of the RP2D. The BOIN design can then be extended in different forms to include time-to-event outcomes and personalized utility functions to the decision algorithm.[38,39]

Introducing the variable "time" into the decision-making process of the RP2D might also be relevant. Trials with TKIs have pointed out at the relevance of long-term toxicities. For example, in the phase I trial testing ceritinib in adults with ALK positive non-small-cell lung cancer, 62% of the patients treated at the MTD required a dose reduction after cycle three.[40] Other interesting insights come from a review which analyzed data from 445 patients treated with molecular target agents for a total of 1566 cycles. In total, 57% of grade 3 and 4 toxicities occurred after cycle one, and 50% of patients presented their worst-grade toxicity after cycle one.[41] For these reasons, the European Organization for Research and Treatment of Cancer (EORTC) arranged a DLT and toxicity assessment recommendation group for phase I/II studies of targeted therapies (DLT-TARGETT) recommending the use of pooled data from all cycles to establish the RP2D.[42] Also the ITCC has advocated for a redefinition of RP2D criteria for molecularly targeted agents, and in particular for the need of assessing toxicity data collected beyond cycle one.[43] Also in our bosutinib trial five patients (18%) discontinued the treatment for tolerability issues beyond cycle 1.

Obviously these innovative designs carry a significant amount of complexity into clinical research that requires statistically trained research personnel, but the benefits seem to outweigh the cons under certain circumstances.[44] First, the fact that most drugs have already been tested in adults when investigated in children may create conditions to combine previous knowledge with new data under a Bayesian framework (particularly when the research team is able to get access to the adult data). Second, the literature on simulation studies (in particular from the adult population) tends to corroborate the fact that the performance of model-based designs, based on CRM and Bayesian methods, are superior in terms of operating characteristics to more simplistic rule-based approaches, such as rolling-6, which main advantage is to be computationally economic. More precisely, model based designs have been shown to be more precise at selecting the correct dose, to be shorter (in some cases even more than one year shorter) than traditional rule-based approaches in case of testing several dose levels, and also to increase the probability of approval when the new drug is more effective or less toxic.[45–47] Of interest is the fact that longer duration of rule-based design seems attributable also to the need of amending the protocol or adding intermediate dose levels, reflecting a more rigid structure of the protocol when compared to adaptive designs. Indeed, in absence of such delays, rule-based design might last no longer than other designs.[45]

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On the other hand, it is worth mentioning also some limitations of applying model-based design in pediatric phase I trials. Probably, the limited number of dose levels normally tested in pediatric phase I trials represents a major one. As literature shows, pediatric phase I trials usually test a limited number of dose levels which tend to cluster around three or four, while in adults the range is on average much larger (six to ten).[28] For example, when comparing the CRM and the 3+3 method for a number of dose levels inferior to five, the two designs tended to yield similar performances in terms of time to reach the MTD and total sample size.[48] Another issue might be the fact that in a Bayesian framework, the small sample size of pediatric trials will determine an "*overweight*" of the prior which in this case is usually based on adults. To prevent that large differences between adult and children might be overlooked, in cases when the data collected from pediatric patients and the prior distribution are significantly discordant, more weight should be given to the data (robustness with respect to the prior). In order to reflect the conditions under which most pediatric dose-finding trials are conducted, Zocholl et al simulated the operating characteristics of 1 and 2 parameters CRM designs assuming small sample sizes (n=10) and a limited number of dose levels (n=4, 70%, 100%, 130%, and 160% of the adult MTD). The simulation showed that this approach might be applicable to pediatric studies as well. It is therefore important to investigate further the applicability of these new statistical methods in pediatric early phase clinical trials as most evidence is currently confined to simulation studies rather than real-life applications.[49]

A second major challenge that is only partially addressed by most early-stage pediatric trials is the large variability in terms of drug disposition that is sometimes observed in the age range defined as "*pediatric*". Enrolling in the same phase I trial children with age ranging from 1 to 21 years requires to account for the fact that the metabolic processes the compound undergoes might differ greatly among patients. For example, for agents administered orally (e.g. TKIs such as bosutinib, cabozantinib, crizotinib, dabrafenib, gefitinib, ponatinib, sorafenib), the intraluminal pH can affect their bioavailability, with infants having a less acidic lumen (pH > 4) compared to older children.[50,51] As the liver is the main site of metabolism and excretion for many drugs including TKIs, the development of hepatic function might also affect the disposition of drugs. Particularly the expression pattern of P-450 cytochromes, and to a lesser extent phase II enzymes, is known to vary over the first years of life.[50] Similar considerations can be made for the body composition, with a higher percentage of total body water in the first year, and for the development of the renal function, despite the latter tend to stabilize at adult-like values already at 8-12 months of age and therefore both parameters (total body water and renal function) are mostly relevant for infant patients which are usually treated separately under dedicated protcols.[50]

In this sense, in silico PK simulation studies have the advantage that they can be performed before testing the drug on children, and can inform about the optimal starting dose level. TKIs represent an example of compounds to which this approach can be applied successfully. As TKIs target specific molecular structures, such as the BCR-ABL transcript, the dose-response and dose-toxicity relationships can be partially derived from previous clinical trials, and PK parameters

(for example the trough concentration) can be used to guide the RP2D selection.[52,53] An example is the trial ITCC-054/COG AAML1921 presented in chapter 5, where a specific AUC target was used to defined the RP2D for newly diagnosed and for R/I patients (chapter 6). As a dose-response relationship was not characterized yet at the time the trial was designed, the target concentration in children was therefore defined as the AUC achieved in adults at the approved dose of bosutinib, allowing for a 20% up and down margin. Starting from adult data collected across three trials with bosutinib, allometrically scaled PK pediatric parameters were calculated. Initially, the 500 mg/day adult dose was simply transformed in 300 mg/m^2 in children, therefore starting at 100% of the RP2D in R/I adults. Subsequently, the AUC for three dose levels (250, 300 and 350 mg/m^2) were simulated and the fraction of simulated trials yielding an AUC in the target range (power), assuming a sample size of 6 to 10 patients per dose level, was calculated.[54] Then alternative sampling schedules and cohort sizes were simulated to maximize the proportion of trial simulation producing an exposure within the target range for each dose level. From here the justification to use 6 + 4 design rather than the traditional rolling-6.[54] As clinical research is mostly empirically driven, simulations can aid rational decision making and help conduct "*what if*" analysis preserving the safety of patients and shortening the development of new drugs.

Finally, it remains an open question whether we still need to develop pediatric drugs in a consecutive manner after adult trials are completed, or if we might opt for a parallel development approach. For example, by developing protocols inspired to the Master Protocol philosophy which contemplate pediatric testing since the inception of the trial in adults, for instance extrapolating pediatric doses from adult PK analysis as soon as a sufficient sample size in older patients treated at the RP2D is reached. There are several examples of recent studies where the age inclusion criteria is dropped after the adult phase I data are collected, for example the Augment-101 trial (NCT04065399) testing revumenib (menin e KMT2A inhibitor), and trial NCT05086315 testing the CD123-NK cell engager SAR'579. This is also in line with the FAIR principles (foster age-inclusive research) advocated by a Working Group of the ACCELERATE platform aiming at increasing the access of adolescents and young adults to innovative treatments as soon as available.[55] Secondly, it might be worth exploring the possibility to combine pediatric phase I and phase II, having efficacy and tolerability as a co-primary objective. In this regard, current designs offer two main options, either a dose-escalation stage followed by an expansion cohort (with and without re-assessment of the MTD in the expansion cohort), or one stage efficacy–toxicity designs (e.g. BOIN-I/II). Applications of this strategy in pediatrics might be represented by the AcSe′-ESMART trial mentioned earlier, in which ten parallel arms are designed with a dose-escalation stage based on the CRM principles, followed by an expansion cohort assessing preliminary efficacy.[56] Interestingly, the dose-escalation part, is integrated in a three stage Ensign's design, the facto combining a dose escalation phase I trial with a Simon's two stage phase II trial.[57,58] Indications on which approach might work better in pediatrics are limited to simulation studies, which seems to show that the therapeutic window should inform the selection of the design.[59] For drugs with a narrow therapeutic index, sequential designs (like the AcSe′-ESMART

trial) perform better and in particular those with re-assessment of the MTD in the expansion cohort.[59] While one stage designs, based on efficacy–toxicity trade-off, seem more appropriate for drugs with larger therapeutic index and a well-established safety profile.[59]

6.2 Future Perspectives in Relapsed/Refractory Pediatric ALL

ALL is a rare disease overall, but among pediatric malignancies is the most common, and relapsed ALL is more common than many other newly diagnosed cancers in children.[60] Over the last five decades, research brought the overall survival of children with ALL from 20% to 90%, but given its incidence, ALL still accounts for most deaths due to cancer in pediatrics.[61,62] Certainly, the margin for improvement has shrunk, but progresses can still be achieved by testing new combinations of existing drugs, refining the risk stratification, and identifying new molecular targets. A second fundamental goal is to diminish the therapy intensity for those that can easily be cured with current ALL therapy, and who need replacement of toxic therapeutic agents by less noxious but equally effective strategies.

