# Determinants of clinical outcome in *MLL*-rearranged infant acute lymphoblastic leukemia

Emma Driessen

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**Determinants of clinical outcome in *MLL*-rearranged infant acute lymphoblastic leukemia**

Determinanten van klinische uitkomst bij zuigelingen
met acute lymfatische leukemie met een *MLL*-herschikking

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## CONTENTS

# Chapter 1

General introduction

## LEUKEMIA

In the bone marrow the normal hematopoeisis (formation of blood cells) takes place. The main function of the bone marrow is to maintain the number of mature blood cells in the peripheral blood at a constant level throughout life. Leukemia is a type of cancer, characterized by abnormal growth of immature white blood cells in the bone marrow. This overgrowth of non-functional leukemic cells compromises the formation of normal mature blood cells, leading to anemia (due to the loss of functional red blood cells), infections (due to the loss of functional white blood cells), bleedings or bruising (due to the loss of functional white blood cells). Once the bone marrow is overgrown, leukemic cells are capable of infiltrating other organs, like the spleen, liver, skin, testes and central nervous system. Leukemia can be classified in several subtypes, based on cell-growth and immunophenotype. Fast-growing leukemias are characterized as "acute" and slow-growing as "chronic". Based on immunophenotype leukmias are classified into "lymphoblastic" or "myeloid", depending on the type of with blood cells the leukemic cells rises from.

## INFANT ACUTE LYMPHOBLASTIC LEUKEMIA

Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) is a rare and aggressive malignancy with early onset, high frequencies of early relapse during treatment, and a poor clinical outcome[1, 2]. Infant ALL accounts for approximately 4% of all pediatric ALL cases. Infants have a distinctive biology compared to older children with ALL. Translocation of the *Mixed Lineage Leukemia* (*MLL*) gene are found in ~6% of childhood ALL cases, of which 80% is associated with infant ALL[3]. The presence of an *MLL*-rearrangement in their leukemic cells, is a strong independent predictor of poor outcome in infant ALL patients[1-3]. More than 70 different *MLL* partner genes have been identified[4]. The most common *MLL* translocation in infant ALL is t(4;11), in which the N-terminus of *MLL* (chromosome 11q23) fuses to the C-terminus of *AF4* (chromosome 4q23). Other recurrent in-frame *MLL*-rearrangements found among infant ALL patients are t(11;19) and t(9;11), giving rise to the fusion proteins MLL-ENL and MLL-AF9 respectively[2, 5]. *MLL* germline infant ALL patients are patients, who lack an *MLL*-translocation in their leukemic cells.

The past decades survival rates for pediatric ALL patients have improved dramatically, with event-free survival (EFS) of 80% in older children with ALL. In contrast with the ~50% EFS for infant ALL patients (Figure 1), regardless of the more aggressive chemotherapy treatment they usually recieve[2, 6]. Although most infant ALL patients (~90-95%) achieve morphological complete remission (CR) after initial induction therapy, their prognosis is hampered by high relapse-rates. Most relapses occur very early, i.e. within the first year

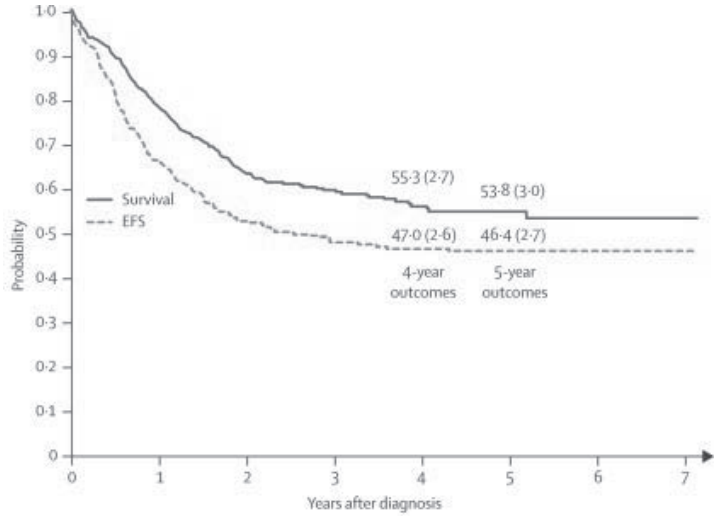


**Figure 1: Clinical outcome of infant ALL patients** (Kaplan-Meier curve is adapted from Pieters *et al.* 2007).

after diagnosis[2, 7-9]. The poor outcome of infant ALL patients in general is caused by the very poor outcome of infants which harbor an *MLL*-translocation in their leukemic cells (EFS of ~30-40%). This is in contrast to the outcome of infants lacking *MLL*-rearrangement in their leukemic cells, which nearly reaches the survival rates of older children with ALL (Figure 2)[2]. Other prognostic factors in infant ALL, associated with poor outcome are, high white blood cell (WBC) counts at diagnosis, very immature pro-B ALL phenotype, and glucocorticoid resistance[2, 3, 10-12]. More insights in the prognostic factors, molecular biology and clinical outcome of infant ALL, and in particular *MLL*-rearranged infant ALL, are needed in order to improve risk stratification and therapeutic strategies.

Insights into the biology and pathogenesis of *MLL*-rearranged ALL are hampered by the lack of genuine animal models accurately recapitulating this severe malignancy. Various attempts have been made to develop mouse models mimicking leukemogenesis of human t(4;11)-positive ALL, these mice displayed propensities towards developing lymphomas or leukemia with phenotypes that significantly differ from those found in humans[13-15]. Recent studies suggest involvement of the *RAS*-pathway, additional to the *MLL*-fusion, in the pathogenesis of *MLL*-rearranged leukemia [16-19]. The *RAS*-pathway regulates diverse cellular functions including cell proliferation, survival, differentiation, angiogenesis, and migration [20, 21]. Studies on the incidence of *RAS* mutations (*NRAS* and *KRAS*) in *MLL*-rearranged ALL demonstrate various frequencies 10-34% in *MLL*-rearranged and 26-63.9% in t(4;11)-positive childhood leukemia [16, 17, 22-24]. Deregulation of the *RAS*-pathway is a common event in childhood ALL and may guide new therapy strategies for *MLL*-rearranged infant ALL[21].

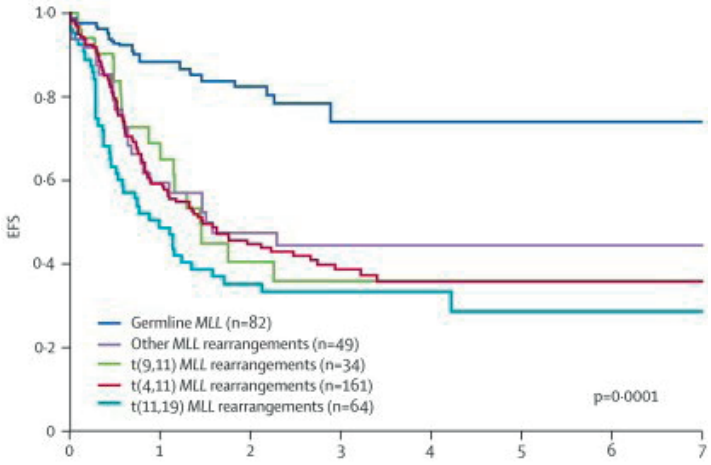


**Figure 2: Clinical outcome of infant ALL patients, dissected by type of *MLL*-translocation** (Kaplan-Meier curve is adapted from Pieters *et al.* 2007).

## OUTLINE OF THIS THESIS

In **Chapter 2** we detail the clinical outcome and prognostic factors of infant ALL patients, who relapsed on or after receiving the Interfant-99 treatment protocol. Relapsed infant ALL is assumed to be inevitably fatal. However, published data on outcome of relapsed infant ALL is limited. In **Chapter 3** we report on the correlation of *in vitro* drug sensitivity with clinical outcome in a relatively large cohort of primary *MLL*-rearranged infant ALL cases. To further identify prognostic factors in infant ALL patients we investigated the frequencies and prognostic value of *RAS* mutations in our cohort of primary infant ALL cases. As we report in **Chapter 4** we found that the presences of a *RAS* mutation in *MLL*-rearranged infant ALL cases is an independent prognostic factor for outcome. Therefore we hypothesized that inhibiting the *RAS*-pathway could be beneficial for infant ALL patients. In **Chapter 5** we report the effect of MEK inhibitors targeting primary *RAS*-mutated *MLL*-rearranged infant ALL cells *in vitro* and the relation with prednisolone sensitization. **Chapter 6** describes the role of Casitas B lineage lymphoma (CBL), a protein involved in the *RAS*-pathway, in acute myeloid leukemia and *MLL*-rearranged infant ALL. In **Chapter 7** we address our study of the relation of high *Versican* expression and clinical outcome in *MLL*-rearranged infant ALL patients. In **Chapter 8** we report on the effect of minimal contaminating stromal cells in *in vitro* co-culture experiments. **Chapter 9** summarizes this thesis and comprises a general discussion and **Chapter 10** covers a layman's summary in Dutch.

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# Chapter 2

## Outcome of relapsed infant acute lymphoblastic leukemia treated on the Interfant-99 protocol

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## INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) is an aggressive disease. About 80% of infant ALL patients harbor a *Mixed Lineage Leukemia* (*MLL*) translocation, which is a strong independent predictor of poor outcome[1]. Furthermore, infant ALL is associated with higher white blood cell (WBC) counts at diagnosis, very immature pro-B ALL phenotype, and glucocorticoid resistance[1, 2]. Although most infant ALL patients (~95%) achieve morphological complete remission (CR) after induction therapy, due to high relapse rates their outcome is poor[1]. Most relapses occur early, within the first year after diagnosis. The largest series of infant ALL, described by the Interfant-99 study group, reported a relapse rate of 34%. Relapsed infant ALL is generally assumed to be inevitably fatal. Published data on outcome of relapsed infant ALL is very limited, and available ones report on small cohorts.[3] Here, we describe the clinical outcome of 202 infant ALL patients, who relapsed on or after receiving the Interfant-99 treatment protocol[1].

## METHODS

Design and inclusion criteria of the Interfant-99 trial have been described previously[1]. Therapy after relapse differed in participating countries according to local policy and individual choice. We retrospectively asked the physicians whether the therapy was based on curative or palliative intent.

Outcome measure was overall survival (OS), defined as time from relapse until death from any cause. Curves were estimated with the Kaplan-Meier method and compared by subgroups with the log-rank test. Follow-up was updated at December 31 2009 and median follow-up (range) was 5.2 years (1 month–10.1 years). Survival analyses regarding hematopoietic stem cell transplantation (HSCT) were corrected for waiting time to transplantation. Association between prognostic characteristics at first diagnosis of ALL and at relapse with risk group and with assignment to curative or palliative care was evaluated with the Fisher exact test. To account for imbalances in characteristics between patients, a propensity score was derived, reflecting the probability that a patient would undergo curative treatment, by performing a multivariable logistic regression analysis. Any residual association between the covariates and treatment allocation was assessed by the Cochran–Mantel–Haenszel test. Cox model was used to evaluate outcome of curative and palliative treatment and of HSCT vs chemotherapy alone as curative treatment after relapse. Hazard ratios were tested according to Wald. SAS version 9.2 statistical software was used for data analysis.

## RESULTS

Out of 478 patients, 448 patients achieved first CR, 70.1% patients were allocated in the standard risk (SR, i.e. good prednisone response) and 29.9% in high risk (HR, i.e. poor prednisone response) group. Two-hundred and two patients (45.1%) relapsed, 37.6% and 62.7% in the SR and HR group, respectively. 56.9% (115/202) of the relapses occurred in the first year after diagnosis, with a median time to relapse of 10.0 months (range: 1.7-50.7 months). Most relapses occurred in the bone marrow (BM, 71.8%), while others were isolated extramedullary (n=23) or combined relapses (n=32). Patients in the HR group relapsed earlier than patients in the SR group (median: 9 vs. 12 months, $p$=0.042).

The 3-year OS after relapse was 20.9% (SE 3.5%). Infants with germline-*MLL* ALL relapsed later (>24 months) than infants with *MLL*-rearrangements ($p$=0.03). There was no difference in site of relapse (BM involvement vs. extramedullary) between germline-*MLL* and *MLL*-rearranged infant ALLs.

Out of 202 patients, 159 (78.7%) received relapse treatment with curative intent and 32 (15.8%) received supportive care only. For 11 patients (5.5%) treatment after relapse was not reported and these were excluded from further analyses. The 3-year OS after relapse of patients treated with curative intent was 24.9% (SE 4.0%), of whom 76.1% (121/159) died due to progressive ALL (n=93), HSCT-related causes (n=21) and other, mainly infectious, causes (n=7). All patients who received palliative care (n=32) died within 1 year after relapse, due to disease progression (Figure 1A, $p$<0.0001). As expected, this straightforward outcome comparison is biased by imbalances in the characteristics of the two groups. Curative treatment was more likely to be adopted for patients with a long duration of first CR ($p$=0.0005).

All patients (n=28), who relapsed after 24 months received curative treatment. 84.4% of patients receiving palliative care relapsed in the first year, compared to 50.3% of curative treated patients. Almost all patients carrying germline-*MLL* underwent curative treatment, whereas 87.5% of patients with palliative care had an *MLL*-rearrangement. Similarly, patients with higher WBC counts (>300x$10^9$/L) at first diagnosis received palliative care more frequently. In order to account for these imbalances, we derived a propensity score which predicted whether a patient would undergo curative treatment as a function of clinical characteristics likely affecting treatment choice. We used the propensity score in a stratified Cox model in order to perform a fair comparison of outcome in the two groups. The model which was also adjusted by relevant covariates, revealed that curative treatment was associated with a significantly superior OS (hazard ratio=0.17, 95% CI (0.10–0.27), $p$<0.0001). Interestingly, the time of

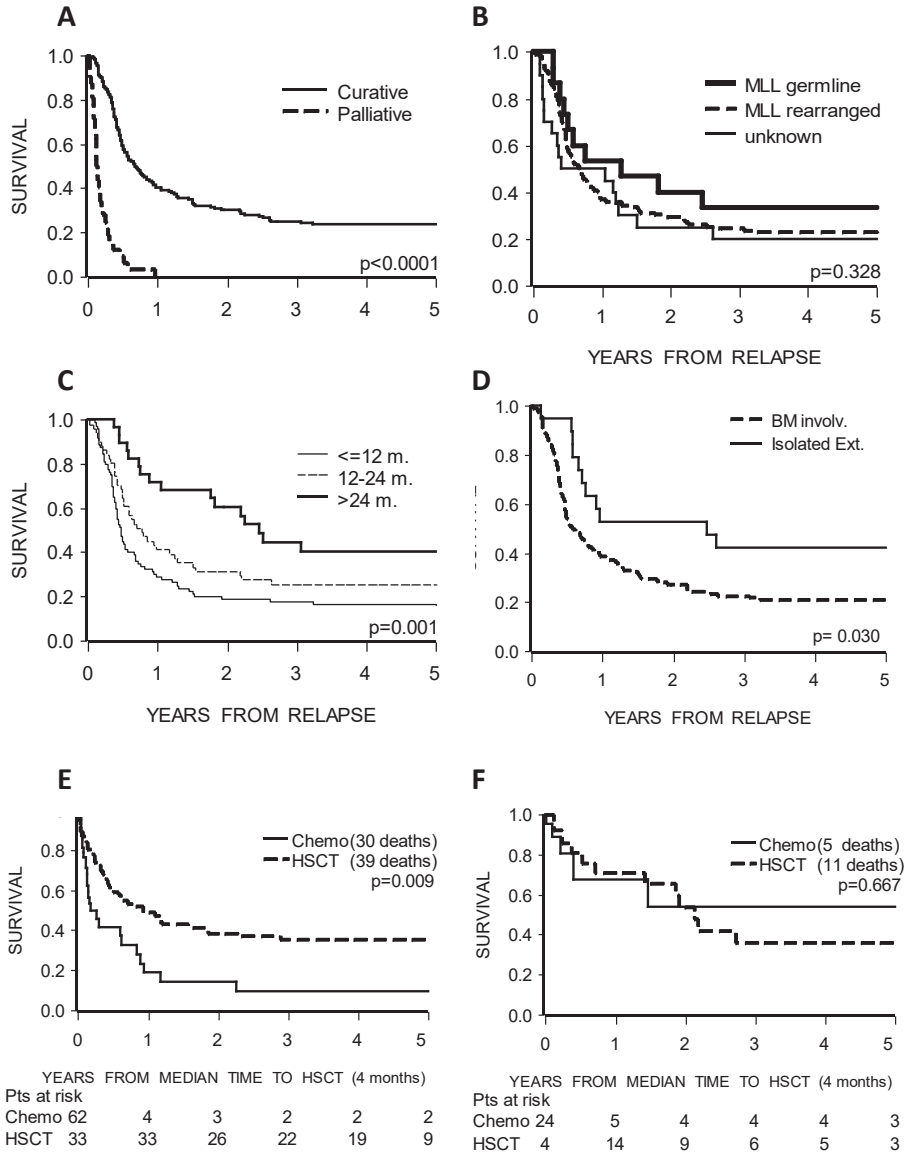


**Figure 1: Overall survival of relapsed infant ALL patients**

Overall survival, defined as time from relapse until death, (A) by treatment given after relapse, (B) of patients treated with curative intents by MLL status at initial diagnosis, (C) of patients treated with curative intents by time at relapse after initial diagnosis, (D) of patients treated with curative intents by site at relapse, (E) of patients treated with HSCT vs. chemotherapy only of early relapses (within 24 months from initial diagnosis), (F) of patients treated with HSCT vs. chemotherapy only of late relapses (beyond 24 months from initial diagnosis). Curves were estimated using the Kaplan-Meier method and analyzed by Log-rank tests. Follow-up was updated at December 31 2009 and median follow-up(range) was 5.2 years(1 month–10.1 years). Survival analyses regarding HSCT were corrected for waiting time to transplantation.

relapse retained prognostic relevance. Patients who relapsed within 24 months after diagnosis had a 1.5-fold increase in the risk of death (hazard ratio=1.40, 95% CI (0.97–2.01), $p$=0.016).

We then restricted our attention to the outcome of 159 patients treated with curative intent (Table 1 and Figure 1B-F). We did not observe a significant difference in OS after relapse between infants with and without 11q23 abnormalities ($p$=0.328). Infants with a t(4;11)-re-

**Table 1: Outcome of relapsed infant ALL patients treated with curative intent**

| | Patients | Deaths | 3-year OS (SE) | *p*-value |
|---|---|---|---|---|
| **Risk group** | | | | 0.487 |
| SR | 94 | 72 | 25.3 (5.2) | |
| HR | 65 | 49 | 24.4 (6.2) | |
| **11q23 abnormalities** | | | | 0.328* |
| *MLL* germline | 15 | 10 | 33.3 (12.9) | |
| t(4;11) | 65 | 54 | 16.9 (6.2) | |
| t(9;11) | 18 | 15 | 27.8 (11.8) | |
| t(11;19) | 21 | 14 | 32.7 (10.9) | |
| Other positive | 20 | 12 | 40.0 (11.2) | |
| Not known | 20 | 16 | 20.0 (11.2) | |
| **Age at diagnosis** | | | | 0.078 |
| < 3 months | 42 | 34 | 19.1 (7.7) | |
| 3 - 6 months | 50 | 41 | 16.8 (7.1) | |
| 6 - 9 months | 39 | 28 | 30.8 (8.0) | |
| 9 – 12 months | 28 | 18 | 39.3 (9.4) | |
| **WBC counts (cell/L)** | | | | 0.017 |
| < 100 x $10^9$ | 58 | 38 | 36.2 (6.6) | |
| 100-300 x $10^9$ | 50 | 38 | 25.1 (7.1) | |
| ≥ 300 x $10^9$ | 51 | 45 | 11.8 (7.0) | |
| **Time of relapse** | | | | 0.001 |
| 0-12 months | 80 | 67 | 17.5 (5.6) | |
| 12-24 months | 51 | 38 | 25.5 (7.0) | |
| > 24 months | 28 | 16 | 44.4 (9.7) | |
| **Site of relapse** | | | | 0.030§ |
| Bone marrow | 113 | 91 | 20.2 (4.7) | |
| Bone marrow + other | 26 | 18 | 34.2 (9.8) | |
| Isolated Extramedullary | 19 | 11 | 42.1 (11.3) | |
| Not known | 1 | 1 | - | |

Overall survival, defined as time from relapse until death, of curative treatments after relapse by relevant prognostic factors at initial diagnosis and at relapse, including risk group (based on prednisone response), 11q23-abnormalities, age at initial diagnosis, WBC counts at initial diagnosis, time of relapse, defined as time from initial diagnosis until relapse, and site of relapse. Subgroups were analyzed by using the Log-rank test. *p-value comparing positive vs. negative. §p-value comparing BM involvements vs. extramedullary.

arrangement showed a worse 3-year OS compared to those with other 11q23-abnormalities ($p$=0.037). Age at diagnosis had a significant impact on outcome after first relapse, with a 3-year OS of 17.9% (SE 5.2%) in patients below 6 months of age and 34.3% (SE 6.1%) in older patients ($p$=0.012). Higher WBC count at diagnosis was associated with inferior OS after relapse ($p$=0.017), while risk group stratification by initial prednisone response showed no impact ($p$=0.487). In addition, we found that infants relapsing within one year after diagnosis had a 3-year OS of 17.5% (SE 5.6%) compared to 25.5% (SE 7.0%) and 44.4% (SE 9.7%) of those who relapsed between 12-24 and beyond 24 months after diagnosis, respectively ($p$=0.001). Patients with relapse involving BM, either isolated or combined, had a worse OS compared to patients with isolated extramedullary relapse ($p$=0.030).

Among patients treated for relapse, 87 (54.7%) underwent HSCT, the majority in second CR (n=66, 75.9%) and from an HLA-matched unrelated donor (n=41, 47.1%). The median time from relapse to HSCT was 4.2 months (range: 1.1-9.1). According to the results of the Cox model corrected for waiting time to transplantation, patients receiving HSCT after relapse had a better OS compared to patients receiving chemotherapy alone ($p$=0.021); similar results were found after adjusting for known prognostic factors at initial diagnosis. The advantage of HSCT over chemotherapy did not change significantly over time of follow-up, contrary to what was observed for HSCT in CR1 in the Interfant-99 study[4]. Interestingly, there was a significant interaction between treatment and time at relapse ($p$=0.010), while the interaction between treatment and site of relapse was not significant. The analysis in subgroups defined by the time at relapse showed that HSCT significantly reduced the risk of death compared to chemotherapy alone in 131 patients who relapsed within 24 months from initial diagnosis (hazard ratio=0.52, 95% CI(0.32–0.85), $p$=0.009), while no advantage over chemotherapy was observed for patients who relapsed later (hazard ratio=1.28, 95% CI(0.420–3.89), $p$=0.667). After fitting the Cox model to subgroups of early relapses, site of relapse retained its significant impact over survival (hazard ratio for BM and combined vs. extramedullary relapses was 2.00, 95% CI(1.04-3.85), $p$=0.038).