6.2.1 Inotuzumab Ozogamicin Combined with Blinatumomab and Chemotherapy

The evidence presented in this thesis (chapter 2, and 3) corroborate the efficacy and tolerability of InO in heavily pre-treated children. Nonetheless, there are still open questions regarding how to use InO in combination with other drugs and whether it is efficacious also in newly diagnosed patients. Indeed, InO has to be collocated in a landscape that already includes chemotherapy and other immunotherapies like blinatumomab and CAR-T cell therapy.

As explained in the introduction, blinatumomab showed its superiority to standard chemotherapy, both in front-line (added to chemotherapy in infants) and re-induction settings, but in the former case the remission rate remains confined in the 40% range.[63,64] On the other hand, InO has shown that is very effective as (re-)induction drug, with remission rates approximating 80% (chapter 2 and 3) with 70-80% MRD-negative CR rates in heavily pretreated patients.[65] In addition, there seems to be a correlation between tumor burden and toxicities induced by blinatumomab. For example, in patients with more than 25% blasts in the bone marrow, neurological toxicities were experienced by 24% of patients while cytokine release syndrome by 11% of patients.[63] Differently, the drug is better tolerated in consolidation settings in patients which already are in morphological CR (<5% blasts).[66,67]

Is it possible to combine these two drugs? In which patients? Data from ALL adult patients already point at this possibility where, due to the poor outcome of especially ALL in the elderly, pilot studies are more feasible than in pediatrics, where the outcome is already very favorable. For example, the M.D. Anderson Cancer Center is testing hyper-CVAD (hyper-fractionated

cyclophosphamide, vincristine, doxorubicin, and dexamethasone) with sequential blinatumomab with or without InO (NCT02877303) in newly-diagnosed ALL adult patients and adolescents aged 14 years and older. Preliminary results from the latter study showed that all patients in the InO group were alive and in remission at one year.[68] In addition the same center is planning to test mini-hyper-CVD (cyclophosphamide, vincristine, and dexamethasone), intensified IT therapy, plus rituximab, InO, and blinatumomab (cRIB) in R/I children (NCT05645718).[69] Results are, at the moment, limited but point out that mini-hyper-CVD with cRIB (InO at 1.2 mg/m^2/cycle: 0.6 + 0.3 + 0.3 mg/m^2) might be well-tolerated also in heavily pretreated pediatric patients.[65] In adults, mini-Hyper-CVD was administered with InO at a dose of 1.3 - 1.8 mg/m^2 in cycle 1, which was later amended to lower dosages to mitigate the risk of liver toxicities.[70] Rituximab was added in CD20+ patients only (which occurs in approximately 40-50% of children), and patients subsequently received consolidation with blinatumomab. The combination yielded a remission rate of 89%, and the five-year progression-free survival was 44.0% (95%CI: 31.2 - 54.3), in elderly newly diagnosed patients (n=80, median age 68, IQR: 63-72); while in younger subjects (n= 31, median age 25, range: 18-57) the remission rate and one-year OS probability were both 100%, although three patients (10%) had isolated CNS relapse (NCT01371630).[69]

Another research question concerns the role of traditional chemotherapy. In upcoming trials, InO will be compared to standard chemotherapy (UKALL -R3) in a phase III randomized trial for first relapse high-risk ALL children who did not receive HSCT or CAR-T therapy before (NCT05748171). In adults, InO is also being tested in newly diagnosed CD22+ ALL patients (18-39 year-old) combined with chemotherapy (NCT03150693), but the study was recently halted due to safety concerns. In older patients, which do not tolerate multi-agent chemotherapy, there are attempts to develop "*chemotherapy-free*" regimens based on InO. Trial NCT03739814 tests InO in induction followed by blinatumomab as consolidation in patients > 60 years.[71] This strategy though is not currently pursued in children, where the role of standard chemotherapy seems still relevant given the current survival rates which make it more difficult to perform chemo-free or highly experimental pilot studies compared to settings in the elderly. Indeed, while in pediatrics we also have examples of InO used in front-line, it is not in the induction phase but rather in consolidation as an add-on to standard chemotherapy. For example, the COG AALL1732 trial will test InO added post-induction (arm III) to COG-modified BFM chemotherapy versus chemotherapy alone, for high-risk newly diagnosed ALL pediatric patients (NCT03959085). Specifically, two cycles of single agent InO at 1.2 mg/m^2 will be given after standard induction and post-induction chemotherapy. Following consolidation, patients with MRD > 0.01% were randomized 1:1 (n=50) to chemotherapy (Arm A) or chemotherapy plus 2 cycles of InO (Arm B), one before the high-dose methotrexate interim maintenance and the other before proceeding to the delayed intensification blocks.[72] From an interim analysis, no differences in grade ≥3 ALT or bilirubin elevations were recorded between arm A and B, but patients treated with InO showed a significant higher incidence of neutropenia (87.5% vs 50%) and sepsis during delayed intensification (10 cases in arm B, 1 case in arm A), as well as SOS (4 cases in arm B, 0 in arm

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A). The enrolment was halted and treatment was amended to mitigate toxicity during post-InO chemotherapy blocks.[72] Similarly, the "ALLTogether1" group will test InO, in a front-line setting, as additional consolidation treatment (arm R3-InO) before the maintenance chemotherapy block in intermediate-high risk patients (NCT04307576).[73] Subjects randomized to InO will be treated with two cycles of 0.5 mg/m^2/week for six weeks.

Taken together, these evidence underscores the new paradigm for ALL treatment which is going to rely less on chemotherapy and more on immunotherapy (and ADCs like InO) combined with targeted therapy (for example TKIs in Philadelphia-positive ALL or menin inhibitors in *KMT2A*-rearranged positive cases). Potentially, this might also shape a chemotherapy-free future for pediatric patients with ALL.

6.2.2 The Role of CAR-T in R/R ALL Patients

CAR-T cell therapy is probably the other game-changer in ALL. Studies already pointed at a high efficacy of this therapy with MRD-negative remission rates around the 80% area in CD19+ relapsed or refractory B-cell ALL pediatric patients treated with tisagenlecleucel, although relapsed do occur, with currently 44% EFS probability at three years.[74,75] Initially, CD19 was selected as target over CD20 and CD22 based on its relatively higher expression in B-cell malignancies and results obtained in animal models.[76,77] Currently, also other targets are being researched, such as CD22 directed CAR-T therapy (NCT02315612) which has been proven effective (remission rate was 70%) also in children who already relapsed after CD19 CAR-T therapy, despite a relatively short median relapse-free survival (6.0 months; 95% CI: 4.1 to 6.5 months).[78] Indeed, among the major limitations of CD19 directed CAR-T cell therapy there is the fact that CD19 negative relapses occur as well as CAR T-cell loss/lack of expansion.[79] The problem of CD19-negative relapse is currently addressed in trials by either infusing CD19 and CD22 directed CAR-T cells (sequentially or mixed in the same cocktail), or by designing tandem CAR-T cells which connect two single-chain variable fragments (binding different epitopes) on the same transgenic receptor.[80] Data collected from trials adopting CD19 directed CAR-T cell therapy (NCT03919240), and compared to trials opting for a CD19/22 tandem CAR-T cell strategy (NCT03614858), seem to show a higher remission rate with the latter CAR-T cell product (98.0%, 50/52 vs 83.0%, 122/147; p=0.006).[81]

It remains to be established which strategy will yield the most favorable long-term EFS probability, especially in pediatrics, where the follow-up is currently relatively short and published data from most CAR-T trials in children seem to point out at a clustering of relapses within the first two years.[82] In this context, new strategies to prevent these early events are needed. Understanding of antigen escaping mechanisms might help to tackle the problem. For example, using animal ALL models and CD19 CAR-T cells, it has been observed that tumor cells might be able to decrease the density of the CAR-T target on their surface by transferring it to the T cells (trogocytosis) and inducing fratricide killing.[83] Other mechanisms include either the

selection of cells missing the extracellular antigen/epitope targeted by the CAR-T cell product (e.g. CD19) already present at diagnosis, or the acquisition of *de-novo* variants generated via alternative splicing mechanisms.[84] Targeting multiple antigens simultaneously (e.g. tandem CAR-T cells), developing high-affinity CARs, or even increasing target density on the tumor cell surface (e.g. CD22 stimulation with Bryostatin1) might mitigate the occurrence of early relapses with CAR-T cell therapy.[84,85]

A second aspect of using CAR-T cell products, even if less problematic than averting early relapse, is the risk management of cytokine release syndrome (CRS) and neurological side effects in general. At present it is mostly managed with anti-cytokine (tocilizumab) or corticosteroid therapy, but can also be controlled by lowering disease burden prior to cart infusion.[86] As observed with blinatumomab, also CAR-T shows a correlation between probability of adverse events and tumor burden. In overt disease settings, CRS was experienced by 80%-90% of patients, neurologic toxicity up to 40% and 20%-40% required intensive care.[87,88]

Finally, while CAR-T cell therapy has its current main application subsequent to debulking treatment and often after HSCT, particularly in those patients already transplanted or unfit to stand a conditioning regimen, another potential use of CAR-T cell therapy might be as replacement of HSCT. Currently many physicians opt for first treating with CAR-T and rescue by HSCT in those that subsequently relapse. Better strategies will strongly depend on the development of new CAR-T cell products able to induce a sustained remission and potentially "*cure*" the cancer in larger percentages of children. The main advantage of this approach would be to spare the long-term toxicity caused by HSCT, despite having risk of complications such as hypogammaglobulinemia, and potentially other long term side effects currently not yet known. For example, the FDA has recently issue an investigation over the risk of secondary malignancy following BCMA- and CD19-directed CAR-T cell treatment and suggested life-long monitoring for new malignancies in treated patients.[89]

The possibility to solve these challenges will also depend on the availability of CAR-T products for academic research groups, currently limited also due to the fact that the European legal framework requires pharmaceutical licensing of CAR-T-cell products which might not align with the financial needs of private manufactures. In these regard, alternative models were proposed in which academic institutions might act as producers of CAR-T products, either in a centralized or decentralized framework.[90] In the first case, one single hospital performs leukapheresis, production, administration of the CAR-T cells, patient management, and follow-up. Patients are therefore referred to this center that acts as a hub and as license holder. The second model can be defined as a "*decentralized academic manufacturing and distribution platform*" in which academic hospitals produce the CAR-T product and distribute it to the hospitals part of the network which then administer the product and manage the patients (in this case the producing hospital basically functions as the current standard industry manufacturers). Despite this models might overcome the current lack of interest for projects with limited financial scope (for example

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rare pediatric tumors), also academic institutions will be in need to find alternative funding strategies to carry on CAR-T production, including product licensing and post-marketing tasks.[90]

6.2.3 Beyond CD22 and CD19: Alternative Targets

In addition to test different combinations with the already existing agents, research is also investigating alternative targets other than CD22 and CD19. Among them there are for example CD38 and CD123. CD38 is expressed by ALL and AML cells.[91] Daratumumab is a cytotoxic agent directed against CD38, initially developed for multiple myeloma, but now being tested in combination with chemotherapy also in T-cell ALL pediatric and young adult patients (NCT03384654).[92] Preliminary data from the DELPHINUS study, conducted in R/R T-ALL and lymphoblastic lymphoma patients aged 1-30 years, reported a CR rate of 83.3% in pediatric ALL and 40% in lymphoblastic lymphoma patients.[92] Isatuximab is another potentially effective drug that targets CD38 and is under investigation in children with first or second relapse T-ALL, B-ALL and AML and in adults usually administered after Daratumumab. An updated interim analysis of the ISAKIDS trial based on the first 67 patients (27 B-ALL, 13 T-ALL, 27 AML) showed that remission was achieved by around 50% of patients in all cohorts, namely 52.0% in the B-ALL cohort, 45.5% in the T-ALL cohort, and 60.9% in the AML cohort.[93] Therefore, efficacy did not meet prespecified criteria to proceed to stage two and the trial will not go further.[93] ADCs directed against CD123 are investigated in adults with AML, alone and in combination with venetoclax and/or azacytidine (NCT04086264 and NCT03386513) and might in future also be used in pediatric patients which express CD123.[94,95] Indeed, higher expression of CD123 in pediatric AML seems to correlate with a higher prevalence of high risk genetic abnormalities such as *KMT2A* rearrangements and *FLT3-ITD* mutations, and also with lower EFS and OS.[96] CD123 directed CAR-T cell products have been produced and are being tested in children with AML (NCT04318678).[97] By contrast, CD123 seems to correlate with a better prognosis in ALL patients and therefore it might be a less attractive target for this form of cancer.[98,99]

6.2.4 The Role of Risk-Stratification

In terms of risk stratification, most trials opened until 1995 stratified risk based on age and WBC counts.[100] Despite still possible to call at "*standard risk*" a patient aged 1 to 10 years and with WBC of <50,000 per cubic millimeter at diagnosis, it became necessary to also distinguish risk among those relapsing. The time to relapse, the site of relapse, and the immunological lineage of the disease (B-cell precursor versus T-cell ALL) are widely used prognostic factors.[101] IntReALL risk stratification classifies patients with relapsing BCP-ALL as "*standard risk*" when patients have a late relapse (≥ 6 months after completion of primary therapy) or early relapse (>18 from diagnosis and < 6 months after completion of primary therapy) with isolated EM or combined EM/BM relapse; and "*high risk*" those with very early relapse (< 18 months from diagnosis) regardless the site of relapse and those with early isolated BM relapse (see table below).[101] While patients with T-cell ALL are considered at "*high risk*" unless have early/late isolated EM relapse.[102,103]

Not all these risk groups have achieved a satisfactory expectancy of cure nor shown a significant OS improvement in the last 10 years. Patients with very early relapse still show a 10-year EFS of around 15% and those not achieving remission during second induction also have dismal chances of surviving.[104,105] Furthermore, some risk stratifications used outside clinical trials do not consider known genetic mutations which confer higher risk of relapse and death.

Namely, ALL with *KTM2A/AF4, E2A/TCF3-PBX1, E2A/TCF3-HLF* rearrangements, severe hypodiploidy and/or TP53 mutations seem to confer particularly low probability of sustained remission and therefore can be used to distinguish these patients at "*very-high risk*".[106–109] For this reasons, and based on a post-hoc analysis of the ALLR3 and ALL-REZ BFM 2002 trials, the IntReALL group has recently proposed a new risk-stratification for the previously "*high-risk*" BCP-ALL patients with isolated BM or combined EM/BM very-early relapse and those carrying high risk mutations regardless the time of relapse. These patients are now considered "*very-high risk*" (see table below) and are currently enrolled in the stratum III of our InO/ITCC-059 trial which was added to the protocol via an amendment in March 2022 and that are being treated with a single agent strategy at 1.8 mg/m²/cycle, ideally followed by CAR T-cell therapy to test a new paradigm of treatment.

Table 1. Risk Stratification IntReALL 2020 and Applied to Trial ITCC-059 stratum III

Immunophenotype B-cell precursor ALL			
Time point and cytogenetic characteristics vs Site of relapse	Isolated EM relapse	Combined BM/ EM	Bone marrow isolated
Very early < 18 months after initial diagnosis	HR	**VHR**	**VHR**
Early ≥ 18 months after initial diagnosis and < 6 months after completion of initial therapy	SR	SR	HR
Late ≥ 6 months after completion of initial therapy	SR	SR	SR
Presence of **TP53 mutation and/or deletion Hypodiploidy(< 40 chromosomes) t(1;19) TCF3-PBX1 or (17;19) TCF3-HLF KTM2A/AF4**	Refer to time point of relapse	**VHR** Independently from timing	**VHR** Independently from timing

VHR: Very High Risk

6.3 Future Perspectives in Pediatric CML

The treatment of CML, in both adults and children, was changed by one of most important drug discoveries of recent years, imatinib (and TKIs in general), and today both populations have access to an expanding list of these compounds which represent the first-line treatment of CML and other types of cancer.