## DISCUSSION

Here, we demonstrate in a large cohort that relapsed infant ALL is not inevitable fatal. We found a 3-year OS of 20.9% for all relapsed infant ALL patients and 24.9% for those treated with curative intent. This is in concordance with the previous reported 5-year OS of 25.6% of Tomizawa *et al.*[3]. Even after adjusting for imbalances of prognostic factors in the treatment groups, patients treated with curative intent had a significant better outcome. Young age and a high WBC count at initial diagnosis were associated with inferior outcome after first relapse, but response to prednisone prophase during first line treatment did not. Fur-

thermore, patients who relapsed earlier and with patients BM involvement (either isolated or combined) had a worse outcome. These findings are consistent with previous studies in other ALL subtypes[5-7].

The use of HSCT in infant ALL is a matter of debate. Previous studies demonstrated that routine use of HSCT does not improve outcome for infants[1, 8-10]. However, data from the Interfant-99 study group suggested that a small subset with high risk features (young age combined with poor response to prednisone prophase or high WBC) may benefit from HSCT in first CR[4]. The present study showed that HSCT improved outcome of infants who relapsed 'early' (i.e. within 2 years after initial diagnosis), a subset which in our cohort accounts for 82% of all relapses.

In conclusion, we demonstrated that relapsed infant ALL was not invariably lethal and underline the relevance of offering treatments with curative intents. New therapeutic strategies, such as FLT3 inhibitors, epigenetic drugs, glucocorticoid sensitization and *RAS*-pathway inhibition, could be beneficial for infant ALL patients[11-15].

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# Chapter 3

## Relation between *in vitro* drugs responses and prognostic markers in *MLL*-rearranged infant acute lymphoblastic leukemia

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## ABSTRACT

Despite the current successful treatment results in pediatric acute lymphoblastic leukemia (ALL), the prognosis for *MLL*-rearranged infant ALL patients remains poor, and novel strategies are needed to identify and predict patients at high risk of therapy failure. We explored to what extent *in vitro* drug resistance is associated with clinical outcome and established prognostic markers, such as age <6 months, *MLL* rearrangement and white blood cell count (WBC) at diagnosis.

We found that *in vitro* response to none of the drugs tested by itself is capable of predicting clinical outcome, although an association between *in vitro* glucocorticoid (i.e. prednisolone and dexamethasone) resistance and a poor outcome was observed. Also, *in vitro* resistance to glucocorticoids was associated with age <6 months and high WBC. The combined *in vitro* response to prednisolone, vincristine and L-asparaginase (PVA) was found to be predictive for an adverse outcome in *MLL*-rearranged infant ALL. PVA-resistant *MLL*-rearranged infant ALL patients are at high risk of therapy failure. PVA-resistant *MLL*-rearranged infant ALL patients showed cross-resistance towards dexamethasone but not to other drugs.

Taken together, we conclude that *in vitro* PVA-sensitivity testing in *MLL*-rearranged ALL may be useful to stratify patients at high risk of therapy failure, and that this approach allows identification of patients urgently requiring effective novel treatment options.

## INTRODUCTION

Over the last decades, the outcome for childhood acute lymphoblastic leukemia (ALL) in general has improved tremendously. Yet, the clinical outcome for infants (< 1 year of age) diagnosed with ALL remained dismal[1-5]. Infant ALL is characterized by a high incidence of balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene located on chromosome 11, which are detected in ~80% of the cases[6-8]. As a result of such translocations, the N-terminal region of *MLL* fuses to the C-terminal region of one of its many translocation partner genes. Among infant ALL patients, the most recurrent *MLL* translocations are t(4;11), t(11;19) and t(9;11), which generate the chimeric fusion proteins MLL-AF4, MLL-ENL and MLL-AF9, respectively. The presence of leukemia-specific *MLL* translocations on its own represents a strong predictor of an unfavorable clinical outcome[2, 3, 5, 9, 10]. Additional parameters predicting a poor outcome include age at diagnosis (<6 months of age and white blood cell count (WBC) at disease presentation[5]. Moreover, infant ALL cells are resistant to glucocorticoids and L-asparaginase[11, 12]. The *in vitro* responses of prednisolone, vincristine and L-asparaginase combined (known as the PVA score), appeared highly predictive for clinical outcome in childhood ALL[13, 14]. To date it remains unclear whether *in vitro* drug resistance patterns obtained in primary infant ALL cells influence prognosis, or to what extent cellular resistance is associated with abovementioned prognostic factors. Therefore we generated *in vitro* cytotoxicity profiles in a cohort of infant ALL patients enrolled in the Interfant treatment protocols, and investigated the relation between *in vitro* drug responses, clinical outcome, and known prognostic factors, in order to attain evidence-based progression towards personalized medicine.

## METHODS

### Patient samples

In the present study we used a total of 67 samples obtained from infant ALL patients (<1 year of age) enrolled in either the Interfant-99 or the Interfant-06 study, for whom a complete overview of clinical parameters and follow up data was available, as well as sufficient material for *in vitro* cytotoxicity testing. This cohort of infant ALL patients consists of *MLL*-rearranged infant ALL cases ($n$=55) as well as patients carrying germline (or wild-type) *MLL* genes (i.e. no translocation ($n$=12). The MLL-rearranged infant ALL patient group consists of patients carrying t(4;11) ($n$=28), t(11;19) ($n$=18) and other 11q23 translocations ($n$=9), as determined by fluorescence *in situ* hybridization (FISH) and RT-PCR analyses. Bone marrow aspirates or peripheral blood was collected at the Erasmus MC-Sophia Children's Hospital, and within 24 hours leukemic cells were isolated by density gradation centrifugation (Lymphoprep; density 1.077 g/ml, Nycomed Pharma, Oslo, Norway). Samples were

enriched for leukemic blasts by removal of contaminating normal cells using monoclonal antibodies linked to magnetic beads as described by Kaspers *et al.*[15]. Consequently, all samples used for *in vitro* drug testing contained >90% leukemic cells, as determined morphologically on May-Grunwald Giemsa (Merck, Darmstadt, Germany) stained cytospins.

### *In vitro* drug response and in vivo prednisone response

For all samples, *in vitro* drug cytotoxicity was assessed by MTT assays as described previously[16]. Briefly, leukemic cells were incubated for 4 days in a humidified incubator at 37°C and 5% $CO_2$, both in the absence and presence of a range of drug concentrations. Then, 10 µl of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide (MTT) was added to the cells, following an additional 6-hour incubation under the same conditions, during which the yellow MTT is reduced to blue formazan crystals by viable cells only. Next, the blue formazan crystals were dissolved using HCL-isopropyl alcohol, allowing spectrophotometrical measurement of the optical density. Culture medium without cells nor drugs served as blanks (i.e. 0% survival), and leukemic cells cultured in the absence of drugs (i.e. controls) were adjusted to 100% survival and used to calculate the $LC_{50}$-value. Results were considered evaluable only if the mean control OD, after correction for the background (as determined by the blanks), at day 4 exceeded 0.05 arbitrary units. The following drugs and concentration ranges (6 dosages per drug) were used: prednisolone, 0.08-250 ug/mL (PRED; Bufa, Uitgeest, The Netherlands); Vincristine, 0.05-50 ug/mL (VCR; Pharmacy Erasmus Medical Center, Rotterdam, The Netherlands); L-Asparaginase, 0.003-10 IU/mL (L-ASP; Nycomed BV, Hoofddorp, The Netherlands); Daunorubicin, 0.002-2.0 ug/mL (DNR; Sanofi Aventis, Gouda, The Netherlands); Cytarabine, 0.04-2.5 ug/mL (Ara-C; Hospira Benelux BVBA, Brussel, Belgium) and Dexamethasone, 0.0002-6.0 ug/mL (DEX; Pharmacie Erasmus Medical Center, Rotterdam, The Netherlands). The PVA score was based on the *in vitro* drug responses (i.e. $LC_{50}$-values) of prednisolone, Vincristine and L-Asparaginase, in which sensitivity towards each drug individually was scored as 1, intermediate responses as 2, and resistance was scored as 3. For this, the cut-off $LC_{50}$-values were: prednisolone sensitivity: <0.1 µg/mL and resistance: >150 µg/mL; vincristine sensitivity: <0.3906 µg/mL and resistance: >1.7578 ug/mL; L-asparaginase sensitivity: <0.033 IU/mL and resistance: >0.912 IU/mL. Hence, the PVA scores vary between 3 (sensitive to all three drugs) and 9 (resistant to all three drugs).

The *in vivo* response to prednisone was determined after one intrathecal dose of methotrexate and a 7-day window of prednisone mono-therapy (before the initiation of combination chemotherapy). Patients were defined as prednisone poor responders (PPRs) when >1000 leukemic cells/µL remained present in the peripheral blood[17]. When the amount of leukemic cells dropped below 1000/µL patients were defined as prednisone good responders (PGRs).

### Statistical analysis

Differences in the distribution of variables between patient groups were analyzed using the Mann-Whitney *U*-test or the Kruskall-Wallis test. Cross–resistance patterns were studied by correlating the $LC_{50}$ values for different drugs using the Kruskall-Wallis test and the Spearman's Rho-test. The probability of event-free survival (EFS) was calculated using the Kaplan-Meier method and the Log-rank (Mantel-Cox) test was performed to analyze differences in outcome between patient groups. Furthermore, a correlation between *in vitro* prednisolone response and age at diagnosis was analyzed using the Spearman's Rho-test. The EFS rate is defined as time from diagnosis to death in induction, failure to achieve complete remission after induction, disease relapse, the emergence of secondary malignancies, or death in complete remission. Patients who did not achieve complete remission were assigned an event at time-point zero in the EFS analyses. All analyses were two-tailed and differences with $p$-value <0.05 were considered statistically significant.

## RESULTS

### Prognostic relevance of in vitro PVA scoring in infant ALL

The combined *in vitro* responses to prednisolone, vincristine and L-asparaginase (i.e. PVA score) has been shown to be predictive for clinical outcome in pediatric ALL (i.e. children >1 year of age)[13, 14, 17]. We analyzed whether this also accounts for infants with ALL (<1 year of age). Patients were divided into a sensitive (PVA scores of 3-4), a intermediate (PVA scores of 5-6), or a resistant (PVA scores of 7-9) group. PVA-resistant infant ALL cases (including both patients with and without *MLL* translocations) tended to have a worse outcome when compared to patients with sensitive and intermediate PVA scores (Figure 1A); although this did not reach statistical significance ($p$=0.0686). As the presence of *MLL* translocations represents a strong and independent prognostic factor[5], we also analyzed the influence of the PVA score on clinical outcome excluding patients with wild-type *MLL* genes. PVA-resistant *MLL*-rearranged infant ALL patients showed worse EFS compared with both PVA-sensitive and PVA-intermediate *MLL*-rearranged infant ALL cases (Log-rank; $p$<0.05, Figure 1B). The 5-year EFS for PVA-resistant *MLL*-rearranged infant ALL patients is with ~10% very poor. Furthermore, we also analyzed whether *in vitro* sensitivity towards single chemotherapeutics (prednisolone, vincristine, L-asparaginase, daunorubicin, cytarabine or dexamethasone) is predictive for EFS. *MLL*-rearranged infant ALL patients *in vitro* resistant to these single chemotherapeutics have worse outcome when compared to their sensitive counterpart. However, these differences were not significant (prednisolone, $p$=0.1756, L-asparaginase, $p$=0.2970, daunorubicin, $p$=0.1396).

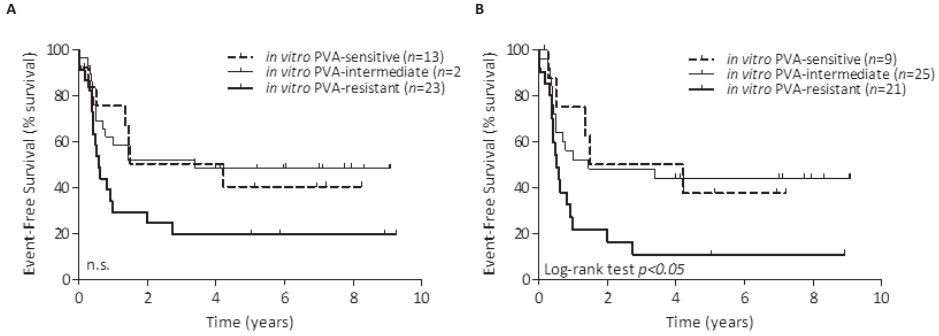


**Figure 1: Prognostic relevance of prednisolone, vincristine and L-asparaginase (PVA) in infant acute lymphoblastic leukemia**

EFS analysis of (A) total infant ALL patients (with and without *MLL*-rearranged infant ALL) and (B) *MLL*-rearranged infant ALL cases only. Infant ALL patient samples were divided based on their *in vitro* response to prednisolone, vincristine and L-asparaginase (PVA): PVA-sensitive (score 3 or 4), PVA-intermediate (score 5 or 6) and PVA-resistant (score 7-9). Differences in outcome were statistically analyzed using the Log-rank test.

Next, we analyzed whether PVA resistance showed cross-resistance to other drugs. In *MLL*-rearranged infant ALL patients, PVA resistance was correlated to dexamethasone resistance, but not to resistance to daunorubicin or Ara-C (Figure 2A). Interestingly, when analyzing cross-resistance between prednisolone, vincristine and L-asparaginase, only a correlation was found between prednisolone and vincristine (Spearman's Rho-test=0.5520, $p<0.005$) (Figure 2B); no correlation was found between prednisolone and L-asparaginase (Spearman's Rho-test=0.044, $p=0.7494$) nor between vincristine and L-asparaginase (Spearman's Rho-test=0.247, $p=0.0688$). Furthermore, *MLL*-rearranged infant ALL patient samples which were *in vitro* sensitive to prednisolone or vincristine only displayed *in vitro* sensitivity to the glucocorticoid dexamethasone (Spearman´s Rho-test prednisolone-dexamethasone=0.8951, $p<0.0001$; Spearman´s Rho-test vincristine-dexamethasone=0.6611, $p<0.0001$) and the antracycline daunorubicin (Spearman´s Rho-test prednisolone-daunorubicin=0.4036, $p=0.0025$; Spearman´s Rho-test vincristine-daunorubicin=0.3681, $p=0.0061$) (Figure 2C). No cross-resistance or cross-sensitivity was observed for neither L-asparaginase nor Ara-C with other drugs (data not shown).

Both the *in vitro* prednisolone response, as well as the *in vivo* prednisone response have been identified as prognostic makers in pediatric ALL[17] and infant ALL[2, 5]. Here, we investigated to what degree the clinical prednisone response correlates with *in vitro* prednisolone response as determined by MTT assays. As shown in Figure 3, PGRs are more frequent sensitive to prednisolone *in vitro* as compared with PPRs. Similar results are obtained when analyzing infant ALL in general (Figure 3A), or when analyzing only *MLL*-rearranged infant ALL cases (Figure 3B). Although the observed differences are statistically significant

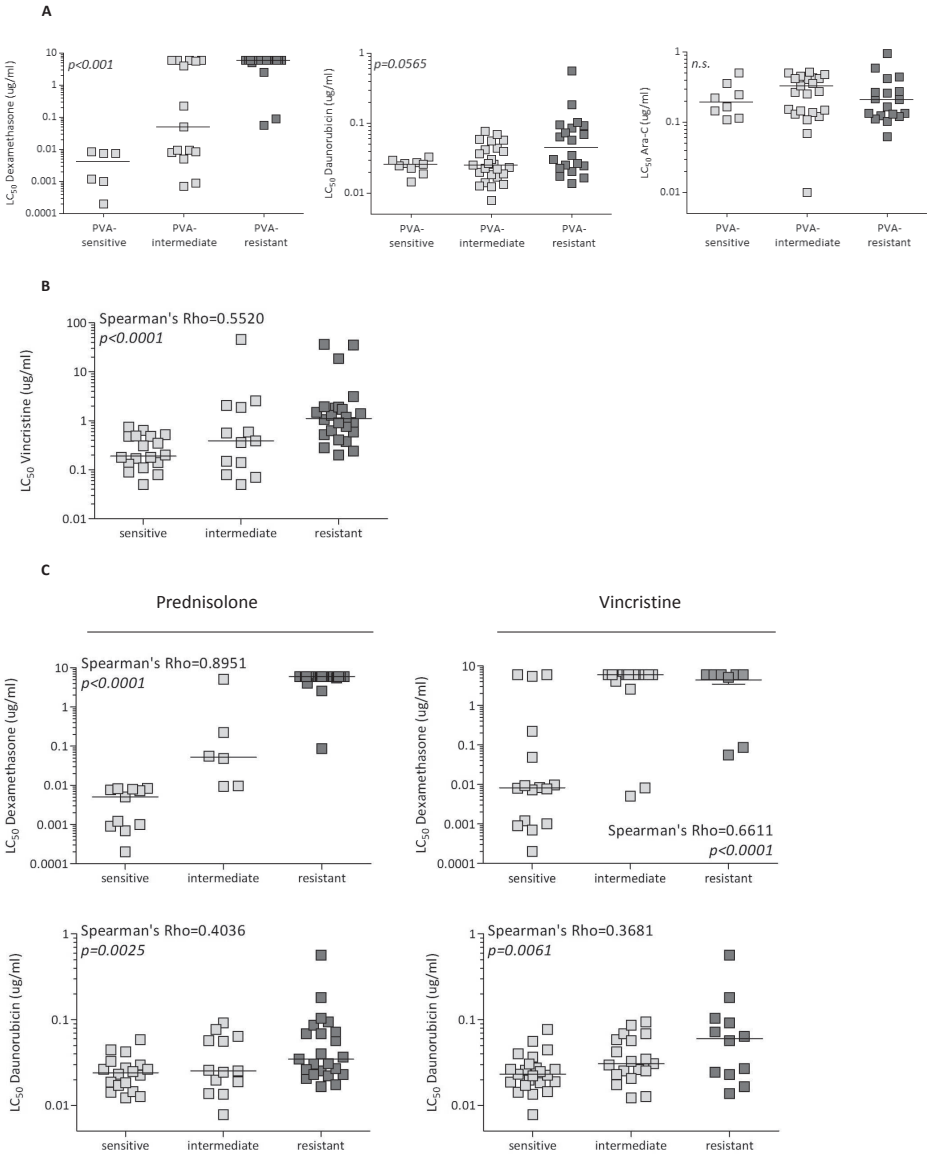


**Figure 2: *In vitro* cross-resistance of PVA to other induction-therapeutic drugs**

(A) $LC_{50}$ values of dexamethasone, daunorubicin or cytarabine (Ara-C) of *MLL*-rearranged patient samples divided into *in vitro* sensitive, intermediate or resistant to PVA. *P*-values were determined by the Kruskall Wallis-test. (B) $LC_{50}$ values of vincristine of *MLL*-rearranged patient samples divided into *in vitro* sensitive, intermediate or resistant to prednisolone. The indicated Spearman´s Rho index was determined by comparing $LC_{50}$ values of prednisolone with $LC_{50}$ values of vincristine. (C) $LC_{50}$ values of dexamethasone, daunorubicin or cytarabine (Ara-C) of *MLL*-rearranged patient samples divided into *in vitro* sensitive, intermediate or resistant to either prednisolone or vincristine. Indicated *p*-value was determined by Kruskall Wallis test. Correlation index was determined by Spearman's Rho-test, in which $LC_{50}$ values of both indicated drugs were correlated to each other.

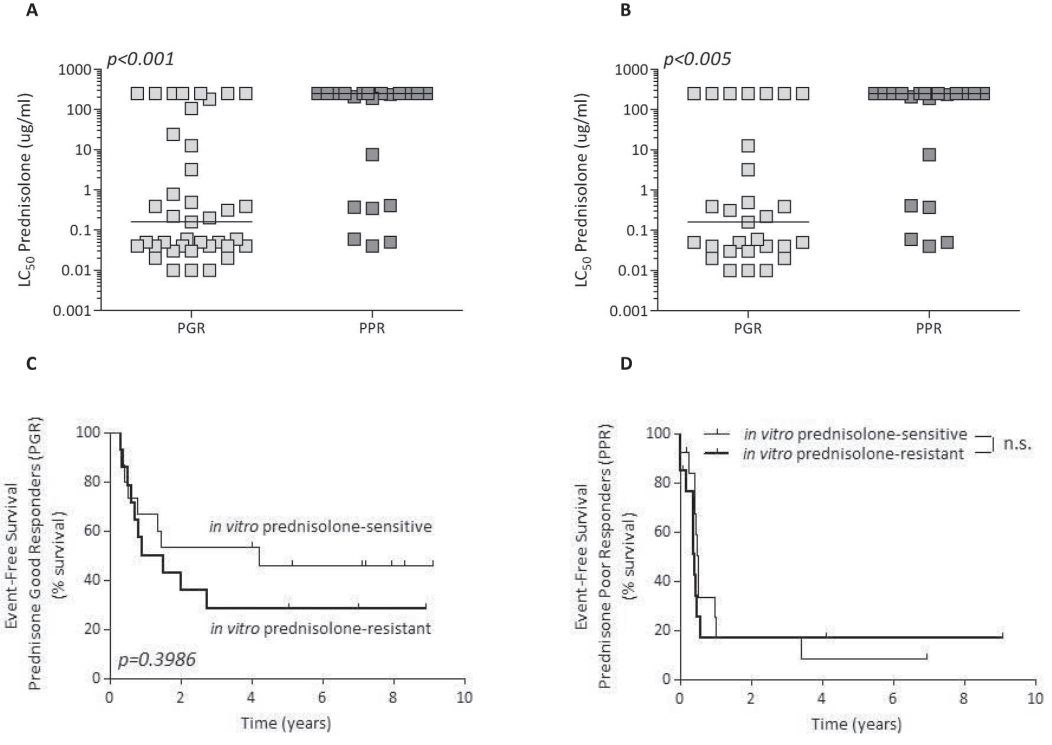


**Figure 3: Correlation of *in vivo* and *in vitro* prednisolone resistance**

$LC_{50}$ values of prednisolone of (A). infant ALL or (B). *MLL*-rearranged infant ALL that are *in vivo* prednisone good responders (PGR, *n*=39) or *in vivo* prednisone poor responders (PPR, *n*=29). *In vivo* prednisone response is based on the WBC count after one-week of prednisone monotherapy. PPRs demonstrate to be *in vitro* prednisolone resistant whereas PGRs are *in vitro* sensitive to prednisolone. *P*-values were determined by the Mann–Whitney *U*-test. EFS analysis of prednisolone-sensitive and prednisolone-resistant within (C) PGRs and (D) PPRs. The median *in vitro* prednisolone response was used as a cut-off. *In vitro* prednisolone response is non-significantly predictive in PGRs, and not in PPRs.