A current area of intense research is whether or not the possibility of discontinuing the treatment in those patients showing a deep and sustained molecular response exist. Indeed, if TKIs are effective and less toxic than chemotherapy, they require prolonged administration. Interrupting TKIs in CML has been proven possible in adults, and some (for example Mahon et al, 2017) also proposed this criterion as an end-point for future trials.[110,111] But it remains to be proven whether stopping treatment can be an option in children. On the one hand, they would benefit the most from this approach, due to their longer life expectancy, but on the other hand, they are also exposed to the risk or relapse for a longer period. In adults, the European Stop Kinase Inhibitor (NCT01596114) study analyzed 755 adult patients and showed that interrupting treatment in those on treatment for at least three years of which at least one in deep molecular response is safe when the patient is monitored closely. The trigger to re-start the treatment is an increase in BCR-ABL1 transcripts >0.1% IS.[111] It was shown that approximately 50% of patients will relapse within six months from treatment interruption with no significant difference among different TKIs.[111]

In children, encouraging evidence were observed in small pediatric cohorts (n=14) already more than five years ago, and were conformed more recently in slightly larger studies (n=22) in which, after achieving molecular response 4.0 (MR4.0) and maintaining it for ≥ 2 years, the treatment-free remission (TFR) rate at 12 months was 50.0%, and all those who lost the MR4 response regained it after treatment was restarted.[112] Nevertheless, additional evidence from the pediatric STOP IMAPED study showed that the molecular relapse-free survival at six months from treatment interruption (imatinib) in those that previously maintained MR4 for two years was just 29%.[113] Therefore, the question on discontinuing TKIs in children remains fundamentally unanswered, but at least for imatinib it seems that most patients might not be eligible for treatment interruption or will anyway need to re-start the treatment at a certain point. Of interest will be the results of the COG sponsored trial NCT03817398, which will aim at determining the two-year TFR rate of children, adolescents, and young adults (< 25 year-old) affected by CML which, under any TKI, maintained MMR for at least two years at time of screening. The trial is expected achieve completion in 2026.

The other big challenge in the treatment of CML in pediatrics is how to curb the side effects of TKIs related to longitudinal growth. It is well known that the administration of TKIs during the developmental age, and the growth spurt in particular, can negatively affect the longitudinal growth of bones.[114,115] This seems to be correlated to an off-target binding of the TKIs to several receptors implied in the regulation of the bone matrix metabolism as well as hormonal signaling disruption.[116,117] Bosutinib seems to have a more tolerable profile from this point of view, as it has low affinity for c-KIT and for PDGFR, and animal models showed limited or no statistically significant impact on bone growth compared to controls, and anyway better results when compared to imatinib and second generations TKIs.[118] This needs confirmation in human subjects and particularly in newly diagnosed patients. Possibly, insights will emerge from the newly diagnosed patients currently enrolled in the ongoing trial ITCC-054/COG

AAML1921 discussed in chapter 5. If pre-clinical data are confirmed, this aspect might play a big role in choosing bosutinib as first line treatment over the other TKIs for children and adolescents. However, alternative dosing regimens different from the one now tested in the ITCC-054/COG AAML1921 study may be needed – as reported for adults – for example with ramp-up dosing for better gastro-intestinal tolerance.

Another open question is the role of HSCT in CML. Currently, this treatment is used in patients with blast crises or in case of a T315I mutation, or when TKIs treatment fails. The reluctancy to use HSCT in these cases is related to its morbidity and mortality. Nevertheless, when successful, HSCT offers a possibility of definitive cure for children with CML, while TKIs require long-term administration and, potentially, life-long. Two diriment facts can aid at establishing the role of HSCT. First the results from trials stopping TKIs and calculating long-term relapse-free survival rates. Secondly, little is known about long-term toxicities in children in which virtually all second generations TKIs were approved less than 10 years ago. If TKIs should show some toxicities in the long term, this might change the role of HSCT.[119]

In terms of compliance, it is important to deliver drugs that can be dosed once daily and with an absorption minimally affected by food intake. For example, while nilotinib showed to be effective in the DIALOG trial (NCT01844765), it does require *bis in die* administration on empty stomach making its long term use difficult to comply with, especially in younger children.[120] All other TKIs require daily administration and some, for example bosutinib, have an absorption influenced by food intake and gastric pH levels which is affected by commonly used co-medications such as proton pump inhibitors.[121] Developing TKIs with a less frequent administration pattern might be helpful to increase the compliance to the treatment which, in children, is bound to last years.

Finally, it is worth mentioning how the landscape of TKIs is expanding rapidly. Currently, two other 3rd generation TKIs under investigation in pediatrics. Namely, a phase I pediatric trial (NCT04925479) of asciminib (targeting the ABL Myristoyl Pocket) and another phase I/II trial testing ponatinib (NCT03934372).[2,122] Furthermore, another molecule, olverembatinib, has recently developed in China, a BCR-ABL1 inhibitor effective against several mutations including T315I. Olverembatinib was approved in China in 2021 for TKI-resistant chronic-phase CML or accelerated-phase CML harbouring the T315I mutation, based on the trials NCT03883087 and NCT03883100 in which approximately 50% of patients achieved CCyR.[123] A trial in patients with CML or Ph+ ALL is ongoing also in the US (NCT04260022).

In adults, the phase III ASCEMBL trial reported a MMR rate after six months of 25.5% with asciminib and 13.2% with bosutinib, and treatment discontinuation due to toxicities occurred in 6% and 21% cases respectively.[2] Ponatinib is effective in patients carrying the T315I mutation, but at the expense of more frequent cardiovascular events than with other TKIs, particularly arterial occlusive events. As showed in the PACE study (NCT02467270), and confirmed in the OPTIC study (NCT02467270), both performed in adults resistant to at least

two other TKIs or carrying the mutation T315I.[124,125] In the OPTIC study, the achievement rate of MR3 at 12 months was 44.1% (95%CI: 31.7-57.0) in the 45-mg cohort, 29.0% (95%CI: 18.4-41.6) in the 30-mg cohort, and 23.1% (95%CI: 13.4-35.3) in the 15-mg cohort.[125] Treatment-emergent arterial occlusive events were observed at all dose levels, but slightly less frequently at lower dosages (9.6%, 5.3%, and 3.2% for the 45-, 30-, and 15-mg cohorts, respectively), despite a higher rate of discontinuation for lack of efficacy in the 15-mg cohort compared with the 45-mg cohort was observed.[125] Nevertheless, the highest dose level yielded a 26.3% improvement in the response rate when compared to the lowest (25.3% to 51.6%).[125] therefore, the current dosing strategy recommends a 45-mg starting dose and then decrease to 30 and 15 mg once response is achieved.[124,125] Using it at a higher dose for a shorter course to induce remission prior to HSCT is also a viable strategy in children with a T315I mutation, although cardiac monitoring for ejection fraction is warranted.

In summary, long-term data might help to clarify the efficacy of TKIs as curative alternative to HSCT. While, to understand the effect of bosutinib on growth, we need to wait for the data from the phase II arm of trial ITCC-054/COG AAML1921 in newly diagnosed patients.

6.4 Conclusions

The studies presented in this thesis showed some of the developments in treating leukemia in children. They portrayed which challenges this field presents, and possible approaches to overcome them.

From a research methodology perspective, working with limited sample sizes is a challenge that today still does not receive sufficient attention. Therefore, innovative trial designs are needed and the once recently developed (e.g. BOIN) need to be promoted and popularized. Dose selection strategies might need a rethinking, for example relying more on adult PK data when the drug is tested in this population first, but also considering abandoning the sequential development (first adults then children) in favor of a joint approach.

Secondly, it also emerged how fruitful global collaboration can be in terms of accelerating drug development. Not only collaboration among research groups, but also among regulatory authorities as well as between academia and industry. These efforts, together with new regulatory incentive systems (as described at the beginning of this chapter), can support a faster drug development in pediatrics. Such multistakeholder meetings may become the cornerstone of the new Pharmaceutical Legislation which is under consideration in Europe.

With regards to ALL, the next step will be to move the newly developed agents (for example InO) in front-line settings where their efficacy compared to standard chemotherapy has not been tested yet. Furthermore, attention should be paid on developing new CAR-T cell therapies targeting different and multiple antigens with the intent of preventing early relapse. In the end, preventing relapse rather than curing it, with minimal long-term toxicity, is the ultimate goal.

In the CML field, the portfolio of TKIs is expanding and is currently counting on third generation TKIs which already proved themselves particularly effective in adults (e.g. asciminib). Challenges for pediatrics mostly articulates in three domains. The first is to bring to the market agents which do not impact on longitudinal growth. The second is to invest in child-friendly formulations to improve compliance with the therapy. The third is to establish the long-term safety and efficacy of second and third generation TKIs and, the potential to have treatment-free intervals, or when that is not possible, to re-assess the role of HSCT in selected cases.

Overall, the outlook for pediatric hemato-oncology is expected to be less dependent on chemotherapy and more reliant on targeted therapy and immunotherapy which appears less toxic but more effective.

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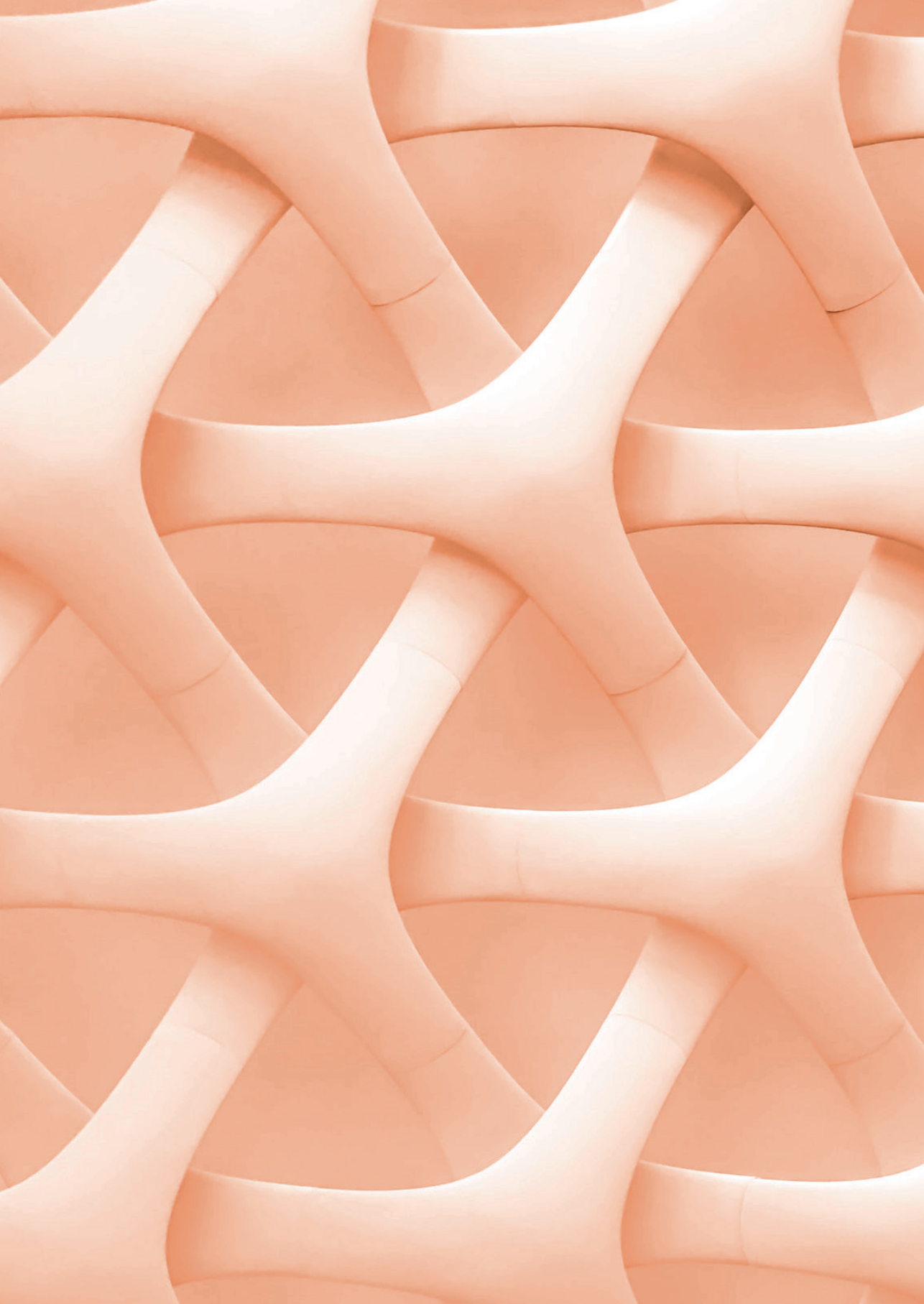
Chapter 7

Summary/Samenvatting

Summary

This thesis reports the results of early stage trials ITCC-059 and ITCC-054/COG-AAML1921 investigating inotuzumab ozogamicin (InO) and bosutinib, respectively used to treat Acute Lymphoblastic Leukemia (ALL) and Chronic Myeloid Leukemia (CML). Both studies were registrational trials developed in the context of a Pediatric Investigation Plan in collaboration with the marketing authorization holder (MAH), and with the involvement of international research centers in continental Europe and the United States.

In **Chapter 2**, the results from the phase II cohort of the ITCC-059 trial, investigating the preliminary activity of InO as a single agent for patients with relapsed and refractory CD22+ B cell precursors (BCP) ALL, are presented. While significant progress was made in treating pediatric ALL, this was achieved mostly with the refinement of chemotherapy regimens and risk group stratification rather than with the development of new agents. InO represents one of the alternatives to traditional chemotherapy for these patients, and particularly for the re-induction phase of relapsed and refractory cases. Using a single-stage design, the trial tested a null hypothesis of an overall response rate (ORR) ≤ 30% against an ORR > 55% as alternative hypothesis. To achieve 80% power at 0.05 significance level, the sample size was at least 25 patients. Thirty-two patients were enrolled, 28 were treated, 27 were evaluable for response. The estimated ORR was 81.5% (95%CI: 61.9%-93.7%), and 81.8% (18/22) of the responding subjects were minimal residual disease (MRD) negative. Therefore, the null hypothesis was rejected and the drug was considered promising. One-year event free survival (EFS) was 36.7% (95% CI: 22.2%−60.4%), and one-year overall survival (OS) was 55.1% (95% CI: 39.1%−77.7%). The cumulative incidence of non-relapse death was 22.6% (95% CI 8.8-40.2) at one year, including post hematopoietic stem cell transplant (HSCT) follow-up. The drug was generally well tolerated despite seven (25%) cases of sinusoidal obstruction syndrome (SOS) were reported; one grade 2, four grade 3 and two grade 4. Six cases occurred after HSCT post-treatment with InO, confirming data collected in previous studies. Despite virtually all patients experienced laboratory abnormalities such as white blood cell decrease, thrombocytopenia and neutropenia, these toxicities were mostly transient and rarely precluded the possibility of completing the treatment. Therefore, the data suggest that InO can be safely administered also in these heavily pretreated patients yielding remarkable response rates.

Chapter 3 elaborates on the results from the phase IB of the ITCC-059 trial in which InO was combined with a modified version of the UKALL-R3 re-induction regimen, with the aim to replace mitoxantrone, which is well known for its toxicities. InO was combined with 1.5 mg/m^2 of vincristine (on days 3, 10, 17, 24), 20 mg/m^2 of dexamethasone (two 5-day blocks, then amended), and intrathecal therapy. A rolling-6 design was used testing InO from 0.8 to 1.8 mg/m^2/cycle. Overall, 30 patients were treated, and 29 were evaluable for dose limiting toxicities (DLTs). After amending the dose of dexamethasone, reducing it to 10 mg/m^2, it was possible to escalate InO up to 1.8 mg/m^2/cycle (1.5 mg/m^2/cycle once remission is achieved). The pooled response rate was 80% (24/30; 95%CI: 61.4% to 92.3%) and, among responders, 66.7% achieved MRD negativity.

At one year, the EFS probability was 41.7% (95%CI: 27.1-64.3) and the OS probability was 62.3% (95%CI: 46.9-82.8). The cumulative incidence of non-relapse death was 10.2% (95% CI 2.5-24.3) at one year, including post-HSCT follow-up. Alanine aminotransferase increase (ALT) occurred in 23 patients (76.%) of which 15 (50%) were ≥ grade 3. Aspartate aminotransferase (AST) increase occurred in 22 patients (73.3%) of which 10 (33.3%) were ≥ grade 3. Overall, 24 (80%) patients had either AST and/or ALT elevation. Seven patients (23.3%) had bilirubin increase; of which six (20%) at grade 1-2, and one (3%) at grade 3. None met Hy's law criteria. In total, five (17%) patients developed SOS. Four following HSCT (one grade 4 and three grade 3). In conclusion, preliminary efficacy and safety data underscore the possibility to combine InO up to 1.8 mg/m^2 with vincristine, dexamethasone and IT therapy in a safe manner. Nevertheless, a noticeable advantage of this combination regimen in terms of ORR, when compared to the single agent arms of the same trial, was not observed in these heavily pretreated patients.

Chapter 4 outlines the pharmacokinetic (PK) behavior of InO as single agent. Indeed, when moving from adult to children, it is important to understand how children's metabolic system differs from adults. Genetic factors, food intake, drug formulation and concomitant medications are all factors that can alter the drug disposition. Furthermore, the pediatric population is actually a rather heterogenous group in terms of absorption, distribution, metabolism and elimination of drugs given the differences in the maturation of kidneys and liver observed in neonates (0-28 days), infants (>28 days to 12 months) and older children. The goal of this chapter is to provide an overview of the factors influencing InO disposition in children and its relationship with clinical outcomes. A published adult population PK model for InO, with a two-compartments and linear and time-dependent clearance, was adapted to describe the pediatric data obtained from the trial ITCC-059 in which ALL patients were treated with a single agent regimen. The dataset was combined with adult data provided by the MAH. For ALL patients, an increase in age was associated with a decrease in k_{des} (decay coefficient) and in CL_t (time-dependent clearance), reflecting that the target-mediated drug clearance declines more rapidly in children (possible due to a faster decrease in leukemic blasts during the first weeks of treatment). An increase in peripheral blood blasts count was related to an decrease in k_{des}; hence a decrease in the decline rate of CL_t despite its clinical relevance remains untested. In addition, patients with lower lean body mass showed lower values of linear clearance. Interestingly, cumulative area under concentration-time curve (AUC) was higher among responders (n = 42) versus non-responders (n = 10) at the end of first cycle (26.1 [IQR 18.9 - 35.0] vs. 10.1 [IQR 9.19 - 16.1], *10^3 ng*h/mL), despite the study was not powered to detect this difference. Based on simulations, we found no parameters based on which proposing dose modifications in children, and the exposure achieved at 1.8 mg/m^2/cycle appeared appropriate based on this PK model and on clinical data regarding efficacy and toxicity presented in chapters 2 and 3.