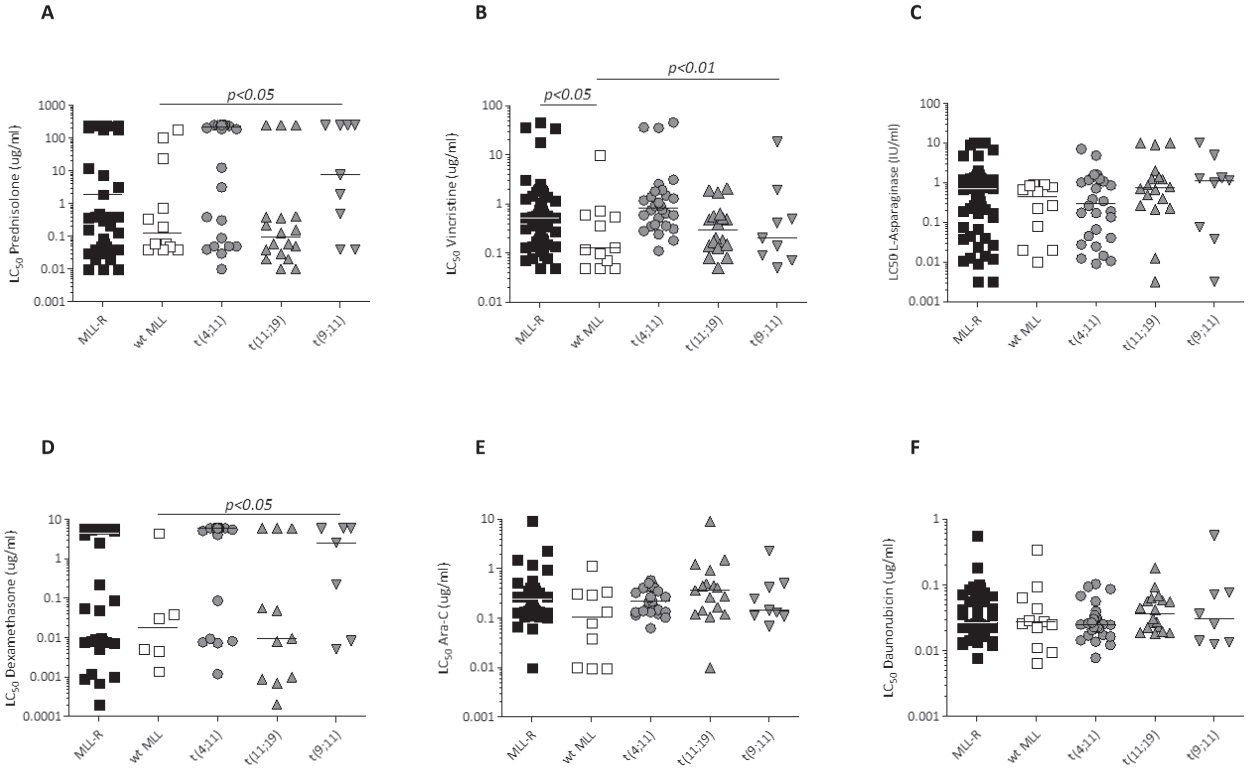


**Figure 4. *In vitro* drug response and the type of *MLL* rearrangement**

$LC_{50}$ values of (A) prednisolone, (B) vincristine, (C) L-asparaginase, (D) dexamethasone, (E) cytarabine (Ara-C) and (F) daunorubicin in infant ALL patient samples with (*MLL*-R; in black) or without an *MLL* rearrangement (wild-type *MLL*, denoted as wt *MLL*; in white). *P*-values were determined by the Mann–Whitney *U*-test, when comparing *MLL*-R and wt *MLL* patient samples. *In vitro* drug response was also depicted in *MLL-AF4*-rearranged ("t(4;11)"), *MLL-ENL*-rearranged ("t(11:19)") and other *MLL*-rearranged ("11q23") infant ALL (in grey). *P*-values were determined by the Kruskall-Wallis test, when comparing $LC_{50}$ values of the groups "wt MLL", "t(4;11)", "t(11;19)" and "11q23".

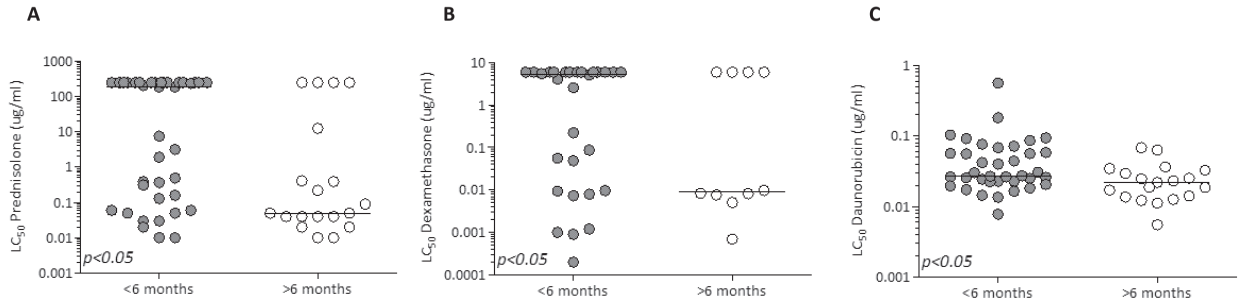


**Figure 5: Relationship between age and *in vitro* drug resistance in *MLL*-rearranged infant ALL**

*In vitro* drug response of *MLL*-rearranged infant ALL aged <6 months were compared with their counterpart, *MLL*-rearranged infant ALL with aged >6 months. All $LC_{50}$ values are depicted in the figures for (A) prednisolone, (B) dexamethasone and (C) daunorubicin. The median is shown as a horizontal bar. *P*-values were determined by the Mann–Whitney *U* test.

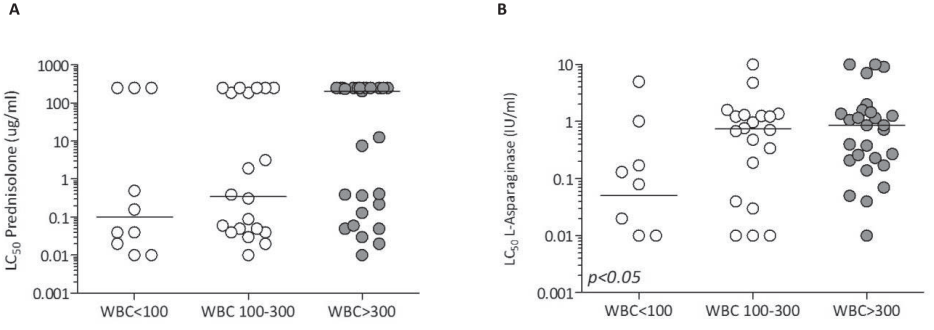


**Figure 6: Correlation of WBC and *in vitro* drug response in *MLL*-rearranged infant ALL**

*In vitro* drug response of *MLL*-rearranged infant ALL with WBCs $<100*10^6$ cells/mL were compared with their *MLL*-rearranged infant ALL with WBCs $100\text{-}300*10^6$ or $>300*10^6$ cells/mL. All $LC_{50}$ values are depicted in the figures for (A) prednisolone and (B) L-asparaginase. The median is shown as a horizontal bar. *P*-values were determined by the Kruskall-Wallis test.

($p$<0.005) and the median $LC_{50}$ values of the patient groups show that PPRs are >1000-fold more resistant to prednisolone *in vitro*, a discrepancy was found in 8/39 of the PGRs that appeared to be *in vitro* resistant to prednisolone, whereas 3/21 of the PPRs appeared to be sensitive to prednisolone *in vitro*. We found a trend of a better outcome in *MLL*-rearranged infant ALL patients that were PGR and *in vitro* sensitive for prednisolone (not significant, Figure 3C). Among PPR patients no differences in outcome between *in vitro* prednisolone respons was found (Figure 3D).

### *In vitro* drug responses and MLL translocations

*MLL*-rearranged infant ALL patients have an inferior outcome when compared with infant ALL patients who do not carry translocations of the *MLL* gene[5]. We compared *in vitro* drug responses between *MLL*-rearranged and wild-type *MLL* infant ALL samples. *MLL*-rearranged infant ALL appeared to be more resistant to vincristine, and a trend was found for increased resistance to glucocorticoids (i.e. prednisolone and dexamethasone) and to cytarabine (Ara-C) (Figure 4). Analysis by type of *MLL* rearrangement showed that t(4;11)-positive infant ALL samples were significantly more resistant to prednisolone, dexamethasone and vincristine (Figure 4). Samples of t(11;19)-positive infant ALL patients displayed a remarkable sensitivity towards the glucocorticoids prednisolone and dexamethasone, with median $LC_{50}$ values comparable to that of wild-type *MLL* infant ALL samples.

### Relation between in vitro drug response and prognostic factors in *MLL*-rearranged infant ALL

Among *MLL*-rearranged infant ALL patients, predictors of an adverse outcome identified in the Interfant-99 study, include young age, high WBC and poor prednisone response[5]. Central nervous system (CNS) involvement and a highly immature (CD10 negative) pro-B cell immunophenotype have been identified as factors adversely influencing outcome[3, 18], but were not significantly contributing to prognosis in the Interfant-99 study[5]. Infant ALL patients <6 months of age have a worse outcome than infant ALL patients >6 month of age[2-5] and therefore we analyzed *in vitro* drug sensitivity in *MLL*-rearranged infant ALL samples in both age groups. Significant differences in *in vitro* drug response were only observed for the glucocorticoids prednisolone and dexamethasone and for the anthracycline daunorubicin (Figure 5).

*MLL*-rearranged infant patients with high WBC (>300x$10^6$ cells/mL), had a worse outcome when compared with to patients with low WBC numbers (<100x$10^6$ cells/mL) or intermediate WBC numbers (100-300x$10^6$ cells/mL)[3-5]. Here we show that samples from *MLL*-rearranged patients with high WBC numbers are *in vitro* more resistant to L-asparaginase when compared to samples from patients with low WBC numbers (Kruskall-Wallis Test $p$<0.05), and tend to be more resistant to prednisolone (Figure 6; Kruskall-Wallis Test $p$=0.1446).

## DISCUSSION

*In vitro* cytotoxicity testing (MTT assays) has been very informative in childhood ALL, providing an indication of therapy effectiveness before the initiation of actual treatment. Prospective analyses showed that patients who are *in vitro* sensitive to several drugs have a superior prognosis over patients displaying *in vitro* resistance[13, 14].

Here we provide evidence of a marked association between clinical parameters (e.g. WBC, age at diagnosis, *in vivo* prednisone response) and *in vitro* cytotoxicity for a variety of drugs used in the treatment of *MLL*-rearranged infant ALL patients.

We show that the *in vitro* sensitivity to none of the drugs tested individually is capable of predicting clinical outcome. However, *MLL*-rearranged infant ALL patients displaying combined *in vitro* resistance to prednisolone, vincristine and L-asparaginase (i.e. PVA score), had inferior EFS rates. This is in concordance with studies in pediatric ALL[13, 14]. Moreover, the *in vitro* sensitivity to prednisolone appeared to correlate with the clinical response to prednisone. Interestingly, both *in vitro* prednisolone resistance as well as *in vivo* prednisone resistance have been identified as predictors of an adverse prognosis in pediatric ALL[14, 17]. For infant ALL patients the *in vitro* prednisolone is indicative for clinical outcome, but not significantly (data not shown). Additionally, within the PGR patient group, *in vitro* prednisolone sensitivity showed to be predictive for a better prognosis in childhood ALL[17] and in *MLL*-rearranged infant ALL. Hence, the strongest indicator for an adverse outcome in infant ALL patients in terms of drug resistance is the combined *in vitro* resistance to prednisolone, vincristine, and L-asparaginase (PVA), especially for *MLL*-rearranged infant ALL cases.

Furthermore, infant ALL patients samples carrying high risk features such as a t(4;11) translocation or age <6 months, or a WBC >100x$10^6$ cells/mL demonstrated to be *in vitro* resistant to the glucocorticoids prednisolone and dexamethasone. In contrast, infant ALL patients carrying translocation t(11;19) appeared to be more sensitive to these glucocorticoids. Although, t(4;11)-positive and t(11;19)-positive infant ALL patients, as well as patients carrying other types of *MLL* translocations (designated 11q23-rearranged) show a comparable poor clinical outcome[5], no similarity was observed based in terms of *in vitro* drug response profiles. Ramakers-van Woerden *et al.* observed no differences in *in vitro* drug responses in infant ALL patients by age[12]. Here we investigated age at presentation within only *MLL*-rearranged infant ALL patients; and demonstrated that *MLL*-rearranged infant ALL patients <6 months of age at diagnosis were *in vitro* more resistant to the glucocorticoids prednisolone and dexamethasone, when compared to *MLL*-rearranged infant ALL patients >6 months of age at diagnosis.

In summary, we show that for *MLL*-rearranged infant ALL patients the *in vitro* drug cytotoxicity profile can predict clinical outcome, but only by combining the responses of prednisolone, vincristine, and L-asparaginase (PVA). Moreover, established prognostic markers such as high WBC and young age at diagnosis are associated with resistance to prednisolone *in vitro*. We therefore conclude that *in vitro* PVA testing identifies *MLL*-rearranged infant ALL patients at very high risk of therapy failure, and that PVA-scoring may be used to stratify these patients more accurately if confirmed in an independent patient cohort. Clearly, PVA-resistant *MLL*-rearranged infant ALL cases form a patient group that needs alternative, more effective therapeutic strategies.

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# Chapter 4

## Frequencies and prognostic impact of *RAS* mutations in *MLL*-rearranged acute lymphoblastic leukemia in infants

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## ABSTRACT

Acute lymphoblastic leukemia (ALL) in infants represents an aggressive malignancy associated with a high incidence(~80%) of translocations involving the *Mixed Lineage Leukemia*(*MLL*) gene. Attempts to mimic MLL fusion driven leukemogenesis in mice raised the question whether these fusion proteins require secondary hits. *RAS* mutations are suggested as candidates. Earlier results on the incidence of *RAS* mutations in *MLL*-rearranged acute lymphoblastic leukemia are inconclusive. Therefore, we studied frequencies and relation with clinical parameters of *RAS* mutations in a large cohort of infant acute lymphoblastic leukemia patients.

Using conventional sequencing analysis, we screened *neuroblastoma RAS viral (v-ras) oncogene homolog gene(NRAS)*, *v-Ki-ras Kirsten rat sarcoma viral oncogene homolog gene(KRAS)*, and *v-raf murine sarcoma viral oncogene homolog B1 gene(BRAF)* for mutations in a large cohort(n=109) of infant ALL patients and studied the mutations in relation to several clinical parameters, and in relation to *Homeobox gene A9* expression and the presence of *ALL1 fused gene 4-Mixed Lineage Leukemia(AF4-MLL)*.

Mutations were detected in ~14% of all cases, with a higher frequency of ~24% in t(4;11)-positive patients($p$=0.04). Furthermore, we identified *RAS* mutations as an independent predictor($p$=0.019) for poor outcome in *MLL*-rearranged infant acute lymphoblastic leukemia, with a hazard ratio of 3.194(95% confidence interval:1.211-8.429). Also, *RAS*-mutated infants have higher white blood cell counts at diagnosis($p$=0.013), and are more resistant to glucocorticoids *in vitro*($p$<0.05). Finally, we demonstrate that *RAS* mutations, and not the lack of *Homeobox gene A9* expression nor the expression of *AF4-MLL* are associated with poor outcome in t(4;11)-rearranged infants.

We conclude that the presence of a *RAS* mutations in *MLL*-rearranged infant acute lymphoblastic leukemia is an independent predictor for a poor outcome. Therefore, future risk-stratification based on abnormal *RAS*-pathway activation and *RAS*-pathway inhibition could be beneficial in *RAS*-mutated infant acute lymphoblastic leukemia patients.

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) represents an aggressive, early onset type of leukemia characterized by high relapse rates during treatment, and an unfavorable clinical outcome[1]. This poor prognosis is associated with a high incidence of balanced chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene, which occur in ~80% of the infant ALL cases[1]. The most common *MLL* translocation in infant ALL is t(4;11), in which the N-terminus of *MLL* (chromosome 11q23) fuses to the C-terminus of *AF4* (chromosome 4q23). As the joining of *MLL* and *AF4* occurs in-frame, the t(4;11) translocation generates a unique fusion gene encoding the chimeric, and supposedly oncogenic MLL-AF4 fusion protein. Other recurrent in-frame *MLL*-rearrangements found among infant ALL patients are t(11;19) and t(9;11), giving rise to the fusion proteins MLL-ENL and MLL-AF9 respectively. The presence of an *MLL* translocation is the strongest independent predictor of an adverse outcome in infant ALL patients[2].

Over the past decades numerous studies provided important insights into the biology and pathogenesis of *MLL*-rearranged ALL, but so far *in vivo* validation of these achievements are hampered by the lack of genuine animal models accurately recapitulating this severe malignancy. Although various attempts have been made to develop mouse models mimicking leukemogenesis of human t(4;11)-positive ALL, these mice displayed propensities towards developing lymphomas or leukemia with phenotypes that significantly differ from those found in humans[3-5]. Another discrepancy between murine MLL-AF4 models and t(4;11)-positive ALL in infants is disease latency. In human infants, *MLL* translocations arise *in utero* and rapidly lead to the development of overt leukemia, often at or shortly after birth[6]. In contrast, most MLL-AF4 mouse models show mean latency periods of ~12-14 months[3, 5]. Moreover, in *MLL*-rearranged infant ALL, short disease latency is strongly associated with a poor clinical outcome[2, 7].

Collectively, these inconsistencies form the basis of the question whether MLL fusion proteins (like MLL-AF4) alone are sufficient to induce ALL, or that these chimeric proteins require cooperative genetic lesions. Bursen *et al*. recently found that not MLL-AF4 but its reciprocal fusion protein AF4-MLL (independent of the presence of MLL-AF4) was capable of inducing pro-B ALL in mice, suggesting that in t(4;11)-positive ALL both fusions may function as cooperative oncoproteins[8]. Tamai *et al* showed that in a transgenic mouse model the latency period of MLL-AF4-induced B-cell leukemia/lymphoma can significantly be shortened by the addition of a *KRAS* mutation[9]. Moreover, recent observations demonstrated that the MLL-AF4 fusion protein can activate Elk-1 through the *RAS*-pathway, which supports the involvement of *RAS* signaling in the pathogenesis of *MLL*-rearranged leukemia[10]. Based on these findings it may be hypothesized that *RAS* mutations represent important secondary "hits". Recent studies on the incidence of *RAS* mutations in *MLL*-rearranged ALL demon-

strate inconsistent results in limited patient groups. For instance, Liang *et al.* reported *RAS* mutations in 10/20 (50%) of the cases, while Mahgoub *et al.* could not identify *RAS* mutations among thirteen *MLL*-rearranged ALL samples[11, 12]. Besides, Tamai *et al.* speculate that the short latency in their *KRAS* mutation-positive mouse model is likely due to an acceleration of leukemo-lymphomagenesis by a collaborative up-regulation of *HOXA9*[9]. *HOXA* over-expression is often believed to be a hallmark of *MLL*-rearranged leukemia and has recently been proposed to be required for leukemia survival of *MLL*-rearranged acute myeloid leukemia (AML) cells[13]. Our recent gene expression profiling study revealed the presence of two distinctive subgroups of MLL-AF4 positive ALL cases; those with and those without *HOXA* expression, with patients lacking *HOXA* expression being at high risk of disease relapse[14]. Based on this finding, as well as on the report demonstrating a prominent oncogenic role for AF4-MLL[8], and the results demonstrating accelerated MLL-AF4-driven leukemogenesis in the presence of a *KRAS* mutation, Tamai *et al* proposed the following subdivision of t(4;11)-positive ALL: one group representing AF4-MLL-driven and *HOXA*-independent leukemogenesis, and another group displaying MLL-AF4 and *HOXA* dependence requiring additional genetic hits, such as *RAS* mutations, to accelerate leukemogenesis[9].

Yet, the precise frequencies and the potential role (in terms of disease aggressiveness) of *RAS* mutations in *MLL*-rearranged infant ALL, and their relation with *HOXA* expression and/or the presence of *AF4-MLL* remain unclear. Therefore, we screened a large cohort (n>100) of primary infant ALL samples for *NRAS, KRAS* and *BRAF* mutations. To further determine the clinical relevance, these mutations were studied in relation to several clinical parameters, as well as to *HOXA* expression and the presence of *AF4-MLL*.

## METHODS

### Patient samples and cell lines

Bone marrow or peripheral blood samples from untreated infants (below one year of age) diagnosed with ALL were collected at the institutes participating in the international collaborative Interfant protocol[2]. Informed consent was obtained according to the Helsinki declaration, and approved by the Institutional Review Board of the Erasmus University Medical Center. All samples were processed as described before[15].

The t(4;11)-positive cell lines SEM, RS4;11, and MV4-11 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), BEL-1 was a kind gift from Dr. Tang (University Paris, France). The t(11;19)-positive cell line KOPN-8 was purchased from The Global Biosource Center (ATCC, Middlesex, UK). All cell lines were maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine

(Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 10% FCS (Integro, Zaandam, the Netherlands).

### DNA and RNA extraction

Genomic DNA and RNA were extracted from ~5x10$^6$ leukemic cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and quantified on a Nanodrop ND-1000 spectrophotometer (Isogen). The integrity of DNA and RNA was assessed on standard 0.8% or 1.5% agarose gels, respectively.

### Detection of *NRAS, KRAS and BRAF* mutations

Using PCR and sequence analysis, mutation hotspots were screened in *NRAS* and *KRAS* exon one and two, and in *BRAF* exon 15[11, 16]. Amplicons were generated on a 2720 Thermal cycler (Applied Biosystems, Foster City, CA, USA), PCR mixture and cycling conditions are described in Supplementary Table S1. Primer sequences were adapted from previous publications[11, 16] and modified by additional M13 tags (Supplementary Table S1). Sequence analysis of both sense and antisense strands was carried out using M13 primers, and the BigDye terminator v1.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturers' recommendations, and analyzed on an Applied Biosystems 3130x/Genetic Analyzer. The CLC Workbench software (CLCbio, Aarhus, Denmark) was used to analyze the sequences, references are listed in Supplementary Table S2. All mutations were confirmed in replicate sequences.

### *In vivo* prednisone and *in vitro* prednisolone responses

*In vivo* prednisone responses, assessed during a prophase of one week of daily systemic prednisone (60 mg/m$^2$) administration before preceding combination chemotherapy, were available for a subset of patients. Responses are defined as good, when blast counts in the peripheral blood dropped below 1000 cells/μL, and poor when more than 1000 cells/μL remained detectable[2, 17].

*In vitro* drug cytotoxcity was determined using 4-day MTT assays as described elsewhere[18]. Due to our recently published gene expression profiling (GEP) study[14] microarray data (Affymetrix HU133plus2.0) was available for a part of the patient samples used in this study. Generation of these gene expression profiles has been described before[14]. Data was deposited in the GEO database[19] under accession number GSE19475. Because of our interest in the relation of *HOXA* expression and *RAS* mutations, we extracted and studied the expression of *HOXA9* from the existing dataset (probe sets: 209905_at, and 214651_s_at). GEP data was available for 27 of the 38 t(4;11)-positive infant ALL cases.

### Statistical analysis

Fisher's Exact Test was used to compare mutation frequencies in different patient groups and Mann-Whitney U-Test to compare the median age at diagnosis.

Event-free survival (EFS) and overall survival (OS) curves were estimated using the Kaplan-Meier method and analyzed by Log-rank (Mantel-Cox) tests. EFS is defined as time from diagnosis to death in induction, disease relapse, the emergence of secondary malignancies, or death in complete remission. OS is defined as time from diagnosis to death from any cause. Cumulative incidence of relapse (CIR) is defined as time from complete remission to disease relapse, adjusted for death as competing risk. Patients who did not achieve complete remission were allocated an event at time-point zero in the EFS and CIR analyses. Multivariate analysis of prognostic factors was performed by Cox regression model based on EFS and the Wald Backward Test (entry probability $p$=0.05 and removal probability $p$=0.10) was used for the joint analysis of age at diagnosis, white blood cell (WBC) counts, *in vitro* prednisolone response ($LC_{50}$: lethal concentration to 50% of the leukemic cells), *in vivo* prednisone response, and *RAS* mutations. *RAS* mutations and *in vivo* prednisone response analyzed as dichotomous variables, the other variables as continuous.

Infant ALL patients without *MLL*-rearrangements were excluded from these analyses as the prognosis of these patients is significantly more favorable[2]. CIR was computed with the statistical environment R version 2.14.0 using Bioconductor packages (R Development Core Team, 2011). The other analyses, were performed with SPSS Statistics version 17.0 (SPSS Inc. Chicago, IL, USA). All tests were two-tailed and a $p$-value less than 0.05 was considered significant.

## RESULTS

### *RAS* and *BRAF* mutations in infant ALL

*RAS* and *BRAF* mutation screening was performed in 109 primary infant ALL samples, as well as in an additional four matched relapsed samples. Patients characteristics are listed in Supplementary Table S3. Overall, in 15/109 (13.8%) of the patients a *RAS* mutation was detected, comprising 7/109 (6.4%) patients carrying an *NRAS* mutation, and 8/109 (7.2%) patients bearing a *KRAS* mutation (Table 1 and Figure 1). No *BRAF* mutations were found. Among patients carrying *NRAS* mutations two harbored an exon one mutation at codon 12, and five an exon two mutation at codon 61. All observed *KRAS* mutations were located in exon one, of which four at codon 12 and four in codon 13. (Table 1 and Figure 1). One mutation was found among the four matched relapse samples and displayed a *NRAS* Gln-61Lys mutation that was not present in the corresponding primary diagnosis sample. For the *MLL*-rearranged ALL cell lines, only KOPN-8 carried a *KRAS* mutation at exon one, at codon 12 (Gly12Asp) (Figure 1E).

**Table 1: *RAS* mutations**

| Patient | *MLL* | *AF4-MLL* | Gender | Age at Dx (months) | Immuno-phenotype | WBC at Dx ($x10^9$/ L) | *NRAS* mutation | *KRAS* mutation |
|---|---|---|---|---|---|---|---|---|
| **1** | t(4;11) | negative | male | 5.5 | pro-B | 677 | | Gly12Val |
| **2** | t(4;11) | negative | female | 10.8 | pre-B | 813.7 | Gly12Ser | |
| **3** | t(4;11) | positive | male | 1.9 | pro-B | 555 | | Gly13Asp |
| **4** | t(4;11) | positive | female | 4.1 | pro-B | 326 | Gln61Arg | |
| **5** | t(4;11) | negative | female | | pro-B | 1101.1 | Gln61Lys | |
| **6** | t(4;11) | positive | female | 1.6 | pro-B | 358.3 | | Gly13Asp |
| **7** | t(4;11) | positive | male | 3.4 | pro-B | 348.6 | | Gly13Asp |
| **8** | t(4;11) | positive | male | 6.3 | pro-B | 204 | Gln61Lys | |
| **9** | t(4;11) | positive | female | 2.3 | | 204 | | Gly12Ser |
| **10** | t(11;19) | | female | 3.6 | common | 916 | | Gly12Val |
| **11** | t(11;19) | | male | 8.1 | | | | Gly12Asp |
| **12** | t(9;11) | | male | 0.8 | pro-B | 740 | Gln61Lys | |
| **13** | 11q23* | | male | 10.8 | pro-B | 5.1 | Gln61Lys | |
| **14** | 11q23* | | female | 11.8 | | | Gly12Asp | |
| **15** | GL-*MLL*† | | female | 11.0 | common | 1.4 | | Gly13Asp |

*11q23; *MLL*-rearranged infant ALL patients with unknown partner gene,† Germline-*MLL*; infant ALL patients without *MLL*-rearrangement. Mutation in patient 8 occurred in a relapse sample, which was not present in the corresponding diagnostic sample. Abbreviations: Gly; Glycine, Val; Valine, Ser; Serine, Asp; Aspartic acid, Gln; Glutamine, Lys; Lysine.

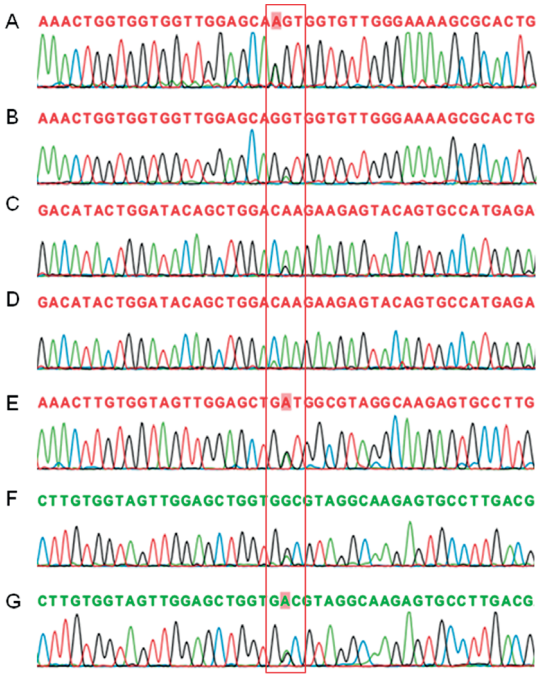


**Figure 1: *RAS* mutations**
(A) *NRAS* exon1 condon12 (Gly>Ser) mutation, corresponding with patient 2, (B) *NRAS* exon1 condon12 (Gly>Asp) mutation, corresponding with patient 14, (C) *NRAS* exon2 condon61 (Gln>Arg) mutation, corresponding with patient 4, (D) *NRAS* exon2 condon61 (Gln>Lys) mutation, corresponding with patient 13, (E) *KRAS* exon1 codon12 (Gly>Asp) mutation, corresponding with KOPN-8 cell line, (F) *KRAS* exon1 condon13 (Gly>Asp) mutation, corresponding with patient 6, (G) *KRAS* exon1 condon13 (Gly> Asp) mutation, corresponding with patient 7.

## Frequency of *RAS* mutations among different infant ALL subtypes

Next we compared the frequencies of *RAS* mutations among different infant ALL subtypes including patients with t(4;11), t(11;19), t(9:11), and infant ALL patients without *MLL* translocations. Interestingly, we found a significantly higher frequency of 9/38 (23.7%) *RAS* mutations in t(4;11)-positive infants ($p$=0.04) compared to the remaining infant ALL cases, with a frequency of 6/71 (7.8%). In the other infant ALL subtypes the frequencies were not significantly different from the total patient cohort (Table 2).

**Table 2: Frequencies of *RAS* mutations in *MLL*-subtypes of infant ALL**

| Patient | *MLL* | *AF4-MLL* | Gender | Age at Dx (months) | Immuno-phenotype | WBC at Dx (x$10^9$/ L) | *NRAS* mutation | *KRAS* mutation |
|---|---|---|---|---|---|---|---|---|
| **1** | t(4;11) | negative | male | 5.5 | pro-B | 677 | | Gly12Val |
| **2** | t(4;11) | negative | female | 10.8 | pre-B | 813.7 | Gly12Ser | |
| **3** | t(4;11) | positive | male | 1.9 | pro-B | 555 | | Gly13Asp |
| **4** | t(4;11) | positive | female | 4.1 | pro-B | 326 | Gln61Arg | |
| **5** | t(4;11) | negative | female | | pro-B | 1101.1 | Gln61Lys | |
| **6** | t(4;11) | positive | female | 1.6 | pro-B | 358.3 | | Gly13Asp |
| **7** | t(4;11) | positive | male | 3.4 | pro-B | 348.6 | | Gly13Asp |
| **8** | t(4;11) | positive | male | 6.3 | pro-B | 204 | Gln61Lys | |
| **9** | t(4;11) | positive | female | 2.3 | | 204 | | Gly12Ser |
| **10** | t(11;19) | | female | 3.6 | common | 916 | | Gly12Val |
| **11** | t(11;19) | | male | 8.1 | | | | Gly12Asp |
| **12** | t(9;11) | | male | 0.8 | pro-B | 740 | Gln61Lys | |
| **13** | 11q23* | | male | 10.8 | pro-B | 5.1 | Gln61Lys | |
| **14** | 11q23* | | female | 11.8 | | | Gly12Asp | |
| **15** | GL-*MLL*† | | female | 11.0 | common | 1.4 | | Gly13Asp |

Differences in frequencies of *RAS* mutations between *MLL*-subtypes. Patient groups were statistically analyzed using Fisher's Exact Test (2-sided) and *p*-value of less than 0.05 was considered significant. *11q23; *MLL*-rearranged infant ALL patients, with unknown or rare partner gene (including one t(1;11)-, one t(3;11)-, and three t(10;11)-positive patients, †Germline-*MLL*; infant ALL patients without *MLL*-rearrangement.

## Time of disease onset and *RAS* mutations

Early onset in *MLL*-rearranged infant ALL is associated with a poor clinical outcome[2, 7]. The median age at diagnosis of primary *RAS* mutation-negative *MLL*-rearranged infant ALL patients (3.8 months; range 0.0–11.5 months) was not different from the *RAS*-mutated group (5.3 months; range 0.8-11.8 months) ($p$=0.89). Likewise, *RAS* mutations did not seem to affect disease latency when we analyzed t(4;11)-positive infant ALL patients alone. Also, dividing patients by their age at diagnosis in the following ordinal categories: <3 months, 3-6 months, 6-9 months, 9-12 months, demonstrated no increased frequencies in any of the age groups for neither the total *MLL*-rearranged cohort ($p$=0,51), nor for t(4;11)-positive patients ($p$=0.31).

### WBC count at diagnosis and *RAS* mutations

High WBC counts at diagnosis have previously been identified as a poor prognostic factor in infant ALL[2]. Interestingly, *RAS*-mutated *MLL*-rearranged infants appeared to have significant higher WBCs at diagnosis ($p$=0.013). Approximately 82% (9/11) of the *RAS* mutation-positive cases showed WBCs higher than 300x10$^9$ cells/L, compared to ~45% (33/73) of the *RAS* mutation-negative infants. Similarly, among t(4;11)-positive cases, WBCs higher than 300x10$^9$ cells/L were found in 87,5% (7/8) of the mutated cases, and in 41.4% (12/29) of the mutation-negative cases ($p$=0.018).

### Drug resistance of *RAS*-mutated infant ALL patients

A poor *in vivo* response to prednisone represents an adverse prognostic factor in *MLL*-rearranged infant ALL[17], and *MLL*-rearranged infant ALL patients cells are highly resistant to prednisolone and dexamethasone *in vitro*[20]. *MLL*-rearranged infant ALL cells bearing a *RAS* mutation at diagnosis appeared significantly ($p$<0.05) more resistant to both glucocortocoids (Figure 2A-B). For t(4;11)-positive samples alone a comparable trend was observed, although the differences did not reach statistical significance (Figure 2C-D). No differences were found comparing the *in vivo* prednisone response of *RAS*-mutated and non-mutated *MLL*-rearranged infant ALL patients ($p$=0.451), nor by comparing *RAS*-mutated and non-mutated t(4;11)-positive cases alone ($p$=0.635). Besides, studying the control cells (without glucocorticoid treatment) in our *in vitro* cytotoxicity assays, we found *RAS*-mutated *MLL*-rearranged infant ALL cells to display significantly ($p$=0.022) higher endogeneous viability (Supplementary Figure S4). Furthermore, we asked whether exposure to glucocorticoids would invoke a positive selection for *RAS*-mutated cells in samples that ostensibly carry subclonal mutations. Therefore we performed a time lapse prednisolone exposure experiment and sequenced the *RAS* mutations in order determine whether the sequence graphs revealed a positive selection of the mutated clone. However, we did not find any signs of positive selection in both patients: the intensity of the peak corresponding to the mutated nucleotide remained equal throughout the experiment (Supplementary Figure S5). Suggesting that, either the subclone was stable during the experiment or that these mutations may not have been subclonal.

### Clinical outcome of *RAS*-mutated infant ALL patients

Clinical outcome data was available for 79 *MLL*-rearranged infant ALL cases, with 33 of them being t(4;11)-positive. The presence of a *RAS* mutation at diagnosis was associated with poor outcome in both the *MLL*-rearranged infant ALL patients, as well as in t(4;11)-positive cases alone. Among all *MLL*-rearranged infant ALL patients, the 5-year EFS rates for the *RAS* mutation-positive and negative cases was 0.0±0.0 % vs. 32.7±6·0% ($p$=0.042), and the 5-year OS was 11.1±10.5% vs. 45.3±6.0% ($p$=0.08), respectively (Figure 3A,B). CIR analysis showed a slight tendency towards a higher relapse risk for *RAS*-mutated cases, with

a 3-year CIR of 66.7±15.7% vs. 48.1±6.1% in *RAS* wild-type patients ($p$=0.119) (Figure 3C). Among the t(4;11)-positive cases comparable, but more distinctive, results were found for the 5-year EFS ($p$=0.019), 5-year OS ($p$=0.020), and 3-year CIR ($p$=0.012) (Figure 3D-F).

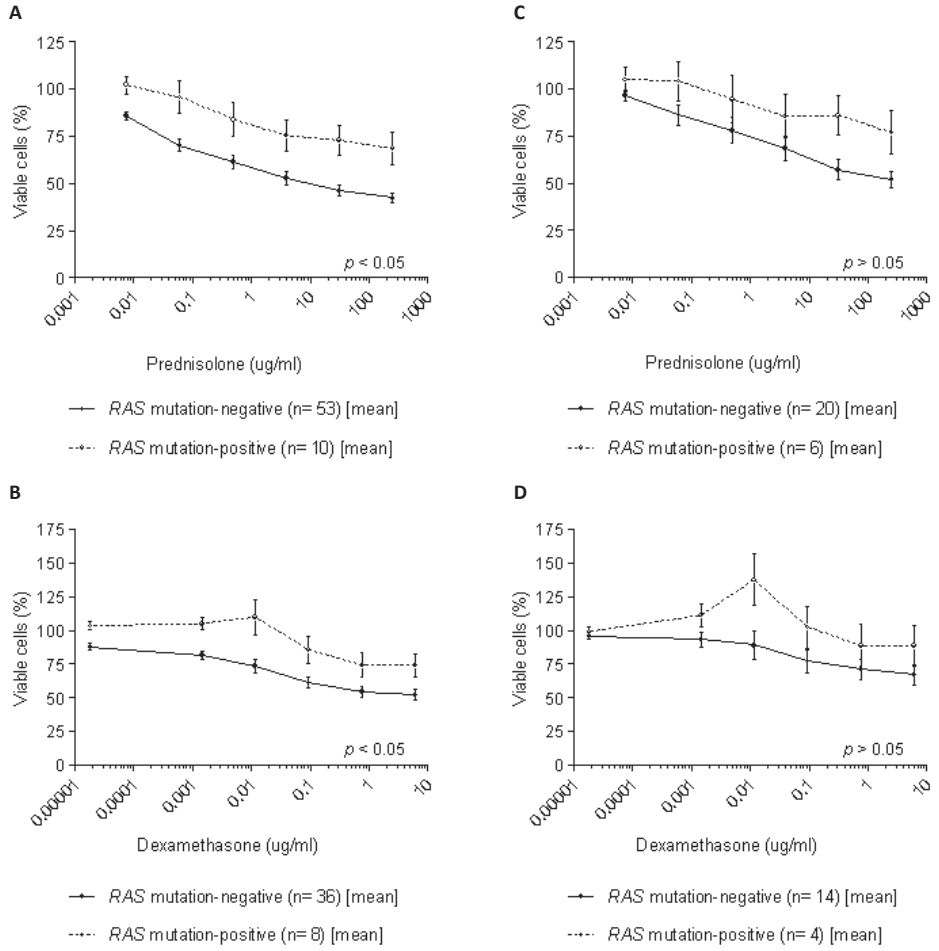


**Figure 2: Drug Cytotoxicity of *RAS*-mutated and non-mutated infant ALL patients**
(A) *In vitro* prednisolone cytotoxicity in *MLL*-rearranged infant ALL patients, (B) *In vitro* dexamethasone cytotoxicity in *MLL*-rearranged infant ALL patients, (C) *In vitro* prednisolone cytotoxicity in t(4;11)-rearranged infant ALL patients, (D) *In vitro* dexamethasone cytotoxicity in t(4;11)-rearranged infant ALL patients. Mean *in vitro* cytotoxicity responses in *RAS*-mutated and non-mutated infant ALL patients were statistically analyzed using Mann-Whitney U-test. Error bars represent standard error of the mean. Cytotoxicity data for prednisolone and dexamethasone was available for 63 and 44 *MLL*-rearranged infants ALL patients and 26 and 18 t(4;11)-rearranged infants respectively.

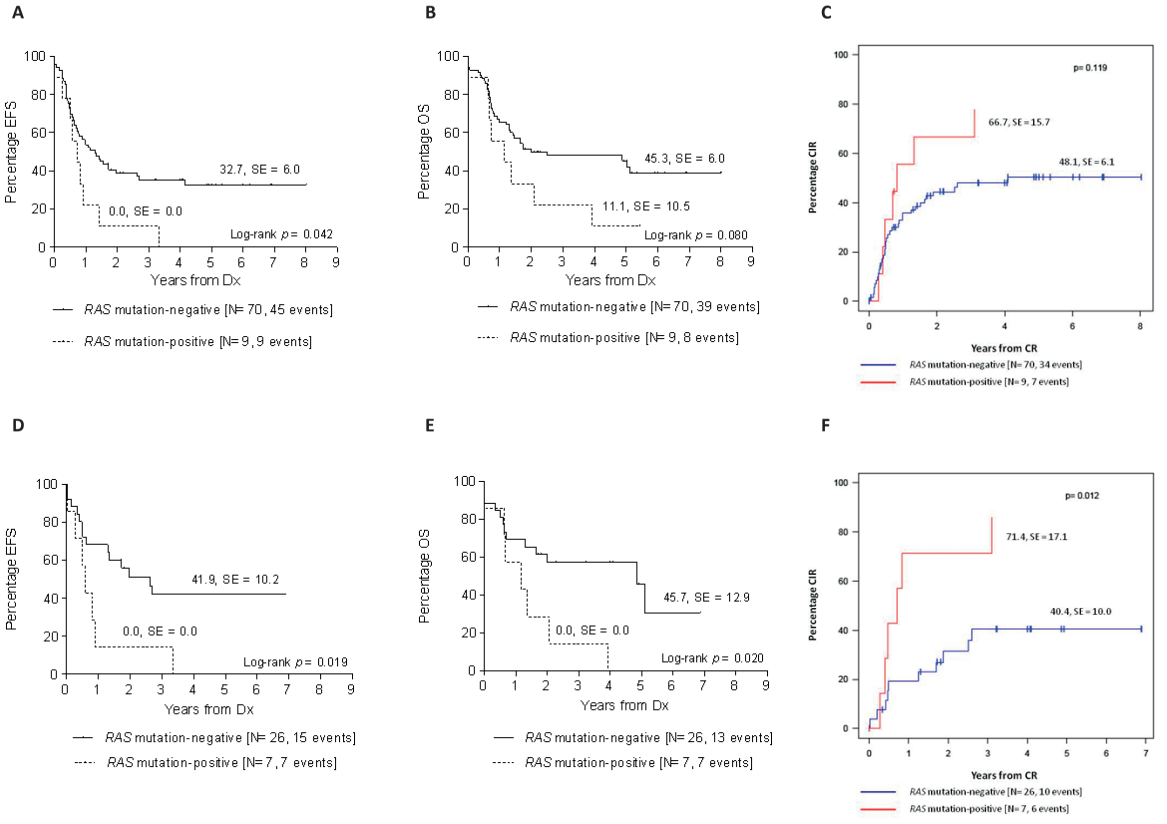


**Figure 3: Survival of *RAS*-mutated and non-mutated infant ALL patients**

(A) 5-year event-free survival (EFS), (B) 5-year overall survival (OS), (C) 3-year cumulative incidence of relapse (CIR) for *RAS*-mutated *MLL*-rearranged infant ALL patients. Survival data was available for 79 of the 91 *MLL*-rearranged infant ALL cases. (D) 5-year EFS, (E) 5-year OS, (F) 3-year CIR for *RAS*-mutated t(4;11)-positive infant ALL patients. Survival data was available for 33 of the 38 t(4;11)-positive infant ALL cases.