The other drug which was object of this PhD project is bosutinib. Patients with CML are now treated almost exclusively with Tyrosine Kinase Inhibitors (TKIs) since imatinib was approved for children in 2003. Among the labeled options, children can be treated with imatinib,

dasatinib or nilotinib (now also bosutinib). The main unmet medical need in this case is two-fold. First, there are subjects that develop resistance to one or more of the currently approved TKIs and that therefore necessitate alternative drugs. Secondly, all the TKIs approved have toxicities which can be intolerable for some patients which are to be treated for a long period of time. Each TKI tends to show a different spectrum of adverse events, therefore expanding the landscape of these compounds will help to meet the individual needs of the these children. Furthermore, all TKIs currently approved showed an impairment of skeletal longitudinal growth. The hope is that with bosutinib this effect will be reduced as anticipated by animal models. The results from a phase I study testing bosutinib for patients with CML which are resistant or intolerant to previous lines of therapy are presented in **Chapter 5**.

In the phase I part of the international, open-label trial ITCC-054/COG-AAML1921 (NCT04258943), children aged 1-18 with Resistant/Intolerant (R/I) (per ELN 2013) Ph+ CML were enrolled using a 6+4 design, testing 300, 350 and 400 mg/m² QD with food. Thirty patients were enrolled; 27 were evaluable for DLT: 6 at 300 mg/m² (0 DLTs), 11 at 350 mg/m² (1 DLT), and 10 at 400 mg/m² (1 DLT). The mean AUCs at 300 mg/m², 350 mg/m² and 400 mg/m² were 2.20 μg·h/mL, 2.52 μg·h/mL and 2.66 μg·h/mL. The RP2D was therefore declared at 300 mg/m² for ND patients and 400 mg/m² for R/I patients, based on AUC targets derived from adults treated at the approved dosage respectively (3.15 ng·h/mL (±20%) for R/I patients, and 2.27 μg·h/mL (±20%) for ND patients). The most common adverse events were diarrhea (93%, n=26), abdominal pain (71%, n=20), vomiting (68%, n=19), nausea (61%, n=12), and maculo-papular rash or other skin disorders (39%, n=11; and 43%, n=12 respectively). The cumulative proportions of complete hematological response (CHR), major cytogenetic response (MCyR), complete cytogenetic response (CCyR), and major molecular response (MMR) by end of treatment, as best response, were 100% (95%CI: 87.7-100), 96.4% (95%CI: 81.7-99.9), 92.9% (95%CI: 76.5-99.1) and 46.4% (95%CI: 27.5-66.1) respectively. Considering only those patients that achieved MCyR, CCyR and/or MMR for the first time on study, the cumulative incidence of MCyR was 71.4% (95%CI: 17.9-93.6%) at six months (no patient at risk after nine months), while for CCyR was 83.3% (95%CI: 40.5-96.4%) at six months, and was maintained at 12 months. The cumulative incidence of MMR was 26.1% (95%CI: 10.3-45.2) at six months and increased to 39.1% (95%CI: 19.4-58.5) at 12 months. The OS was 100% (95%CI: na) at one and two years, and 85.7% (95%CI: 63.3-100%) at three years. Bosutinib was therefore safe and yielded response rates comparable to published data from the other TKIs approved for children with CML.

Chapter 6 provides the conclusions of this work and outlines the author's outlook on early phase studies in pediatric hemato-oncology, including regulatory incentives and design considerations. Overall, the expectation in the field is that future treatments will be less dependent on chemotherapy and more reliant on targeted delivery/therapy and immunotherapy, which appear less toxic but more effective. This in particular for ALL, which is still strongly reliant on multi-agent chemotherapy, while for CML, a chemo-free approach is already available. For CML the challenges consist of developing TKIs which do not impair longitudinal growth, deliver

child-friendly formulations to improve compliance with the therapy, and thirdly establishing the long-term safety and efficacy of second and third generation TKIs when stopping TKIs is not feasible in children. In the latter case the option of HSCT needs to be revisited.

Samenvatting

Dit proefschrift rapporteert de resultaten van vroege stadia van studies ITCC-059 en ITCC-054/ COG-AAML1921 onderzoek naar inotuzumab ozogamicine (InO) en bosutinib, respectievelijk gebruikt voor de behandeling van acute lymfatische leukemie (ALL) en chronische myeloïde leukemie (CML). Beide studies waren registratiestudies die werden ontwikkeld in het kader van een Pediatric Investigation Plan, in samenwerking met de houder van de marketing autorisatie, en met de betrokkenheid van internationale locaties op het vasteland van Europa en de Verenigde Staten.

In **hoofdstuk 2** worden de resultaten gepresenteerd van het fase II-cohort van de ITCC-059-studie, waarin de voorlopige activiteit van InO als enkelvoudig middel voor patiënten met recidiverende en refractaire CD22+ B-celprecursor (BCP) ALL wordt onderzocht. Hoewel er aanzienlijke vooruitgang werd geboekt bij de behandeling van pediatrische ALL, werd dit vooral bereikt met de verfijning van chemotherapieregimes en stratificatie van risicogroepen in plaats van met de ontwikkeling van nieuwe middelen. InO is een van de alternatieven voor traditionele chemotherapie voor deze patiënten, en in het bijzonder voor de re-inductiefase van recidiverende en refractaire gevallen. Met behulp van een eentrapsontwerp testte de studie een nulhypothese van een algemeen responspercentage (ORR) ≤ 30% tegen een ORR-> 55% als alternatieve hypothese. Om een power van 80% te bereiken op een significantieniveau van 0.05, was de steekproefomvang ten minste 25 patiënten. Tweeëndertig patiënten werden geïncludeerd, 28 werden behandeld, 27 waren evalueerbaar voor respons. De geschatte ORR was 81.5% (95%CI: 61.9%-93.7%) en bij 81.8% (18/22) van de reagerende proefpersonen was minimaal residueel van de ziekte (MRD) negatief. Daarom werd de nulhypothese verworpen en werd het medicijn als veelbelovend beschouwd. De eenjaars-overleving zonder event (EFS) was 36.7% (95% CI: 22.2%−60.4%) en de totale overleving na één jaar (OS) was 55.1% (95% CI: 39.1%−77.7%). De cumulatieve incidentie van niet-recidiefsterfte was 22.6% (95% CI 8.8-40.2) na één jaar, inclusief follow-up na hematopoëtische stamceltransplantatie (HSCT). Het medicijn werd over het algemeen goed verdragen, ondanks dat er zeven (25%) gevallen van veno-occlusieve ziekte van de lever (VOD) werden gemeld; één graad 2, vier graad 3 en twee graad 4. Zes gevallen deden zich voor na HSCT na voorgaande behandeling met InO, wat de gegevens bevestigt die in eerdere onderzoeken zijn verzameld. Ondanks dat vrijwel alle patiënten laboratoriumafwijkingen ondervonden, zoals verlaagde aantallen witte bloedcellen, trombocytopenie of neutropenie, waren deze toxiciteiten meestal van voorbijgaande aard en sloten ze zelden de mogelijkheid uit om de behandeling te voltooien. Daarom suggereren de gegevens dat InO ook veilig kan worden toegediend aan deze zwaar voorbehandelde patiënten, wat opmerkelijke responspercentages oplevert.