### *RAS* mutations in relation to *AF4-MLL* and *HOXA* expression in t(4;11)-rearranged infants

We studied the relation between the presence of *AF4-MLL* and *HOXA9* expression in t(4;11)-positive infant ALL samples and the incidence of *RAS* mutations. The occurrence of *RAS* mutations did not differ significantly between cases with *AF4-MLL* (3/15) or without *AF4-MLL* (6/23). Re-analyzing our previously published gene expression profiling data we found that all *RAS* mutation-positive cases lacked *HOXA9* expression (Supplementary Figure S6). Our earlier observations suggested that t(4;11)-positive infants lacking *HOXA* expression have a worse prognosis than patients expressing high *HOXA* levels[14]. However, when excluding the *RAS* mutation-positive cases from this analysis, the association of *HOXA* expression and clinical outcome was lost ($p$=0.857). Also, no association between *AF4-MLL* expression and clinical outcome was detected ($p$=0.354), even after excluding the *RAS*-mutated t(4;11)-positive infants ($p$=0.177). Thus, not the level of *HOXA* nor the presence of *AF4-MLL* expression, but the presence of *RAS* mutations seems to dictate the poor prognosis in these patients. Next, we asked whether *RAS* mutations influenced the previously reported prognostic value of high-level *FLT3* expression as well[21]. Therefore we studied the overlap between high *FLT3* expression and the presence of *RAS* mutations in our patient cohort, but we could not find any correlation between *FLT3* expression and *RAS* mutations at all. The *RAS*-mutated infants are equally divided between the patients with either *FLT3* high or low expression. Because this equal distribution we had no rational for re-analyzing the previous published prognosis data for *FLT3* expression in the same manner as we did with the *HOXA* expression, where all *RAS*-mutated patients had low *HOXA* expression.

### Multivariate analysis of *RAS* mutations and clinical parameters

Because the previously described clinical parameters in this study are interdependent we performed a Cox regression multivariate analysis, to evaluate the independent prognostic value of *RAS* mutations. This multivariate analysis was fitted on *MLL*-rearranged infants (n=50) from whom all prognostic variables were available. We identified the presence of a *RAS* mutation at diagnosis as an independent predictor ($p$=0.019) for poor outcome in *MLL*-rearranged infant ALL, with a hazard-ratio (HR) of 3.194 (95% confidence interval (CI): 1.211-8.429) (Table 3). Besides *RAS* mutations, low age at diagnosis was identified as an independent predictor ($p$=0.006, HR: 0.834, 95%-CI: 0.731-0.950) for poor outcome in our *MLL*-rearranged infant ALL cohort. Other variables in the final model were WBC counts at diagnosis ($p$=0.062, HR: 1.001, 95%-CI: 1.000-1.001) and *in vitro* prednisolone response ($p$=0.069, HR: 0.997, 95%-CI: 0.997-1.000) (Table 3).

**Table 3: Univariate and multivariate analysis of prognostic factors of *MLL*-rearranged infant ALL patients**

| | Univariate analysis | | | | Multivariate analysis | |
|---|---|---|---|---|---|---|
| | **Patients** | **Events** | **5-year EFS (SE)** | ***p*-value** | **HR (95%-CI)** | ***p*-value** |
| ***RAS* mutation** | | | | 0.043 | 3.194 (1.211-8.429) | 0.019 |
| Negative | 70 | 46 | 32.2% (5.9) | | | |
| Positive | 9 | 9 | 0.0% (0.000) | | | |
| **Age at diagnosis (months)** | | | | 0.020 | 0.834 (0.731-0.950)* | 0.006 |
| < 3 | 26 | 22 | 15.4% (7.1) | | | |
| 3-6 | 25 | 17 | 32.0% (9.3) | | | |
| 6-9 | 17 | 10 | 38.6% (12.4) | | | |
| 9-12 | 11 | 6 | 39.8% (16.3) | | | |
| **WBC count (x $10^9$/ L)** | | | | 0.022 | 1.001 (1.000-1.001)† | 0.062 |
| < 100 | 11 | 7 | 34.1% (15.0) | | | |
| 100-300 | 27 | 15 | 40.0% (10.5) | | | |
| >300 | 39 | 31 | 19.0% (6.6) | | | |
| **Response to prednisone prophase** | | | | 0.602 | | |
| Good respons | 34 | 22 | 34.4% (8.3) | | | |
| Poor response | 28 | 17 | 36.7% (9.8) | | | |
| ***In vitro* prednisolone response LC50 (ug/ ul)** | | | | 0.282 | 0.997 (0.994-1.000)§ | 0.069 |
| ≤ 0.100 | 19 | 13 | 33.7% (11.8) | | | |
| > 0.100 < 150 | 12 | 10 | 16.7% (10.8) | | | |
| ≥ 150 | 27 | 17 | 35.3% (9.35) | | | |

Univariate and multivariate analysis of the prognostic factors, including age at diagnosis, white blood cell (WBC) count at diagnosis, *in vivo* prednisone response, *in vitro* prednisolone response and *RAS* mutation status, in *MLL*-rearranged infant ALL patients. Multivariate analysis of prognostic factors was performed by Cox regression model based on EFS and the Wald backward test (entry probability $p$=0.05 and removal probability $p$=0.1). *RAS* mutations and *in vivo* prednisone response were in the Cox regression model analyzed as dichotomous variables, the other variables were analyzed continuous. This multivariate analysis was fitted on 50 *MLL*-rearranged infants from whom all variables were available. *Hazard-ratio (HR) per unit (months) increase of age, †HR per unit (1 x $10^9$/L) increase of WBC, § HR per unit (1 ug/ ul) increase of *in vitro* prednisolone response.

## DISCUSSION

Activating *RAS* mutations, resulting in a proliferative advantage, have been observed in several hematopoietic malignancies including, ALL, AML, chronic myelomonocytic leukemia, and juvenile chronic myelogenous leukemia[22-28]. Here we report a *RAS* mutation frequency of ~14% in a large (n>100) cohort of infant ALL cases, and a frequency of ~24% in infant ALL patients carrying *MLL* translocation t(4;11). These results are not consistent

with previously published studies that reported either high *RAS* mutation frequency of 50%, or a total absence of *RAS* mutations in *MLL*-rearranged ALL[11, 12]. The observed frequencies in these studies may have been compromised by the small patient numbers. However, these frequencies are in concordance with the previously reported frequencies of 6-20.8% *RAS* mutations found in childhood ALL[11, 29-31].

To determine the role of *RAS* mutations in respect of aggressiveness in *MLL*-rearranged infant ALL, we compared several clinical parameters in *RAS* mutation-positive and negative patients. Early onset of a *KRAS* mutation in a MLL-AF4-positive transgenic mouse model was associated with an early disease onset, and therefore suggested to represent a more aggressive leukemia[9]. We could not confirm an association between the presence of *RAS* mutations and an early onset of *MLL*-rearranged infant ALL. However, our data showed that *RAS* mutations independently contribute to a poor outcome in *MLL*-rearranged infant ALL patients. Besides, *MLL*-rearranged infant ALL patients carrying *RAS* mutations also display significantly higher WBC counts at diagnosis, and appeared significantly more resistant to the glucocorticoids *in vitro*.

Although conventional Sanger sequencing certainly is not quantitative, 4/7 (57%) of the *NRAS* mutations and 5/8 (62%) of the *KRAS* mutations appeared to be subclonal in our sequencing graphs. Repeated sequence runs on these samples persistently showed that the peak corresponding to the mutated nucleotide remained markedly smaller than the wild-type nucleotide (e.g. Figure 1D). If indeed a relatively high number of *RAS* mutations is subclonal, suggesting that not all leukemic cells carry the genetic abnormality, it seems plausible that *RAS* mutations are acquired as secondary hits after the MLL-fusions arise (for instance during a MLL-fusion-positive pre-leukemic state, or even during overt leukemia). An alternative explanation could be that *RAS* mutations are necessary for leukemogenesis and that patients harboring the wild-type *RAS* gene carry mutations in other genes supporting MLL fusion driven leukemogenesis. As we only use highly pure leukemic samples (>90% leukemic blasts), this supposed subclonality may not only indicate that a certain portion of the leukemic cells remained unaffected, but also it shows that these mutations are leukemia-specific and are unlikely to be present in germline. Unfortunately, we had no opportunity to validate this, as no germline samples were available. Nonetheless, although several of the identified *RAS* mutations may suggest subclonality, we did not find any differences in clinical parameters or outcome between patients harboring "subclonal" or "clonal" *RAS* mutation (data not shown). In order to confirm subclonality of the *RAS* mutations as implied by our Sanger sequencing results, we used TOPO® TA Cloning (Invitrogen Life Technologies, Breda, the Netherlands) to sequence single PCR-amplified DNA fragments in three patient samples (Supplementary Table S7). We found that in all patients the number of mutated fragments was lower than the expected percentage of ~50% in case the mutation

would have been clonal. Hence, these results demonstrate that *RAS* mutations in infant ALL patients can indeed be of a subclonal nature.

The observed presence of a *RAS* mutation in one of the relapse samples, which was not present in the patient-matched primary diagnostic sample, supports the hypothesis of *RAS* mutations as a secondary hit. In line with this, Case *et al.* recently demonstrated that in matched presentation/relapse samples of childhood ALL patients, *KRAS* mutations are predominantly found at relapse, and were observed at very low levels in the matched diagnostic samples[32]. In combination, these data could suggest that *RAS* mutations represent secondary hits and that *RAS*-mutated clones may very well contribute to disease aggressiveness, progression, and relapse.

Finally, our data indicates that *RAS*-pathway inhibition could be beneficial for infants. Therapy with specific *RAS*-inhibitors would eradicate the *RAS*-mutated leukemic clones, but possibly leave the non-mutated *MLL*-rearranged leukemic cells, especially in the infants that seem to harbor subclonal *RAS* mutations. Although, specific *RAS*-pathway inhibitors maybe not eradicate all leukemic clones, we strongly believe, based on our data, that targeting the *RAS*-mutated clones could lead to a less aggressive disease period and increased survival-rates. Therefore, we would not suggest *RAS*-pathway inhibition as a mono-therapy, but alongside the current infant ALL therapy. Interestingly, several *RAS*-pathway inhibitors, like tipifarib and sorafenib, are already available and currently studied in hematologic malignancies in phase I/II trials. Both compounds are well tolerated, however tipifarib activity did not seem to correlate with *RAS* mutations or *RAS* pathway-dependent activation[33]. On the other hand, phase I/II studies using sorafenib in AML and myelodysplastic syndrome patients, showed promising results and targeted inhibition of both ERK phosphorylation, as well as FLT3 signaling[34-36]. A combined inhibitory effect on both RAS and FLT3 signaling may well be highly effective in the treatment of *MLL*-rearranged infant ALL, as the majority of these patients are also characterized by constitutive FLT3 activation[15].

In conclusion, we demonstrate that *RAS* mutations frequently occur in *MLL*-rearranged infant ALL cases and especially in t(4;11)-positive infant ALL patients, and their presence represents an independent poor prognostic factor. Therefore the *RAS*-signaling pathway could be a potential target for therapeutic intervention, but also provides a rationale for future risk-stratification strategies. However, although *RAS* mutation-positive patients are at high risk of relapse, the prognosis for *RAS* mutation-negative patients remains far from favorable. Thus, a continued search for additional mutations, for instance in other components of the *RAS* pathway, that typify an unfavorable outcome, may be beneficial.

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**Supplementary Table S1:**

| Primer | Sequence (5'-3') |
|---|---|
| *NRAS* Exon 1 Fw | GTTTTCCCAGTCACGACGACTGAGTACAAACTGGTGG |
| *NRAS* Exon 1 Rv | CAGGAAACAGCTAGTACTGCATAACTGAATGTATACCC |
| *NRAS* Exon 2 Fw | GTTTTCCCAGTCACGACCAAGTGGTTATAGATGGTGAAACC |
| *NRAS* Exon 2 RV | CAGGAAACAGCTATGACAAGATCATCCTTTCAGAGAAAATAAT |
| *KRAS* Exon 1 Fw | GTTTTCCCAGTCACGACGGTGAGTTTGTATTAAAAGGTACTGGTG |
| *KRAS* Exon 1 Rv | CAGGAAACAGCTATGACCCTGTATTGTTGGATCATATTCGTCC |
| *KRAS* Exon 2 Fw | GTTTTCCCAGTCACGACGGATTCCTACAGGAAGCAAGTAGTAA |
| *KRAS* Exon 2 Rv | CAGGAAACAGCTATGACCTATAATGGTGAATATCTTCAAATGATTTAGT |
| *BRAF* Exon 15 Fw | GTTTTCCCAGTCACGACTCATAATGCTTGCTCTGATAGGA |
| *BRAF* Exon 15 Rv | CAGGAAACAGCTATGACGGCCAAAATTTAATCAGTGGA |
| M13 Fw | CAGGAAACAGCTATGAC |
| M13 Rv | GTTTTCCCAGTCACGAC |

A PCR mixture 25 µl containing 2.5 units of Amplitaq Gold polymerase (Applied Biosystems), PCR Buffer II (Applied Biosystems), 1.5 mM $MgCl_2$, 0.3 mM deoxyribonucleotide triphosphates (dNTPs) (Promega, Madison, WI, USA), 1 µM of forward and reverse primer, and ~40 ng of gDNA as a template was used. Cycling conditions were: polymerase activation at 94°C for 5 min, following 40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and one additional hold at 72°C for 10 min. *NRAS* Exon 1 primers target the entire exon 1, with the exception of the first 2 nucleotides. *NRAS* Exon 2 primers target the entire exon 2, with the exception of the first15 nucleotides. *KRAS* 1 and *KRAS* 2 primers target the entire exons 1 and 2.

**Supplementary Table S2:**

| Primer | References |
|---|---|
| *NRAS* Exon 1 | ENSE00001364464 |
| *NRAS* Exon 2 | ENSE00001450282 |
| *KRAS* Exon 1 | ENSE00001189804 |
| *KRAS* Exon 2 | ENSE00000936617 |
| *BRAF* Exon 15 | ENSE00002324725 |

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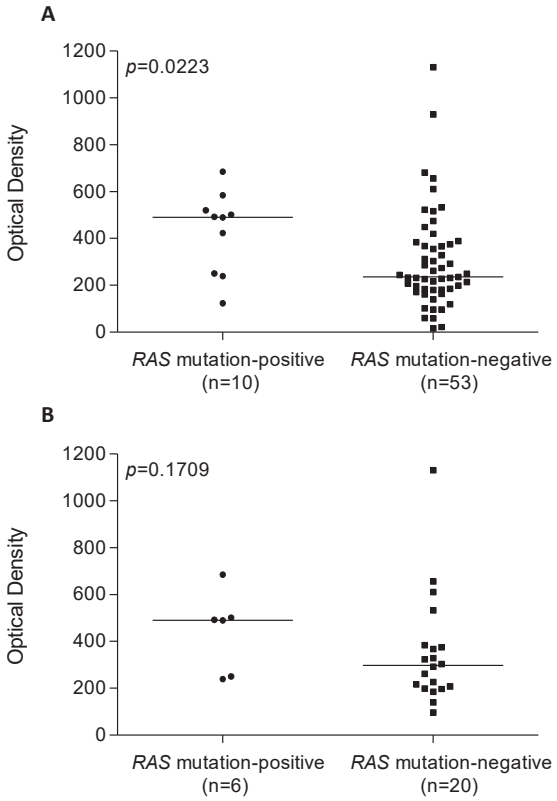
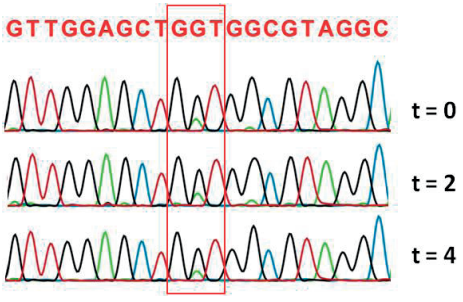
**Supplementary Table S3:**

| | No. of patients (%) |
|---|---|
| **Sex (n=108)** | |
| Male | 43 (39.8) |
| Female | 65 (60.1) |
| **Age (median, range, days, n=108)** | 164 (1-360) |
| **WBC counts at diagnosis x $10^9$/L (median, range, n=101)** | 263 (1.4-1332) |
| **Infant ALL (n=97 samples)** | |
| pro-B | 64 (66.0) |
| common B | 8 (8.3) |
| pre-B | 21 (21.7) |
| B-lineage not classified | 1 (1.0) |
| Biphenotypic | 1 (1.0) |
| T-cell | 2 (2.0) |
| ***MLL*-rearrangement (n=109)** | |
| t(4;11) | 38 (34.9) |
| t(9;11) | 11 (10.1) |
| t(11;19) | 28 (25.7) |
| other 11q23* | 14 (12.8) |
| germline-*MLL*† | 18 (16.5) |
| ***AF4/ MLL* (n=38)** | |
| positive | 23 (60.5) |
| negative | 15 (39.5) |

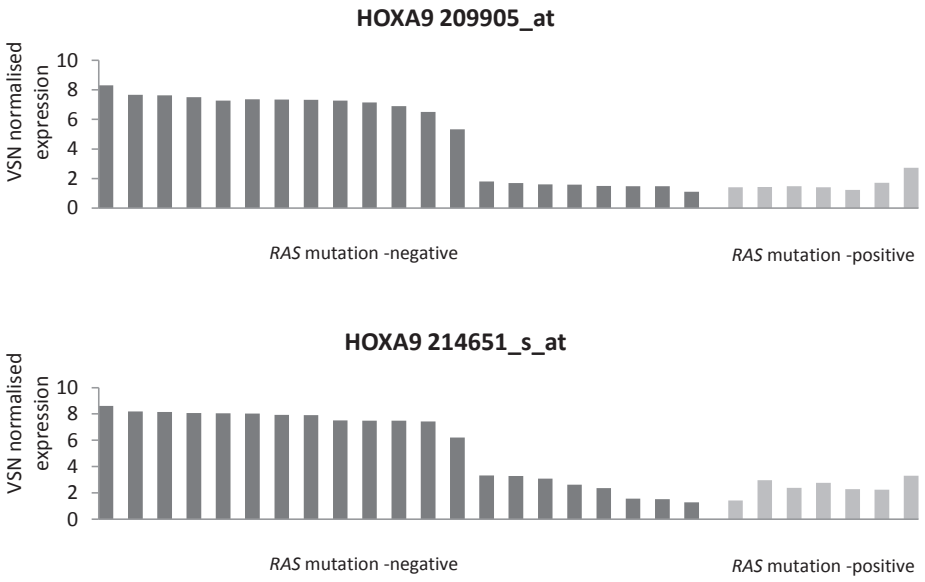
*11q23; *MLL*-rearranged infant ALL patients, with unknown or rare partner gene (including one t(1;11)-, one t(3;11)-, and three t(10;11)-positive patients), common partner genes (t(4;11), and t(11;19)) were excluded by PCR,† Germline-*MLL*; infant ALL patients without *MLL*-rearrangement Abbreviations: WBC; white blood cell.




**Supplementary Figure S4: Optical Density (cell viability) of untreated primary infant ALL cells**
In our *in vitro* cytotoxicity assays (i.e. MTT assays), leukemic cells are cultured in the presence of increasing drug concentrations. Cell viability is determined by measuring the optical density (OD) of the blue formazan derivative of the tetrazolium MTT, which is formed only by viable cells. Here we compared the ODs of control cells (i.e. cultured in the absence of drug) between *RAS*-mutated and non-mutated samples derived from (A) *MLL*-rearranged infant ALL patients and (B) t(4;11)-positive infant ALL patients. Differences in the ODs between *RAS*-mutated and non-mutated samples were statistically analyzed by the Mann-Whitney U-test. The horizontal lines represent the median OD in each group.




**Supplementary Figure S5: *RAS* mutations in prednisolone exposed infant ALL samples**
Representative sequences from time lapse *in vitro* prednisolone exposure experiment (*KRAS* exon1 condon12(Gly> Asp mutation). Two infant ALL samples (that appeared to have subclonal *RAS* mutations) were exposed to various concentrations of prednisolone (0 µg/ml, 0.488281 µg/ml, and 250 µg/ml). Cells were harvested from different time-points (Day 0, Day 2, and Day 4) and sequenced for the *RAS* mutations.




**Supplementary Figure S6: *HOXA9* expression of t(4;11)-rearranged infant ALL patients**
*HOXA9* mRNA expression (Affymetrix HU133plus2.0 microarray data) in t(4;11)-positive infant ALL patients. Differences in expression between *RAS*-mutated and non-mutated were statistically analyzed by the Mann-Whitney U-test, showing significant lower levels of *HOXA9* expression in *RAS*-mutated cases for both probe-sets tested: 209905_at ($p$=0.006) and 214651_s_at ($p$=0.020).

**Supplementary Table S7:**

| Patient | Clonality based on Sanger sequencing | No. of mutated clones (analyzed clones) | Mutated clones (%) | Expected mutated clones if clonal* (%) | *p*-value |
|---|---|---|---|---|---|
| **3** | subclonal | 5 (16) | 31.5 | 48 | 0.069 |
| **6** | subclonal | 12 (43) | 27.9 | 49.5 | 0.003 |
| **7** | clonal | 9 (43) | 20.9 | 48.5 | <0.001 |

In order to confirm subclonality of the *RAS* mutations as implied by our Sanger sequencing results, we used TOPO® TA Cloning (Invitrogen Life Technologies, Breda, the Netherlands) to sequence single PCR-amplified DNA fragments in three patient samples (i.e. patient #3, #6, and #7). For this, multiple PCR fragments from the three patient samples positive for the *KRAS1* (Gly13Asp) mutation were individually ligated into the TA cloning vector (pCR®2.1), and the constructs were transformed into competent *E.coli* cells using heat shock. Transformed cells were then spread LB agar plates containing X-Gal, 100 μg/ml ampicillin, and incubated overnight at 37°C. Next, multiple single colonies (each containing multiple copies of a single PCR fragment) were picked for plasmid isolation using the QiaPrep Spin Miniprep Kit (Qiagen, Venlo, The Netherlands). Finally, the inserts of all plasmids were sequenced by Sanger sequencing as described in the Design and methods section of the manuscript. In all patients the number of mutated DNA fragments was lower than the expected percentage in case the mutation would have been clonal (*corrected for the percentage blasts (96-99%) in the patient samples). The results were statistically analyzed using the one-sided Z-test. Although the results of patient #3 did not reach statistically significance (which may be due to the relatively low number of clones analyzed) these results demonstrate that *RAS* mutations in infant ALL patients can indeed be subclonal. However, results derived from conventional Sanger sequencing must be interpreted with care, as the *RAS* mutation in patient #7 appeared clonal in our initial sequencing data, but clearly turned out to be subclonal when analyzing single PCR fragments.

# Chapter 5

## MEK inhibition is a promising therapeutic strategy for *MLL*-rearranged infant acute lymphoblastic leukemia patients carrying *RAS* mutations

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## ABSTRACT

Acute lymphoblastic leukemia (ALL) in infants is an aggressive malignancy with a poor clinical outcome, and is characterized by translocations of the *Mixed Lineage Leukemia (MLL)* gene. Previously, we identified *RAS* mutations in 14-24% of infant ALL patients, and showed that the presence of a *RAS* mutation decreased the survival chances even further. We hypothesized that targeting the RAS signaling pathway could be a therapeutic strategy for *RAS*-mutant infant ALL patients. Here we show that the MEK inhibitors Trametinib, Selumetinib and MEK162 severely impair primary *RAS*-mutant *MLL*-rearranged infant ALL cells *in vitro*. While all *RAS*-mutant samples were sensitive to MEK inhibitors, we found both sensitive and resistant samples among *RAS*-wildtype cases. We confirmed enhanced RAS pathway signaling in *RAS*-mutant samples, but found no apparent downstream overactivation in the wildtype samples. However, we did confirm that MEK inhibitors reduced p-ERK levels, and induced apoptosis in the *RAS*-mutant *MLL*-rearranged ALL cells. Finally, we show that MEK inhibition synergistically enhances prednisolone sensitivity, both in *RAS*-mutant and *RAS*-wildtype cells. In conclusion, MEK inhibition represents a promising therapeutic strategy for *MLL*-rearranged ALL patients harboring *RAS* mutations, while patients without *RAS* mutations may benefit through prednisolone sensitization.

## INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) represents an aggressive malignancy, associated with high relapse rates and a poor clinical outcome.[1] The majority (~80%) of these patients carry a leukemia-specific chromosomal translocations involving the *Mixed Lineage Leukemia* (*MLL*) gene.[1] *MLL*-rearranged infant ALL patients fare significantly worse than infant ALL patients who do not carry *MLL* translocations, with event-free survival (EFS) rates of 30-40% vs. ~80%, respectively.[2] Recently, we demonstrated that 24% of the infant ALL patients carrying *MLL* translocation t(4;11), the most frequently observed translocation of *MLL* among these patients, also carry a *RAS* mutation. Mutations in *NRAS* were found in 11% and *KRAS* mutations in 13% of cases.[3] Moreover, we showed that the presence of a *RAS* mutation in *MLL*-rearranged patients represented an independent predictive factor for an even worse clinical outcome in this high-risk group. Nearly all *RAS*-mutant t(4;11)$^{+}$ infant ALL patients relapsed within the first year from diagnosis, while still on treatment, and all died within 4 years from diagnosis.[3]

Despite this strong association with an exceedingly poor prognosis, a recent study by Emerenciano *et al*. suggested that *RAS* mutations in *MLL*-rearranged infant ALL may not act as driver mutations and are not required for disease progression, but rather act only at disease onset.[4] Yet, our previous data clearly showed that *RAS*-mutant *MLL*-rearranged infant ALL patients are at extremely high-risk of therapy failure and early death. Moreover, RAS pathway inhibition, including MEK inhibition, was previously shown to effectively inhibit *RAS*-mutant *MLL*-rearranged AML *in vitro*.[5, 6] Therefore, we decided to investigate the potential of *RAS* pathway inhibition and found that *RAS*-mutant *MLL*-rearranged ALL cells are remarkably sensitive to MEK inhibitors.

## METHODS

### Patient samples and cell lines

Bone marrow and peripheral blood samples from untreated infant ALL patients were collected at the Sophia Children's Hospital (Rotterdam, The Netherlands) as part of the international collaborative Interfant treatment protocols.[2] Approval for these studies was obtained from the Erasmus MC Institutional Review Board. Informed consent was obtained according to the Declaration of Helsinki. All samples were processed within 24 hours after sampling as described before, with optional removal of contaminating non-leukemic cells by immunomagnetic beads, to ensure leukemic blast content for all samples was >90%.[7] The t(4;11)-rearranged ALL cell line SEM and t(11;19)-rearranged ALL cell line KOPN8 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ,

Braunschweig, Germany), while the t(4;11)-rearranged ALL cell line RS4;11 was purchased from The Global Biosource Center (ATCC, Middlesex, UK). All cell lines were cultured in suspension in RPMI-1640 with GlutaMAX (Invitrogen Life Technologies, Waltham, MA, USA) supplemented with 10% Fetal Calf Serum, 100 IU/mL penicillin, 100 IU/mL streptomycin and 0.125 μg/mL amphotericin B (Invitrogen Life Technologies) at 37°C under 5% $CO_2$ atmosphere.

### *In vitro* cytotoxicity assay and small molecule inhibitors

The *in vitro* cytotoxicity of MEK162, Selumetinib and Trametinib (MedChem Express, Stockholm, Sweden) was tested by MTS and MTT assays. All inhibitors were weighed, dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C until use. Cytotoxicity assay dilutions were prepared in cell culture medium, keeping final DMSO concentration <0.5%. Final concentrations of the small molecule inhibitors ranged from 50 μM to 0.15 nM, indicated in the respective figures. The *in vitro* sensitivity of cell lines was assessed by using 4-day MTS conversion assays, as described previously.[8] *In vitro* cytotoxicity of patient cells was assessed by using a 4-day MTT conversion assay, as described before.[7] Data was normalized to vehicle (DMSO) controls.

### Western blot analysis

Protein extracts (25 μg) were electrophoretically resolved on pre-cast SDS-polyacrylamide gels (anyKD, TGX, Bio-Rad, Veenendaal, The Netherlands) and transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin and subsequently probed with antibodies directed against total or phosphorylated ERK, MEK, ELK-1, Akt, or p70S6K (Cell Signaling, Danvers, MA, USA). Membranes were counterstained with IRDye® 680/800 conjugated secondary antibodies (Li/COR, Leusden, The Netherlands) and were scanned by an Odyssey imaging system (Li/COR). Membranes were re-probed with mouse monoclonal anti-β-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA) as loading control. Fluorescence was quantified using the Odyssey 3.0 application software.

### RAS activation

RAS activation was analyzed using the RAS Activation Assay Kit (17-218, Merck-Millipore, Amsterdam, The Netherlands). Briefly, $1x10^7$ cells were isolated and lysed with $Mg^{2+}$ Lysis Buffer (MLB), and stored at -80°C until use. GST-fused RAF-1 RBD bead slurry was added to the lysate and incubated for 1 hour at 4°C while agitating. Beads were isolated by centrifugation and washed with MLB, and precipitated protein was denatured with Laemmli buffer at 95°C before immunoblotting. As a positive control, total cell lysate was included in the immunoblotting procedure. The provided RAS antibody (05-516, Merck-Millipore) was used, and GST (Cell Signaling) and β-actin (Sigma-Aldrich) antibodies were used as

loading controls for the beads and total protein, respectively. Fluorescence was quantified using the Odyssey 3.0 application software.

### Annexin-V/7-AAD apoptosis and cell cycle assays

For assessment of early and late apoptosis, the PE Annexin-V Apoptosis Detection Kit (BD Pharmingen, Breda, The Netherlands) was used according to the manufacturer's protocol. Briefly, drug-exposed cells were isolated, washed with PBS and re-suspended in binding buffer. Cells were stained with PE Annexin V and/or 7-AAD for 15 minutes, and sorted using fluorescence activated cell sorting (FACS). Cell cycle progression was assessed by permeabilization of isolated cells through hypotonic lysis. Subsequently, RNAse treatment was performed, and DNA was stained using Propidium Iodide, after which FACS determined DNA content. Data was analyzed using FlowJo software (FlowJo, Ashland, OR, USA).

### Gene expression data

Recently published gene expression data (Affymetrix HU133plus2.0) for part of the $t(4;11)^{+}$ patient samples was available (i.e. for 6 of 9 MEK inhibitor resistant *RAS*-wildtype samples, 4 of 5 sensitive *RAS*-wildtype samples and 3 of 6 *RAS*-mutant samples).[9] This data is available in GEO database19 (accession number GSE19475) and was acquired as previously described.[9] Tyrosine kinase receptor expression was derived from this dataset, using the following probe sets: 206674_at (FLT3), 204406_at (VEGFR-1), 203934_at (VEGFR-2), 234379_at (VEGFR-3), 210973_s_at (FGFR-1), 208225_at (FGFR-2), 204380_s_at (FGFR-3), 204579_at (FGFR-4), 211551_at (EGFR), 210930_s_at (ERBB2), 226213_at (ERBB3), 214053_at (ERBB4), 205463_s_at (PDGFR-A), 217112_at (PDGFR-B), 204891_s_at (Lck) and 213324_at (Src).

### Statistical analysis

Statistical analyses were performed with SPSS Statistics version 17.0 (SPSS Inc. Chicago, IL, USA). All tests were two-tailed and *p*-values < 0.05 were considered significant. The effect of combining drugs (i.e. synergy, additivity or antagonism) was assessed using Berenbaums criteria, as previously described.[10, 11] Briefly, we calculated the Synergy Factor ($F_{Syn}$) with the formula $F_{Syn} = ([\text{Drug X}_{\text{in combination with Y}}]/[\text{Drug X}]) + ([\text{Drug Y}_{\text{in combination with X}}] / [\text{Drug Y}])$ for a particular fractional effect. If the drug combination results in $F_{Syn} < 1$, this is considered synergy.

## RESULTS

### *RAS*-mutant *MLL*-rearranged ALL cells are sensitive to MEK inhibition

Since the previously identified *RAS* aberrations are all activating mutations (at residues G12, G13 or Q61), we wondered whether small molecule inhibitors targeting RAS pathway components could suppress *RAS*-mutant leukemic cells.[3, 12] Therefore, 7 RAS pathway inhibitors, already approved for therapeutical use or under clinical investigation for other malignancies with RAS pathway mutations, were selected as therapeutic strategies for the *RAS*-mutant infant ALL patients. Using 4-day MTS cell viability assays we tested the *in vitro* anti-leukemic potential of Salirasib (RAS localization inhibitor), Vemurafenib (BRAF inhibitor), Sorafenib (pan-kinase inhibitor), Trametinib, Selumetinib and MEK162 (MEK inhibitors) and Temsirolimus (mTOR inhibitor) against *RAS*-mutant *MLL*-rearranged ALL cell line KOPN8, and the *RAS*-wildtype *MLL*-rearranged cell lines SEM and RS4;11. Interestingly, the *RAS*-mutant cell line KOPN8 was more sensitive to the MEK inhibitors MEK162, Selumetinib and Trametinib (Figure 1). Temsirolimus and Sorafenib potently reduced cell viability of both *RAS*-mutant and *RAS*-wildtype cell lines. Additionally, Salirasib and Vemurafenib did not substantially reduce cell viability, even at high concentrations (>10μM). To confirm the efficacy of these inhibitors, we performed 4-day MTT cell viability assays on primary diagnostic *RAS*-mutant (n=6) and *RAS*-wildtype (n=14) t(4;11)$^{+}$ infant ALL samples. Interestingly, compared to *RAS*-wildtype t(4;11)$^{+}$ ALL cases, the *RAS*-mutant t(4;11)$^{+}$ infant ALL cases were significantly more sensitive to all MEK inhibitors (Figure 2A) with median $IC_{50}$ values of <0.1 μM for MEK162 and Selumetinib and <0.01 μM for Trametinib (Figure 2B). Additionally, all other tested inhibitors (Salirasib, Temsirolimus, Sorafenib and Vemurafenib) reached only $IC_{50}$ values of >10μM (Supplementary Figure S1).

Also, we included one matched pair of diagnostic/relapse t(4;11)$^{+}$ samples. For this particular patient, no *RAS* mutation was present at diagnosis, but a *RAS* mutation could be identified at relapse. Indeed, the *RAS*-mutant relapse sample of this patient was more sensitive to all three MEK inhibitors tested than the *RAS*-wildtype diagnostic sample (Figure 2B).

### Enhanced RAS activation in t(4;11)$^{+}$ infant ALL cells carrying *RAS* mutations

The MEK inhibitors MEK162, Selumetinib and Trametinib significantly reduce viability of *RAS*-mutant *MLL*-rearranged ALL cells. Notably, a subset of the *RAS*-wildtype primary t(4;11)$^{+}$ infant ALL samples also responded favorably to the MEK inhibitors (Figure 2B). We wondered whether other biomarkers, besides *RAS* mutation status, could predict MEK inhibitor sensitivity in *MLL*-rearranged ALL. Wildtype RAS proteins are under regulation of upstream signaling events, often involving tyrosine kinase receptors, while mutant RAS proteins are less dependent on upstream activation due to reduced GTPase activity, rendering a surplus of activated GTP-bound RAS. Therefore, we determined the RAS protein levels

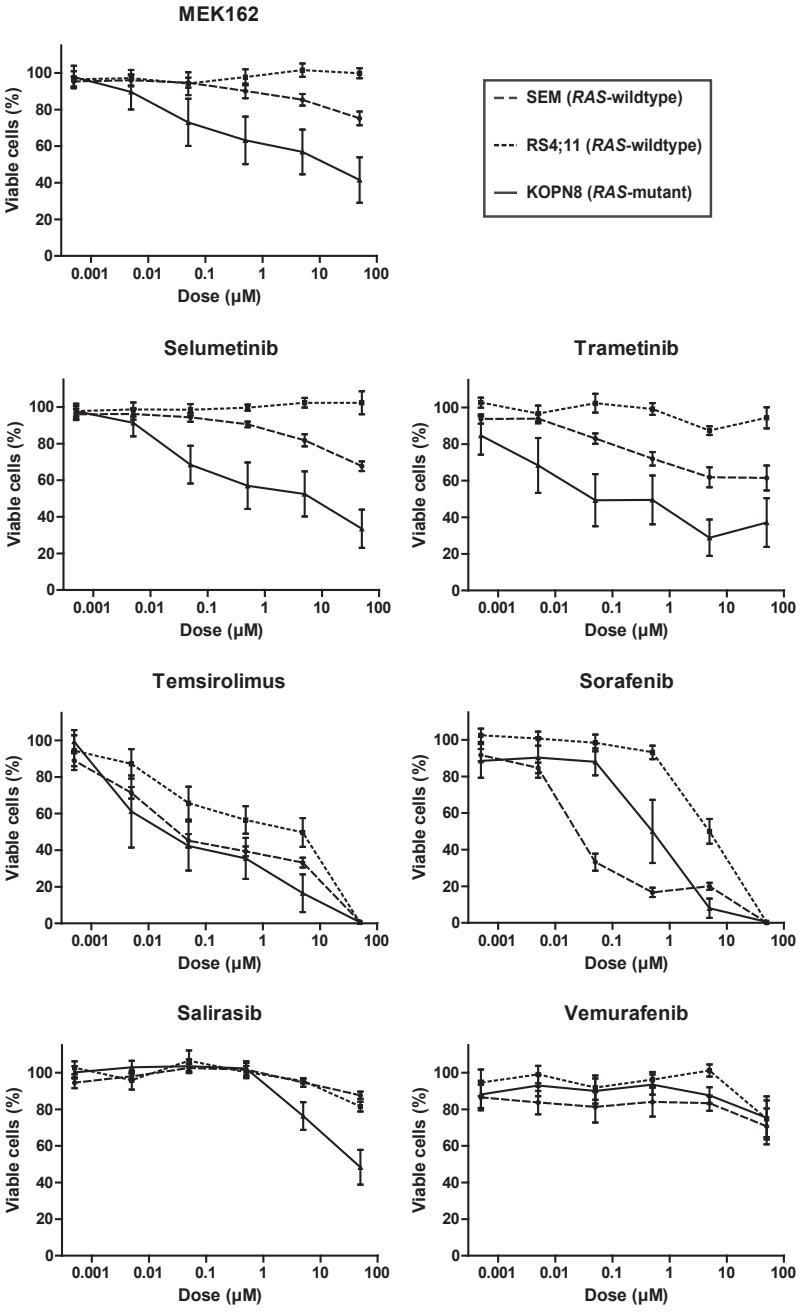


**Figure 1: MEK inhibitors specifically impede *RAS*-mutant *MLL*-rearranged ALL cell line KOPN8**
Cell viability of *MLL*-rearranged cell lines exposed to Sorafenib, Salirasib, MEK162, Selumetinib, Temsirolimus, Vemurafenib and Trametinib. All cell lines respond to Sorafenib and Temsirolimus, while *RAS*-mutant KOPN8 (solid line) is more sensitive than *RAS*-wildtype SEM (large dashed line) or RS4;11 (small dashed line). Data are represented as mean +/- sem. n≥3.

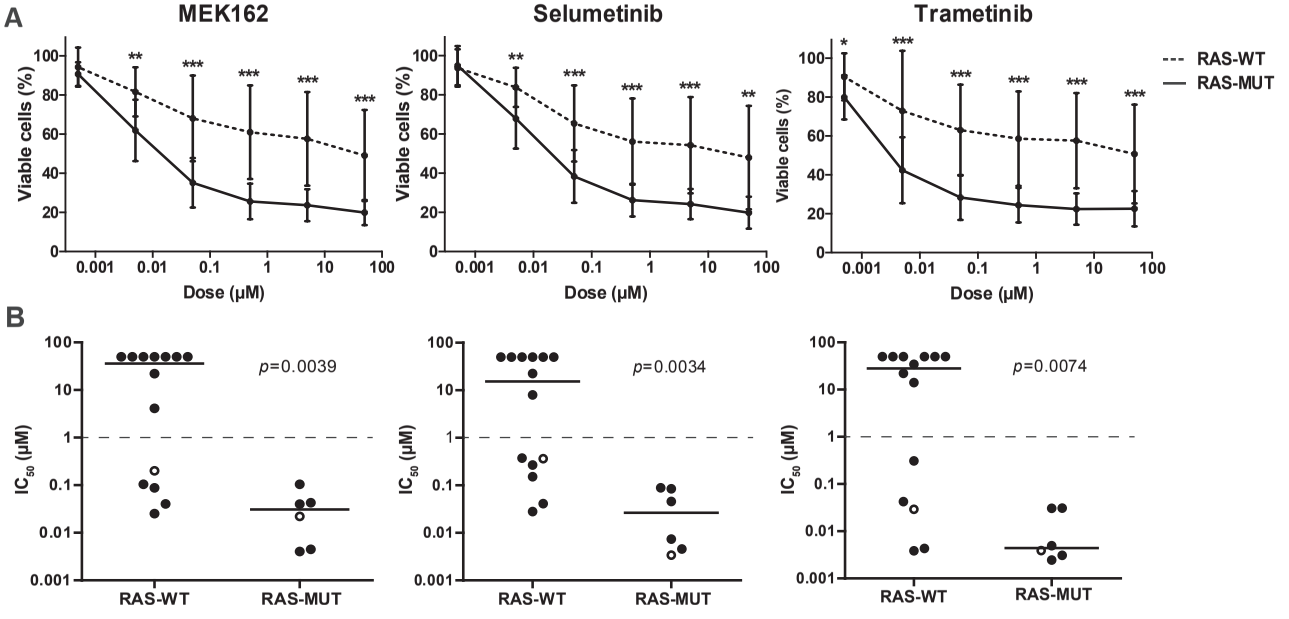


**Figure 2: Primary *RAS*-mutant *MLL*-rearranged ALL cells are sensitive to MEK inhibitors**

(A) Patient derived t(4;11)$^{+}$ infant ALL cells exposed to MEK inhibitors indicate *RAS*-mutant samples (solid line, n=6) are more sensitive compared to *RAS*-wildtype samples (dashed line, n=14). Data are represented as median +/- sd. *0.01<$p$<0.05; **0.001<$p$<0.01; ***$p$<0.001. (B) The $IC_{50}$ (concentration needed to inhibit 50% of the leukemic cell viability) of the individual t(4;11)$^{+}$ infant ALL patient samples shown in A. Median $IC_{50}$ values, represented by horizontal bars, confirm strong sensitivity of *RAS*-mutant patient samples compared to the majority of *RAS*-wildtype samples. Open circles indicate matched diagnosis (wildtype) and relapse (mutant) samples. The tick lines indicate separation between MEK inhibitor sensitive and resistant patient samples ($IC_{50}$<1μM and $IC_{50}$>1μM, respectively).

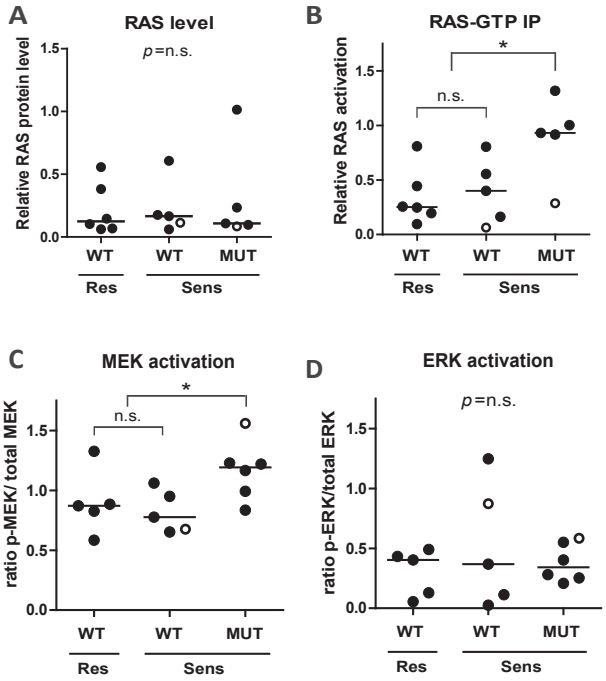


**Figure 3: *RAS*-mutant t(4;11)-positive ALL cells have enhanced downstream activation**
(A) RAS protein level, relative to β-actin, determined by western blotting in t(4;11)$^+$ infant ALL samples, subdivided according to *RAS* mutation status (WT or MUT) and MEK inhibitor sensitivity. No differences in median protein level (horizontal bars) are observed between the different subgroups. (B) Relative RAS activation is enhanced in *RAS*-mutant t(4;11)$^+$ patient samples, though no difference is observed between the MEK inhibitor resistant and sensitive *RAS*-wildtype subgroups. (C) Ratio p-MEK/total MEK in *RAS*-mutant (MUT) and *RAS*-wildtype (WT) t(4;11)-rearranged infant ALL samples shows increased MEK activation in *RAS*-mutant samples, while the MEK inhibitor resistant (Res) and sensitive (Sens) *RAS*-wildtype samples have comparable MEK activation. (D) Ratio p-ERK/total ERK in *RAS*-mutant and *RAS*-wildtype t(4;11)-rearranged infant ALL samples shows no significant differences in ERK activation between subgroups. Open circles indicate matched diagnosis (wildtype) and relapse (mutant) samples. Horizontal bars present group medians. Open circles indicate matched diagnosis (wildtype) and relapse (mutant) samples. *$p$<0.05.

and RAS activity in our primary t(4;11)$^+$ infant ALL cells. No significant difference in RAS protein levels was observed between the *RAS*-mutant and *RAS*-wildtype t(4;11)$^+$ infant ALL samples using Western blot analysis (Figure 3A). Next, we investigated the level of active (GTP-bound) RAS in these samples by precipitation with RAF-1 RAS interaction protein, followed by immunoblotting. As expected, the *RAS*-mutant t(4;11)$^+$ infant ALL samples showed significant ($p$=0.013) higher levels of RAS activation as compared to *RAS*-wildtype samples (Figure 3B). No differences in RAS activation were observed between *RAS*-wildtype samples that were sensitive or resistant to MEK inhibition.