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Hoofdstuk 3 gaat dieper in op de resultaten van de fase IB van de ITCC-059 studie waarin InO werd gecombineerd met een aangepaste versie van het UKALL-R3 re-inductieregime, met als doel mitoxantron, dat bekend staat om zijn toxiciteit, te vervangen. InO werd gecombineerd met 1.5 mg/m² vincristine (op dag 3, 10, 17, 24), 20 mg/m² dexamethason (twee blokken van 5 dagen, daarna aangepast) en intrathecale therapie. Er werd gebruik gemaakt van een rolling-6 ontwerp waarbij InO doseringen werden getest van 0.8 tot 1.8 mg/m²/cyclus. In totaal werden 30 patiënten behandeld en 29 waren evalueerbaar voor dosisbeperkende toxiciteiten (DLT's). Na aanpassing van de dosis dexamethason en verlaging tot 10 mg/m² was het mogelijk om InO te verhogen tot 1,8 mg/m²/cyclus (1.5 mg/m²/cyclus zodra remissie is bereikt). Het gepoolde responspercentage was 80% (24/30; 95%CI: 61.4% tot 92.3%) en van de responders bereikte 66.7% MRD-negativiteit. Na één jaar was de EFS-waarschijnlijkheid 41.7% (95%CI: 27.1-64.3) en de OS-waarschijnlijkheid 62.3% (95%CI: 46.9-82.8). De cumulatieve incidentie van niet-recidiefsterfte was 10.2% (95% BI 2.5-24.3) na één jaar, inclusief follow-up na HSCT. Verhoging van alanineaminotransferase (ALAT) trad op bij 23 patiënten (76%), waarvan 15 (50%) ≥ graad 3. Toename van aspartaataminotransferase (AST) trad op bij 22 patiënten (73.3%), waarvan 10 (33.3%) ≥ graad 3. In totaal hadden 24 (80%) patiënten een AST- en/of ALT-verhoging. Zeven patiënten (23.3%) hadden een toename van bilirubine; waarvan zes (20%) in graad 1-2 en één (3%) in graad 3. Geen enkele voldeed aan de criteria van de wet van Hy. In totaal ontwikkelden vijf (17%) patiënten VOD. Vier na HSCT (één graad 4 en drie graad 3). Concluderend onderstrepen voorlopige werkzaamheids- en veiligheidsgegevens de mogelijkheid om InO tot 1.8 mg/m² op een veilige manier te combineren met vincristine, dexamethason en IT-therapie. Desalniettemin werd een merkbaar voordeel van dit combinatieregime in termen van ORR, in vergelijking met de enkelvoudige middelen van dezelfde studie, niet waargenomen bij deze zwaar voorbehandelde patiënten.

Hoofdstuk 4 schetst het farmacokinetische (PK) gedrag van InO als enkelvoudig middel. Bij de overgang van volwassene naar kind is het inderdaad belangrijk om te begrijpen hoe het metabolische systeem van kinderen verschilt van dat van volwassenen. Genetische factoren, voedselinname, medicijnformulering en gelijktijdige medicatie zijn allemaal factoren die de aanleg van het medicijn kunnen veranderen. Bovendien is de pediatrische populatie een heterogene groep in termen van absorptie, distributie, metabolisme en eliminatie van geneesmiddelen, gezien de verschillen in de rijping van nieren en lever die worden waargenomen bij pasgeborenen (0-28 dagen), zuigelingen (>28 dagen tot 12 maanden) en oudere kinderen. Het doel van dit hoofdstuk is om een overzicht te geven van de factoren die van invloed zijn op de InO-blootstelling bij kinderen en de relatie met klinische uitkomsten. Een gepubliceerd tweecompartimenten PK-model voor de volwassen populatie voor InO, met een lineaire en tijdsafhankelijke klaring, werd aangepast om de pediatrische gegevens te beschrijven die werden verkregen uit de studie ITCC-059 waarin ALL patiënten werden behandeld met een InO monotherapie. De dataset werd gecombineerd met gegevens voor volwassenen die door de houder van de marketing autorisatie waren verstrekt. Voor ALL-patiënten was een toename van de leeftijd geassocieerd met een afname van k des

(vervalcoëfficiënt) en van CL_t (tijdsafhankelijke klaring), wat aangeeft dat de doelgemedieerde geneesmiddelklaring sneller afneemt bij kinderen (mogelijk als gevolg van een snellere afname van leukemische blasten tijdens de eerste weken van de behandeling). Een toename van het aantal perifere lymfoblastenwas gerelateerd aan een afname van k des; vandaar dat een afname van de dalingssnelheid van CL_t, ondanks de klinische relevantie, niet is getest. Bovendien vertoonden patiënten met een lagere vetvrije massa lagere waarden van lineaire klaring. Interessant is dat het cumulatieve gebied onder de concentratie-tijdcurve (AUC) hoger was bij responders (n = 42) versus niet-responders (n = 10) aan het einde van de eerste cyclus (26.1 [IQR 18.9 – 35.0] vs. 10.1 [IQR 9.19 – 16.1], *103 ng*h/ml), ondanks dat de studie niet in staat was om een statistisch significant verschil te detecteren. Op basis van simulaties vonden we geen parameters op basis waarvan dosisaanpassingen bij kinderen werden voorgesteld, en de blootstelling die werd bereikt bij 1.8 mg/m²/cyclus op basis van dit farmacokinetische model en klinische gegevens over werkzaamheid en toxiciteit in de hoofdstukken 2 en 3 geschikt leek.

Het andere medicijn dat onderwerp was van dit promotieonderzoek is bosutinib. Patiënten met CML worden nu bijna uitsluitend met Tyrosine Kinase Inhibitors (TKIs) behandeld sinds imatinib in 2003 werd goedgekeurd voor kinderen. Onder de gelabelde opties kunnen kinderen worden behandeld met imatinib, dasatinib of nilotinib (nu ook bosutinib). De belangrijkste onvervulde medische behoefte is in dit geval tweeledig. Ten eerste zijn er proefpersonen die resistentie ontwikkelen tegen een of meer van de momenteel goedgekeurde TKIs en die daarom alternatieve geneesmiddelen nodig hebben. Ten tweede hebben alle goedgekeurde TKIs toxiciteiten die ondraaglijk kunnen zijn voor sommige patiënten die gedurende een lange periode moeten worden behandeld. Elke TKI heeft de neiging om een ander spectrum van bijwerkingen te vertonen, daarom zal het uitbreiden van het landschap van deze verbindingen helpen om aan de individuele behoeften van deze kinderen te voldoen. Bovendien vertoonden alle TKIs die momenteel zijn goedgekeurd een verslechtering van de longitudinale groei van het skelet. De hoop is dat met bosutinib dit effect zal worden verminderd zoals verwacht door eerder onderzoek met diermodellen. De resultaten van een fase I-studie waarin bosutinib werd getest bij patiënten met CML die resistent of intolerant zijn voor eerdere therapielijnen, worden gepresenteerd in **hoofdstuk 5**.

In het fase I-deel van de internationale, open-label studie ITCC-054/COG-AAML1921 (NCT04258943) werden kinderen van 1-18 jaar met resistent/intolerant (R/I) (volgens ELN 2013) Ph+ CML ingeschreven met behulp van een 6+4-ontwerp, waarbij 300, 350 en 400 mg/m² QD met voedsel werden getest. Dertig patiënten werden geïncludeerd; 27 waren evalueerbaar voor DLT: 6 bij 300 mg/m² (0 DLT) 11 bij 350 mg/m² (1 DLT) en 10 bij 400 mg/m² (1 DLT). De gemiddelde AUCss bij 300 mg/m², 350 mg/m² en 400 mg/m² waren 2.20 µg·h/ml, 2.52 µg·h/ml en 2.66 µg·h/ml. De recommended phase II dose (RP2D) werd daarom gedeclareerd op 300 mg/m² voor ND-patiënten en 400 mg/m² voor R/I patienten op basis van AUC-doelen afgeleid van volwassenen die werden behandeld met de goedgekeurde dosering (respectievelijk 3.15 ng·h/ml (±20%) voor R/I-patiënten en 2.27 µg·h/ml (±20%) voor ND-patiënten). De meest voorkomende

bijwerkingen waren diarree (93%, n=26), buikpijn (71%, n=20), braken (68%, n=19), misselijkheid (61%, n=12) en maculopapuleuze huiduitslag of andere huidaandoeningen (respectievelijk 39%, n=11; en 43%, n=12). De cumulatieve proporties van volledige hematologische respons (CHR), significante cytogenetische respons (MCyR), volledige cytogenetische respons (CCyR) en significante moleculaire respons (MMR) aan het einde van de behandeling, als beste respons, waren respectievelijk 100% (95% CI: 87.7-100), 96.4% (95%CI: 81.7-99.9), 92.9% (95%CI: 76.5-99.1) en 46.4% (95%CI: 27.5-66.1). Rekening houdend met alleen die patiënten die MCyR, CCyR en/of MMR voor het eerst bereikten tijdens het onderzoek, was de cumulatieve incidentie van MCyR 71.4% (95%CI: 17.9-93.6) na zes maanden (geen risicopatiënt na negen maanden), terwijl voor CCyR 83.3% (95%CI: 40.5-96.4) was na zes maanden, en werd gehandhaafd na 12 maanden. De cumulatieve incidentie van MMR was 26.1% (95%CI: 10.3-45.2) na zes maanden en steeg tot 39.1% (95%CI: 19.4-58.5) na 12 maanden. De OS was 100% (95%CI: na) na één en twee jaar, en 85.7% (95%CI: 63.3-100%) na drie jaar. Bosutinib was daarom veilig en leverde responspercentages op die vergelijkbaar waren met gepubliceerde gegevens van de andere TKIs die zijn goedgekeurd voor kinderen met CML.