Subsequently, we determined phosphorylation levels of MEK (p-MEK) and ERK (p-ERK) by immunoblotting (Supplementary Figure S2A and B, respectively). Quantification of the blots indicated a significantly higher level of p-MEK in our *RAS*-mutant samples, compared to *RAS*-wildtype samples ($p$=0.0312, Figure S3C), although there was no difference in p-MEK levels between the MEK inhibitor resistant and sensitive *RAS*-wildtype subgroups. Still, we did find a higher p-MEK level in the mutated relapse sample compared to its matched wildtype diagnosis sample. Additionally, no differences in p-ERK levels were found between *RAS*-wildtype and *RAS*-mutant samples (Figure 3D), nor between *RAS*-wildtype cells that were sensitive or resistant to MEK inhibition.

In *MLL*-rearranged AML, MEK inhibitor resistance can occur through activation of tyrosine kinase receptor (TKR) signaling (i.e. involving VEGFR-2).[5] Furthermore, we previously found *MLL*-rearranged ALL is characterized by high expression of Fms-like tyrosine kinase 3 (*FLT3*).[7] Therefore, we interrogated available gene expression profiles of primary samples for possible differences in TKR expression levels between the MEK inhibitor sensitive and resistant subgroups (Supplementary Figure S3). Interestingly, apart from *FLT3*, expression of TKRs is relatively low in the different patient samples. Surprisingly, *FGFR-1* expression is

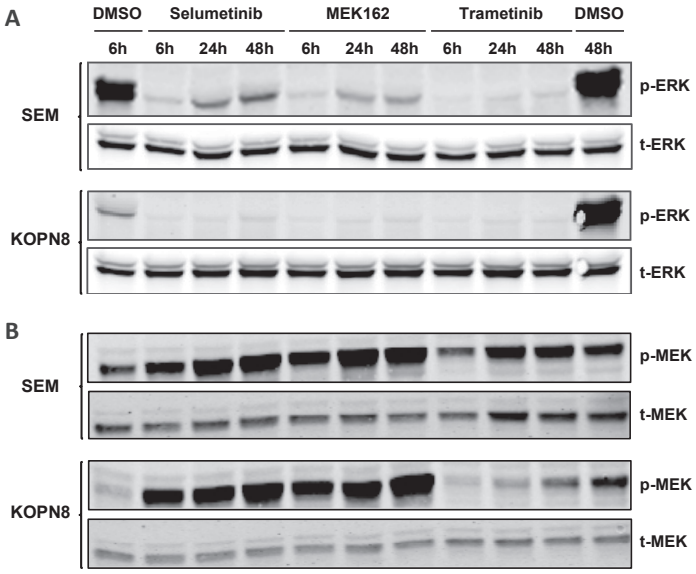


**Figure 4: MEK inhibition results in reduced ERK phosphorylation**
(A) Western blot analysis of SEM and KOPN8 (upper and lower panels, respectively) exposed to 500 nM of MEK inhibitor or vehicle control (DMSO) for 6, 24 and 48 hours. Both cell lines almost completely lose ERK phosphorylation (p-ERK), while total ERK (t-ERK) levels remain unaffected. (B) Analysis of MEK phosphorylation (p-MEK) suggests exposure to MEK162 and Selumetinib results in enhanced MEK phosphorylation in both cell lines, whereas total MEK (t-MEK) levels remain constant.

significantly lower in MEK inhibitor resistant *RAS*-wildtype samples ($p$=0.02), while there are no significant differences in expression of *FLT3*, *VEGFR* (1-3), *FGFR* (2-4), *EGFR* and *ERBB* (2-4), *PDGFR* (A-B) or *Lck* and *Src*.

### MEK inhibition results in reduced ERK phosphorylation

Next, we exposed *MLL*-rearranged ALL cell lines SEM and KOPN8 to the MEK inhibitors (Selumetinib, MEK162 and Trametinib) and determined p-ERK and p-MEK levels by immunoblotting (Figure 4). Interestingly, p-ERK levels were drastically reduced in both SEM and KOPN8, already after 6 hours of exposure, and this effect was sustained for at least 48 hours, regardless of the inhibitor used (Figure 4A). Furthermore, prolonged exposure (24 and 48 hours) to the MEK inhibitors Selumetinib and MEK162 resulted in an increase of p-MEK in SEM and KOPN8 (Figure 4B). Additionally, we determined phosphorylation of ERKs downstream effector ELK-1, but ELK-1 activation was not influenced by MEK inhibition (Supplementary Figure S4A).

Since SEM cells responded modestly to MEK inhibition but did show a significant loss of p-ERK levels, we investigated whether these cells could circumvent loss of ERK activation by upregulating RAS-mediated PI3K-Akt-mTOR signaling. Therefore, the downstream phosphorylation of Akt (Ser437) and p70S6K (Thr389) was assessed by immunoblotting. However, no differences in Akt and p70S6K phosphorylation were observed in response to MEK inhibitor exposure (Supplementary Figure S4B and S4C).

### MEK inhibitors induce apoptosis

Subsequently, we investigated the phenotypic effects of the MEK inhibitors on SEM and KOPN8 through analysis of early and late apoptosis markers (Annexin-V and 7-AAD, respectively), using flow-cytometry. Interestingly, both *RAS*-wildtype SEM and *RAS*-mutant KOPN8 undergo early apoptosis, after treatment with MEK inhibitor (Figure 5A and B, respectively). However, while late apoptosis is barely observed for SEM (Figure 5C), late apoptosis in MEK inhibitor exposed KOPN8 cells is enhanced substantially, especially after prolonged exposure (Figure 5D), suggesting the response to MEK inhibition is characterized by increased apoptosis. Furthermore, MEK inhibitor exposure induced protein levels of pro-apoptotic BIM, most evidently for KOPN8, while p53 levels remained unaffected (Supplementary Figure S5). Additionally, we investigated cell cycle progression under influence of MEK inhibition. Interestingly, no considerable differences in SEM or KOPN8 cell cycle progression are observed after 96 hours exposure to MEK162, Selumetinib or Trametinib (Figure 5E and F, respectively), nor after exposure for 24, 48 and 72 hours (Supplementary Figure S6A-B, C-D and E-F, respectively).

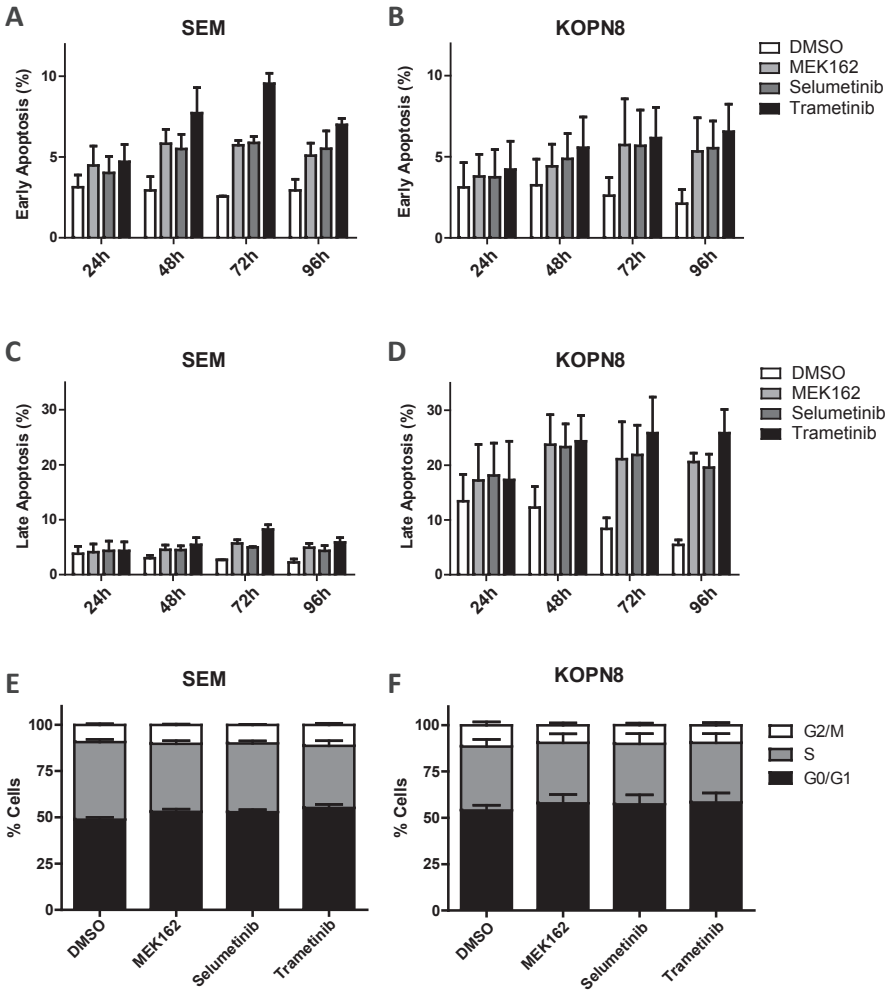


**Figure 5: MEK inhibitors induce apoptosis**

(A and B) Early apoptosis (percentage AnnexinV single positive of total) of SEM and KOPN8 cells (respectively) after exposure to DMSO vehicle (white bars) or 500 nM MEK162, Selumetinib or Trametinib (light grey, dark grey and black bars, respectively), indicates MEK inhibition slightly induces early apoptosis. Data are represented as mean +/- sem. n=3. (C and D) Late apoptosis (percentage AnnexinV and 7-AAD double positive cells of total) of SEM and KOPN8 (respectively) show that while SEM cells have no induction of late apoptosis in response to MEK inhibition, compared to the DMSO controls, KOPN8 clearly undergoes apoptosis, especially after prolonged exposure (>48 hours). Data are represented as mean +/- sem. n=3. (E and F) Cell cycle analysis of SEM and KOPN8 (respectively) after 96 hours exposure to vehicle (DMSO) or 500 nM MEK162, Selumetinib or Trametinib indicates MEK inhibition does not impinge on the cell cycle progression. Stacked bar graph indicates percentage of cells in $G_0/G_1$ (black), S (grey) and $G_2/M$ (white) cell cycle stages. Data are represented as mean +/- sem. n=3.

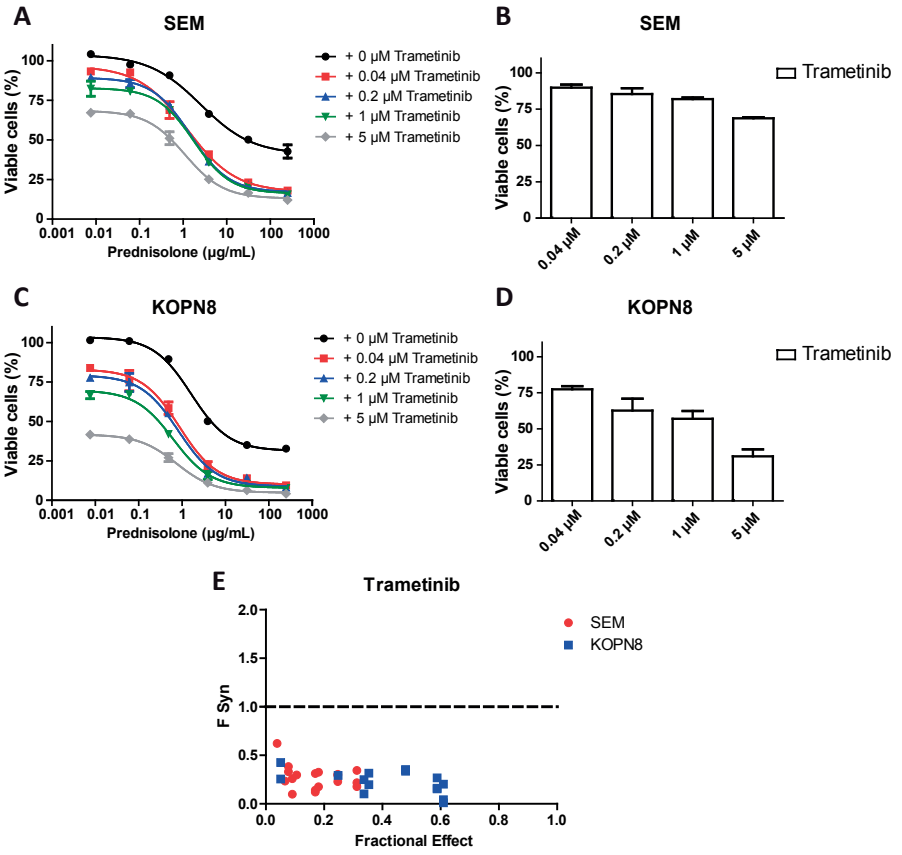


**Figure 6: MEK inhibition enhances prednisolone sensitivity**

(A) Dose-response curves of the SEM cell line exposed to prednisolone alone (black curve) or in combination with 0.04 µM, 0.2 µM, 1 µM or 5 µM Trametinib (red, blue, green and grey curves, respectively). Low concentrations of Trametinib particularly sensitize cells that escape high concentrations of prednisolone. Data are represented as mean +/- sem. n=3. (B) Response of SEM to the single Trametinib concentrations used in A. n=3. (C) Dose-response curves of KOPN8 treated with prednisolone (black curve), or in combination with the aforementioned Trametinib concentrations (shown in red, blue, green and grey, respectively). KOPN8 cells are also sensitized towards prednisolone by co-exposure with low concentrations of Trametinib. Data are represented as mean +/- sem. n=3 (D) KOPN8 exposed to single Trametinib concentrations. Data are represented as mean +/- sem. n=3. (E) Combined exposure to prednisolone and Trametinib (merged data from 3 separate experiments) was quantified using $F_{Syn}$ calculations ($F_{Syn}$<1 indicates synergy) and plotted against fractional effect (i.e. inhibition of cell viability). In SEM (red) moderate to strong synergy was observed, while all combinations of Trametinib and prednisolone result in strong to very strong synergy in KOPN8 (blue).

### MEK inhibition enhances prednisolone sensitivity

In our previous study, we found that *MLL*-rearranged infant ALL patient samples harboring *RAS* mutations are more resistant to prednisolone.[3] Therefore, we examined whether inhibition of MEK could enhance prednisolone sensitivity of *RAS*-mutant cells. As shown in Figure 6A, prednisolone alone decreases cell viability of SEM cells to only ~50%. Interestingly, while Trametinib by itself induces only minor cell viability decrease in SEM cells (Figure 6B), the combination of Trametinib and prednisolone greatly enhanced the efficacy of prednisolone, especially at higher concentrations (Figure 6A). The combination of prednisolone and Trametinib also strongly decreased cell viability in KOPN8 more potently than either drug alone; low concentrations of Trametinib nearly eradicated all KOPN8 cells that did not respond to prednisolone treatment (Figure 6C). A similar sensitizing effect was observed when exposing SEM and KOPN8 to MEK162 or Selumetinib in combination with prednisolone (Supplementary Figure S7A-D and 7F-I, respectively). Since Trametinib alone already effectively decreases viability of KOPN8 cells (Figure 6D), we quantified the combinatorial effect of MEK inhibitors and prednisolone using the synergy factor ($F_{Syn}$) calculation, as previously described.[10, 11] The plot in Figure 6E shows the fractional effect (i.e. the relative decrease of cell viability) induced by the combination of Trametinib with prednisolone, and the corresponding Synergy Factor. Interestingly, in both SEM and KOPN8 cells we observed $F_{Syn}$ values < 0.5, indicating strong synergy between Trametinib and prednisolone. Also combining MEK162 or Selumetinib with prednisolone resulted in moderate to strong synergistic effects (Supplementary Figure S7E and 7J, respectively). Additionally, we investigated whether this enhanced effect was related to differential expression of the glucocorticoid receptor (GR), the target of prednisolone. However, MEK inhibitor exposure did not alter GR protein levels in either SEM or KOPN8 cells (Supplementary Figure S7K).

## DISCUSSION

*MLL*-rearranged ALL in infants is a high-risk hematologic malignancy, characterized by a high incidence of relapse and high mortality rate.[13] Recently, we showed that 14-24% of these patients carry a *RAS* mutation, as an independent predictor of extremely poor outcome.[3] In the present study, we demonstrate that the MEK inhibitors Trametinib, Selumetinib and MEK162 display strong anti-leukemic effects against *RAS*-mutant *MLL*-rearranged ALL cells. Considering the dismal prognosis for infants suffering from *MLL*-rearranged ALL with additional *RAS* mutations, our data supports application of these inhibitors in the treatment of this patient group. Recently, Irving *et al.* already showed that Selumetinib effectively inhibits leukemia progression in an *in vivo* model of *RAS*-mutant BCP-ALL, and Burgess *et al.* found Trametinib to prolong the survival of mice transplanted with $NRAS^{G12D}$ AML cells.[14, 15] Moreover, Trametinib has recently been approved for the treatment of adult *BRAF*-mutated melanoma, while different clinical trials show promising results in adult

patients with *RAS*/*RAF* mutation positive melanoma and non-small-cell lung cancer for Selumetinib and MEK162.[16-20] Even though most clinical trials focused on solid tumors in adult patients, pediatric clinical trials are underway for neurofibromas and gliomas, and could expedite clinical application of these MEK inhibitors in *MLL*-rearranged infant ALL.

Interestingly, while all *RAS*-mutant *MLL*-rearranged ALL patient samples are susceptible to MEK inhibition, patients without *RAS* mutations also might benefit from MEK inhibitor treatment, since a subgroup of *RAS*-wildtype patient samples appears sensitive to MEK inhibition. While in our previous study, we identified *RAS* mutations and found no *BRAF* aberrations, mutations of other upstream regulators, i.e. tyrosine kinase receptors, can occur in other malignancies.[3] Andersson *et al.* recently showed that additional somatic mutations in *MLL*-rearranged infant ALL, like (sub-)clonal RAS/PI3K pathway aberrations, occur in up to 50% of the cases, supporting our previous observation that *RAS* mutations in *MLL*-rearranged infant ALL frequently occur at a sub-clonal level.[3, 21] These findings do not support the hypothesis that other (upstream) mutations are driving RAS-MEK-ERK signaling, but also do not explain observed extensive MEK inhibitor sensitivity of all (sub-clonal) *RAS*-mutant and specified *RAS*-wildtype patient samples. While we found enhanced RAS and MEK activation in *RAS*-mutant samples, these biomarkers could not differentiate MEK inhibitor sensitive and resistant *RAS*-wildtype samples. Interestingly, Kampen *et al.* recently proposed a MEK inhibitor escape mechanism in *MLL*-rearranged AML, which was mediated by VEGFR-2 and PI3K-signaling, and we wondered whether this could play a role in the MEK inhibitor resistance of our wildtype patient cells.[5] However, we observed no difference in downstream PI3K-signalling (i.e. Akt or p70S6 phosphorylation) in response to MEK inhibitor exposure. Additionally, we discovered no significant tyrosine kinase receptor expression differences in *MLL*-rearranged infant ALL patient samples that could explain the MEK inhibitor response of *RAS*-wildtype samples. Surprisingly, *FGFR-1* expression was lower in MEK inhibitor resistant samples, but it is unclear how this would explain MEK inhibitor resistance. Alternatively, Minjgee *et al.* report that *RAS*-mutant transfected cells can induce downstream RAS signaling in a paracrine manner, through excretion of cytokines.[22] Interestingly, Nakanishi *et al.* previously demonstrated that *MLL*-fusion proteins can induce ERK phosphorylation through regulating EphA7 receptor tyrosine kinase expression, but this was not accompanied by increased RAF or MEK phosphorylation.[23] Still, their data shows that leukemic cells carrying the t(4;11) translocation are sensitive to small molecule inhibitors of ERK phosphorylation. These findings indicate alternative regulatory mechanisms for ERK signaling in *MLL*-rearranged leukemia could explain the MEK inhibitor sensitivity we observe in *RAS*-wildtype cells.

Loss of ERK phosphorylation in response to MEK162, Selumetinib or Trametinib exposure confirmed the effect of MEK inhibition. Interestingly, prolonged exposure of cells to MEK162

or Selumetinib resulted in increased MEK phosphorylation. Previously, Hatzivassiliou *et al.* showed that the aromatic fluorine of allosteric MEK inhibitor GDC-0973 interacts with MEK residue S212.[24] Their data indicate this interaction results in exposure of the phosphorylation sites S218/S222, which are then susceptible to RAF mediated phosphorylation. Since MEK162 and Selumetinib both have this aromatic fluorine, the mechanism of interaction with MEK is probably similar to GDC-0973. Hence, although MEK activation in presence of GDC-0973, MEK162 or Selumetinib can still occur, the transduction of the signal by MEK-mediated phosphorylation of ERK is no longer possible, as we show in Figure 4.