Hoofdstuk 6 geeft de conclusies van dit werk en schetst de visie van de auteur op vroege fasestudies in de pediatrische hemato-oncologie, inclusief regelgevende stimulansen en ontwerpoverwegingen. Over het algemeen is de verwachting in het veld dat toekomstige behandelingen minder afhankelijk zullen zijn van chemotherapie en meer afhankelijk zullen zijn van gerichte therapie en immunotherapie, die minder toxisch maar effectiever lijken. Dit met name voor ALL, dat nog steeds sterk afhankelijk is van chemotherapie met meerdere middelen, terwijl voor CML al een chemovrije aanpak beschikbaar is. Voor CML bestaan de uitdagingen uit het ontwikkelen van TKIs die de longitudinale groei niet belemmeren, het leveren van kindvriendelijke formuleringen om de therapietrouw te verbeteren, en ten derde het vaststellen van de veiligheid en werkzaamheid op lange termijn van TKIs van de tweede en derde generatie wanneer het stoppen met TKIs niet haalbaar is bij kinderen. In het laatste geval moet de optie van HSCT opnieuw worden bekeken.

7

A

Appendix

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List of publications

Inotuzumab Ozogamicin as single agent in pediatric patients with relapsed and refractory acute lymphoblastic leukemia: results from a phase II trial.

Pennesi E*, Michels N*, Brivio E, van der Velden VHJ, Jiang Y, Thano A, Ammerlaan AJC, Boer JM, Beverloo HB, Sleight B, Chen Y, Vormoor-Bürger B, Rives S, Bielorai B, Rössig C, Petit A, Rizzari C, Engstler G, Starý J, Bautista Sirvent FJ, Chen-Santel C, Bruno B, Bertrand Y, Rialland F, Plat G, Reinhardt D, Vinti L, Von Stackelberg A, Locatelli F, Zwaan CM.
* contributed equally

Leukemia. 2022 Jun;36(6):1516-1524. doi: 10.1038/s41375-022-01576-3.

Bosutinib in Resistant and Intolerant Pediatric Patients with Chronic Phase Chronic Myeloid Leukemia: Results from the Phase I part of Study ITCC054/COG AAML1921

Brivio E*, **Pennesi E***, Willemse ME, Huitema ADR, Jiang Y, van Tinteren HDR, van der Velden VHJ, Beverloo BH, den Boer ML, Rammeloo LAJ, Hudson C, Heerema N, Kowalski K, Zhao H, Kuttschreuter L, Bautista Sirvent FJ, Bukowinski A, Rizzari C, Pollard J, Murillo-Sanjuán L, Kutny M, Zarnegar-Lumley S, Redell M, Cooper S, Bertrand Y, Petit A, Krystal J, Metzler M, Lancaster D, Bourquin JP, Motwani J, van der Sluis IM, Locatelli F, Roth ME, Hijiya N[†], Zwaan CM[†].
* contributed equally

Journal of Clinical Oncology. 2023 Nov 30:JCO2300897. doi: 10.1200/JCO.23.00897.

*contributed equally; † jointly supervised the work

Inotuzumab Ozogamicin combined with chemotherapy in pediatric B-cell precursor CD22+ Acute Lymphoblastic Leukemia: results of the phase 1B ITCC-059 trial

Pennesi E, Brivio E, Ammerlaan ACJ, Jiang Y, van der Velden VHJ, Beverloo HB, Sleight B, Locatelli F, Brethon B, Rossig C, Engstler G, Nilsson A, Bruno B, Petit A, Bielorai B, Rizzari C, Rialland F, Rubio-San-Simón A, Bautista Sirvent FJ, Diaz-de-Heredia C, Rives S, Zwaan CM.

Haematologica. Published online January 4, 2024. doi:10.3324/haematol.2023.284409

Population Pharmacokinetics of Inotuzumab Ozogamicin in Pediatric Relapsed/Refractory Acute Lymphoblastic Leukemia – results of study ITCC-059

Wu JH*, **Pennesi E***, Bautista F, Garrett G, Fukuhara K, Brivio E, Ammerlaan ACJ, Locatelli F, van der Sluis IM, Rossig C, Chen-Santel C, Bielorai B, Petit A, Starý J, Díaz-de-Heredia C, Rives S, O'Marcaigh A, Rizzari C, Engstler G, Nysom K, Rubio-San-Simón A, Bruno B, Bertrand Y, Brethon B, Rialland F, Plat G, Dirksen U, Sramkova L. Zwaan CM[†], Huitema ADR[†]
* contributed equally; † jointly supervised the work

*Submitted to **Clinical Pharmacokinetics journal***

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Portfolio

PhD Portfolio: Edoardo Pennesi		
Courses & workshops		
	Period	**ECTS**
Clinical Epidemiology [CE02] - NIHES, Erasmus MC University Medical Centre	28 October 2020 – 30 October 2020	**3.7**
Advanced Topics in Decision-making in Medicine [EWP02] - NIHES, Erasmus MC University Medical Centre	25 January 2021 - 6 February 2021	**2.4**
Childhood Cancer: State-of-the-Art Treatment, Innovative (Bio)Medicine and Patient Centered Care - Utrecht University	19 July 2021 - 23 July 2021	**2**
Biostatistics I (CK020S) - NIHES, Erasmus MC University Medical Centre	16 May 2022 – 20 May 2022	**2**
Using R for Statistics in Medical Research (BST02) - NIHES, Erasmus MC University Medical Centre	01 March 2021 – 09 March 2021	**1.4**
Competing Risks and Multi-State Models [EL001] - NIHES, Erasmus MC University Medical Centre	21 February 2022 – 23 February 2022	**0.9**
Repeated Measurements in Clinical Research [EL002] - NIHES, Erasmus MC University Medical Centre	11 April 2022 – 22 April 2022	**1.7**
Scientific Integrity [LLS06] - NIHES, Erasmus MC University Medical Centre	17 May 2022	**0.3**
Joint Models for Longitudinal and Survival Data [ESP72] - NIHES, Erasmus MC University Medical Centre	22 August 2022 – 26 August 2022	**0.7**
Logistic Regression [ESP66]- NIHES, Erasmus MC University Medical Centre	08 August 2022 – 12 August 2022	**1.4**
Introduction to Bayesian Methods in Clinical Research [ESP68] - NIHES, Erasmus MC University Medical Centre	15 August 2022 – 19 August 2022	**1.4**
*BROK (*Basic course Rules and Organisation for Clinical researchers*)*	7 February 2023	**1.5**
	Tot	**19.4**
Symposia & Congresses		
	Period	**ECTS**
New Agents in Leukemia/Lymphoma Symposium (Utrecht)	27-28 October 2020	**1**
New Agents in Leukemia/Lymphoma Symposium (Utrecht)	1-2 November 2021	**1**

18ᵗʰ ITCC (Innovative Therapies for Children with Cancer) Annual Meeting 2021 (Paris)	17-18 November 2021	**1**
63th ASH (American Society of Hematology) Annual Meeting 2021	11-14 December 2021	**1**
54th SIOP (International Society of Paediatric Oncology) (Barcelona) Annual Congress 2022	27 September – 1 October 2022	**1**
64th ASH Annual Meeting 2022	10-13 December 2022	**1**
ASCO (American Society of Clinical Oncology) Annual Meeting 2023 (Chicago)	1-6 June 2023	**1**
20ᵗʰ ITCC Annual Meeting 2023 (Paris)	9-10 November 2023	**1**
	Tot	**8**

Tutoring/Mentoring activities	**4.5**

TOT	**31.9**

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About the author

Edoardo Pennesi was born in Italy on February 2nd 1990. He grew up in Grosseto, a town in southern Tuscany, where he took a major in visual arts at high school. He then studied Medicine at the University of Pisa and graduated in 2017. Subsequently, he moved to the Netherlands and obtained a Master in Business and Administration from the Rotterdam School of Management (MScBA) in 2018. Afterwards, he worked in the field of Health Technology Assessment at the iMTA in Rotterdam and, in 2020, he started a PhD trajectory at the Erasmus MC and Princes Maxima Center for pediatric oncology under the supervision of dr. prof. Christian Michel Zwaan.

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Appendix

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