Recently, we found the presence of *RAS* mutations in *MLL*-rearranged infant ALL cells correlated with prednisolone resistance, an obstacle in the treatment of infant ALL.[2, 3] Remarkably, our present data shows that MEK inhibition strongly enhances the sensitivity of both *RAS*-wildtype and *RAS*-mutant *MLL*-rearranged ALL cells to prednisolone, also further exemplifying the possible value of MEK inhibitors for *RAS*-mutant, as well as *RAS*-wildtype, *MLL*-rearranged infant ALL patients. The prednisolone-sensitizing effect of MEK inhibitors proposes a possible role for RAS-MEK-ERK signaling in the response to glucocorticoids. Recent work by Jones *et al.* shows that MEK plays a key role in drug resistance in relapsed pediatric ALL, and that MEK inhibition can sensitize ALL relapse samples to chemotherapeutics, including methylprednisolone.[25] Moreover, Ariës *et al.* found Trametinib could restore prednisolone sensitivity in *RAS*-mutant BCP-ALL patient samples, whereas Rambal *et al.* showed that MEK activation reduces dexamethasone sensitivity, and the MEK inhibitor PD183452 enhanced dexamethasone responses in ALL cells in a BIM-dependent manner.[26, 27] Activated ERK can phosphorylate BIM, targeting it for proteasomal degradation, and thereby diminishing apoptosis induced by dexamethasone.[28] Moreover, we established that, while glucocorticoid receptor expression remains constant, MEK inhibition upregulates pro-apoptotic BIM, which implies that inhibiting MEK, resulting in abrogation of ERK phosphorylation, may result in prolonged maintenance of pro-apoptotic BIM activity upon prednisolone exposure, leading to enhanced prednisolone sensitivity. This is further supported by our previous study showing that in *MLL*-rearranged ALL, prednisolone sensitization mediated by pan-BCL-2 family inhibitors was largely driven by the up-regulation of pro-apoptotic BID and BIM.[29]

In summary, our data shows that *RAS*-mutant *MLL*-rearranged infant ALL patients may benefit from therapeutic strategies administering small-molecule MEK inhibitors. Furthermore, since MEK inhibition sensitizes *MLL*-rearranged ALL cells to prednisolone regardless of the *RAS* mutations status, *RAS*-wildtype *MLL*-rearranged infant ALL patients may also benefit from MEK inhibitor treatment through enhanced sensitivity to prednisolone.

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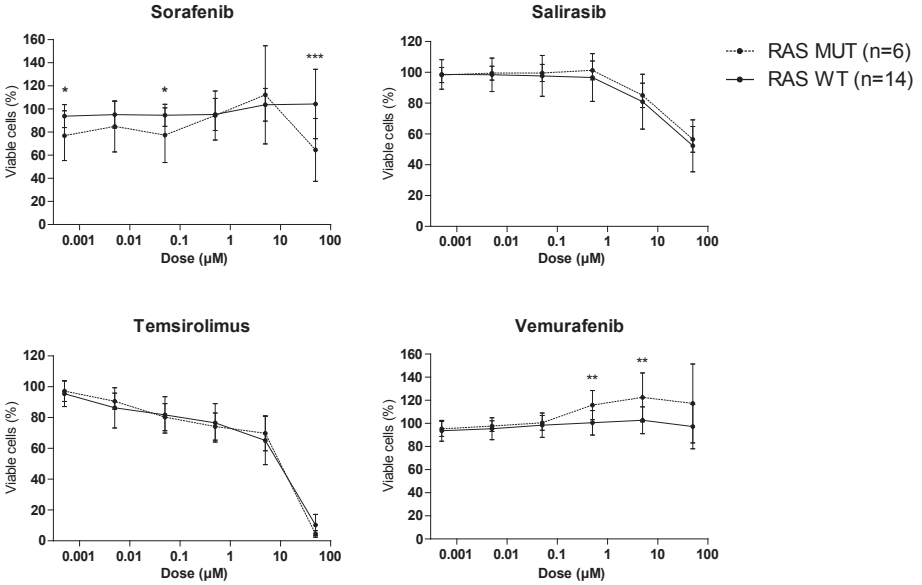
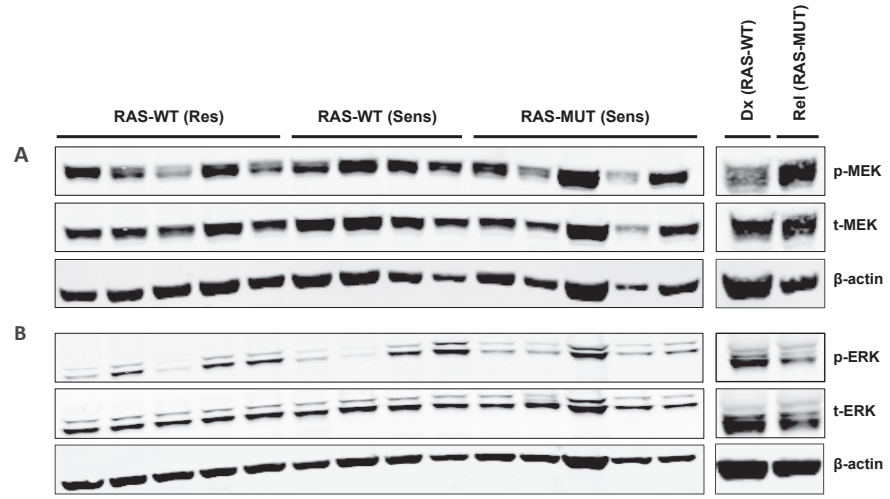
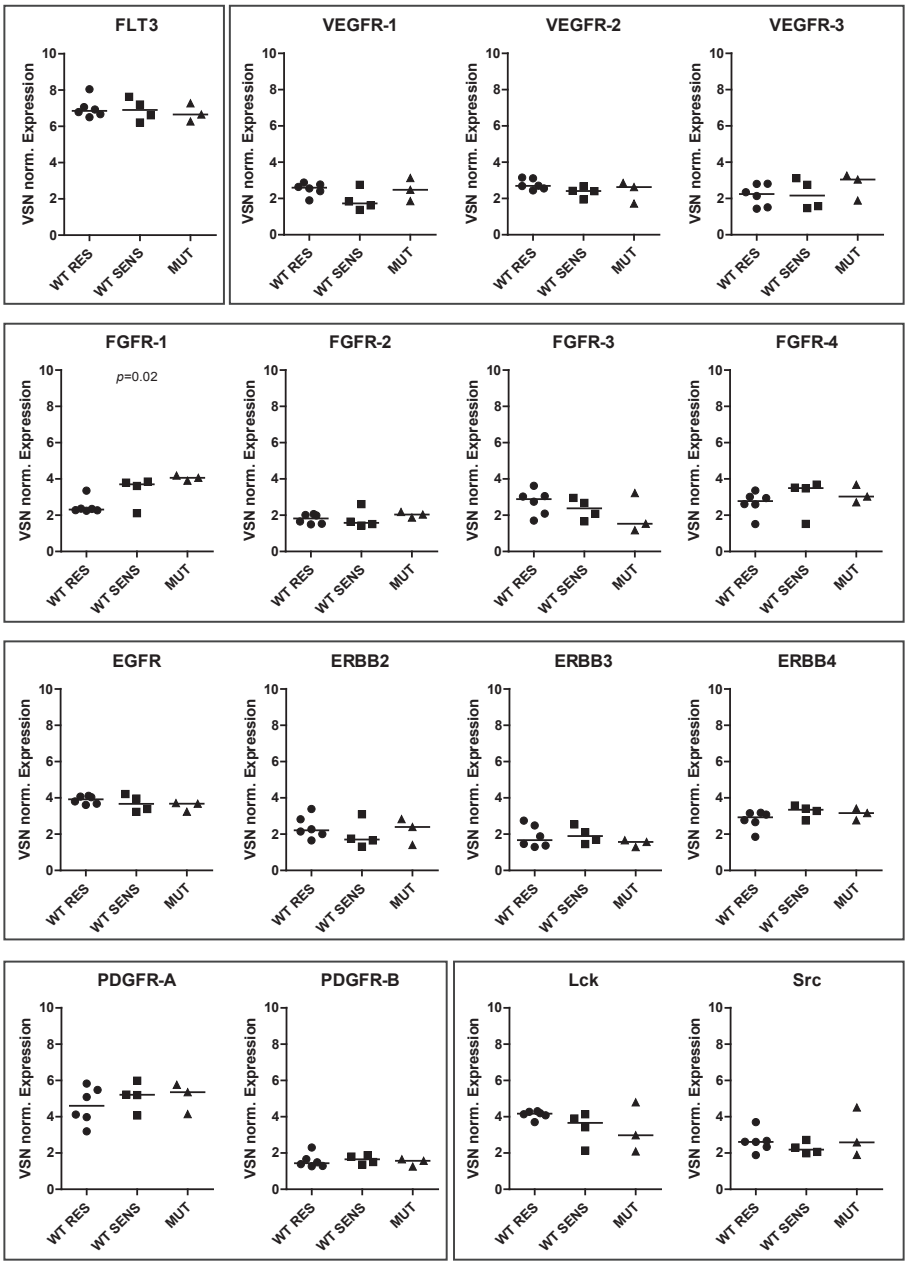



**Supplementary Figure S1: MTT data primary t(4;11)$^{+}$ samples**
MTT assays of patient derived *RAS*-mutant (solid line, n=6) and *RAS*-wildtype (dashed line, n=14) t(4;11)$^{+}$ infant ALL cells exposed to Sorafenib, Salirasib, Temsirolimus and Vemurafenib. Error bars represent standard deviation. *0.01<$p$<0.05; **0.001<$p$<0.01; ***$p$<0.001.

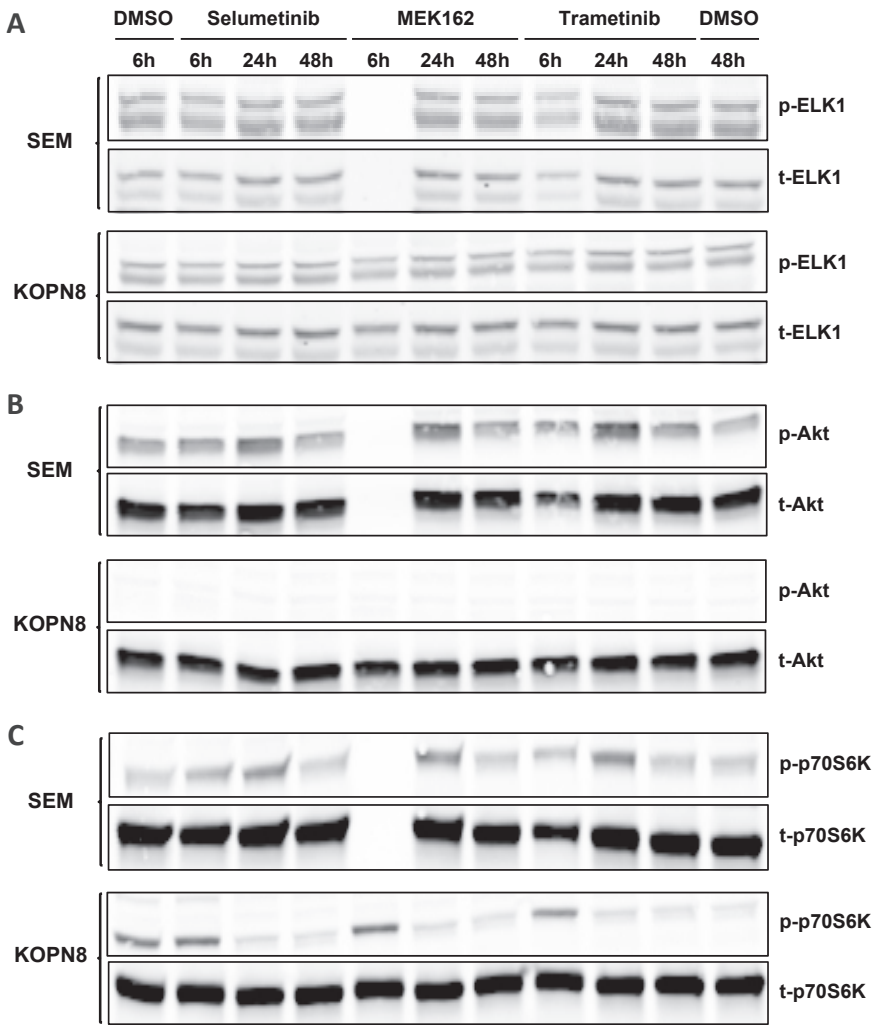



**Supplementary Figure S2: p-MEK and p-ERK immunoblots of patient samples**
(A) Western blots of MEK inhibitor resistant (Res) and sensitive (Sens) *RAS*-wildtype and *RAS*-mutant t(4;11)$^{+}$ patient samples (left) and the matched diagnosis/relapse (Dx/Rel) samples (right) for phosphorylated MEK (upper), total MEK (middle) and β-actin (lower). (B) Western blots of t(4;11)$^{+}$ patient samples for phosphorylated ERK (upper), total ERK (middle) and β-actin (lower).

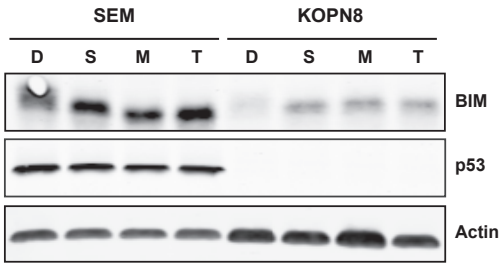



**Supplementary Figure S3: Gene expression of tyrosine kinase receptors**

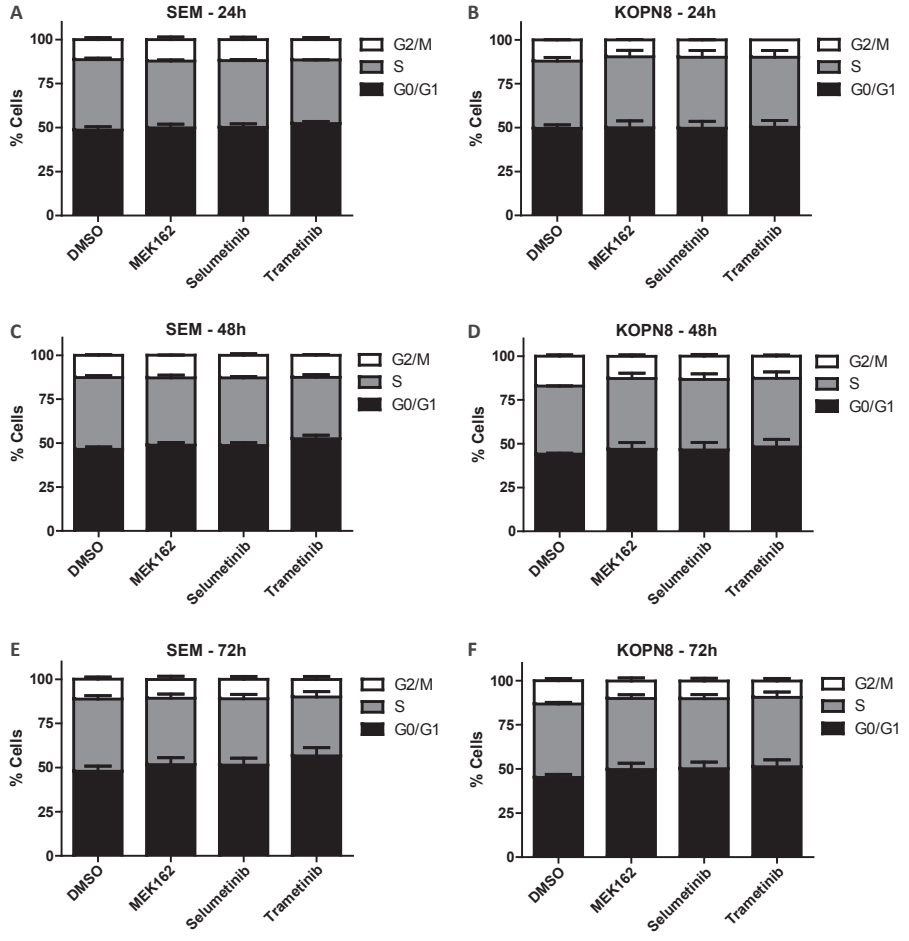
Tyrosine kinase receptor mRNA expression (Affymetrix HU133plus2.0 microarray data) for FLT3, VEGFR-1, VEGFR-2, VEGFR-3, FGFR-1, FGFR-2, FGFR-3, FGFR-4, EGFR, ERBB2, ERBB3, ERBB4, PDGFR-A, PDGFR-B, Lck and Src in MEK inhibitor resistant *RAS*-wildtype (WT RES), MEK inhibitor sensitive *RAS*-wildtype (WT SENS) and *RAS*-mutant (MUT) primary samples.



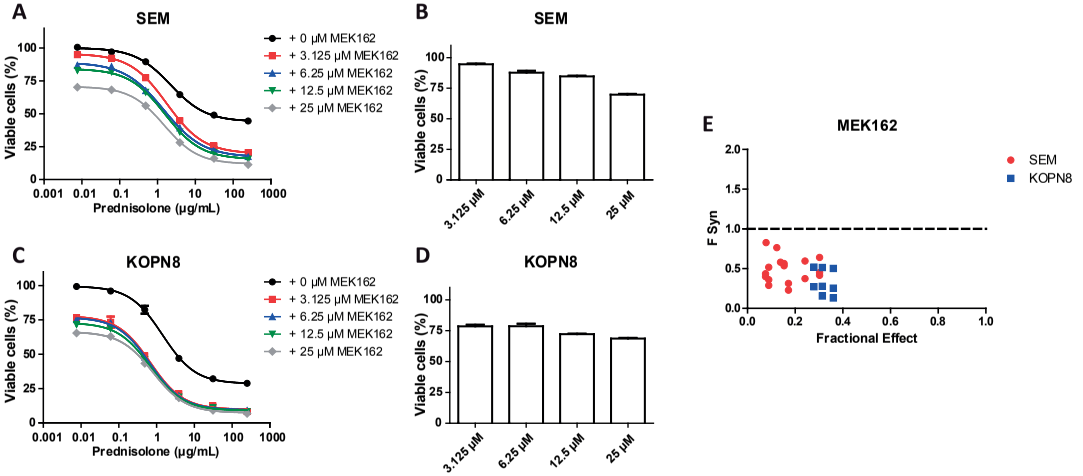

**Supplementary Figure S4: p-ELK-1, p-Akt and p-p70S6K immunoblots of MEKi treated cells**
(A) Western blots of SEM (two upper panels) and KOPN8 (two lower panels), exposed for 6, 24 and 48 hours to either vehicle (DMSO) or Selumetinib, MEK162 or Trametinib, detecting phosphorylated ELK-1 and total ELK-1. (B) Western blots of SEM and KOPN8, exposed to aforementioned MEK inhibitor conditions, for phosphorylated and total Akt. (C) Western blots of SEM and KOPN8, exposed to aforementioned MEK inhibitor conditions, for phosphorylated and total p70S6K.




**Supplementary Figure S5: BIM and p53 immunoblots of MEKi treated cells**
Western blots of SEM and KOPN8 exposed for 48 hours to DMSO (D), Selumetinib (S), MEK162 (M) or Trametinib (T), for determination of BIM and p53 protein levels. Actin was used as loading control.

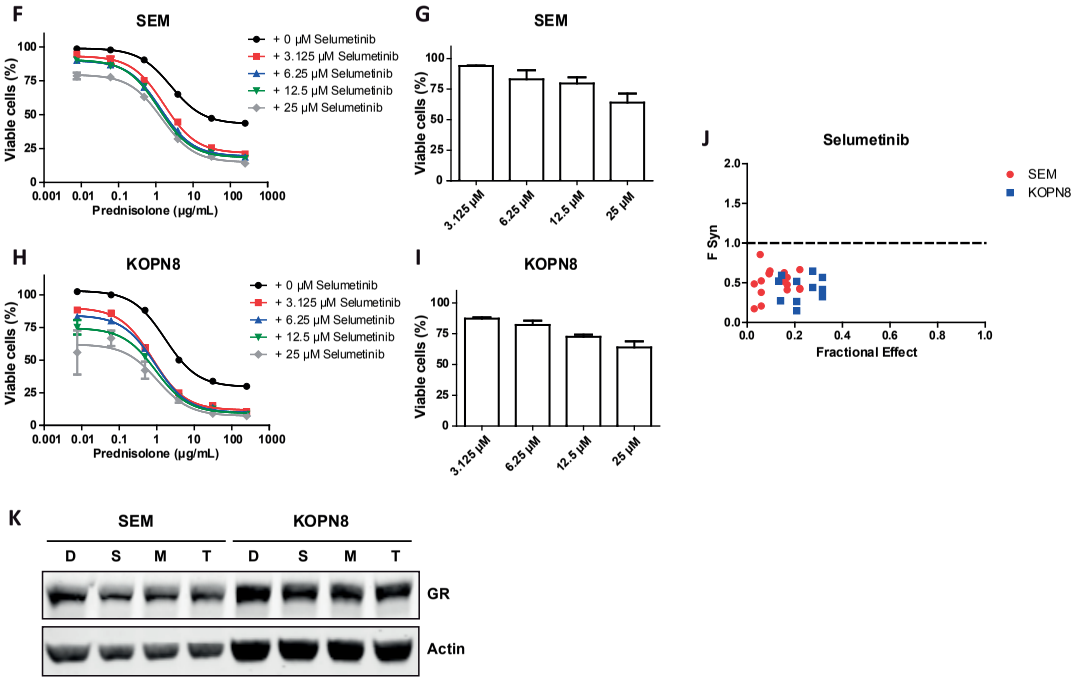



**Supplementary Figure S6: Cell cycle progression after MEK inhibitor exposure**
Representative graphs of percentages SEM (A, C and E) or KOPN8 (B, D and F) cells in G2/M (white), S (grey) or G0/G1 (black) cell cycle stages, as analyzed by DNA staining. Cells were exposed to vehicle control (DMSO) or 500 nM MEK162, Selumetinib or Trametinib for 24, 48 or 72 hours (A and B, C and D, E and F, respectively).




**Supplementary Figure S7: Drug combinations of MEK162 and Selumetinib with prednisolone**

(A) Dose-response curves of the SEM cell line exposed to prednisolone alone (black curve) or in combination with 3.125 µM, 6.25 µM, 12.5 µM or 25 µM MEK162 (red, blue, green and grey curves, respectively). Low concentrations of MEK162 sensitize cells towards prednisolone. (B) Response of SEM to the single MEK162 concentrations used in A. (C) Dose-response curves of KOPN8 treated with prednisolone (black curve), or in combination with the aforementioned MEK162 concentrations (shown in red, blue, green and grey, respectively). KOPN8 cells are also sensitized towards prednisolone by co-exposure with low concentrations of MEK162. (D) KOPN8 exposed to single MEK162 concentrations. (E) Combined exposure to prednisolone and MEK162 (n=3) was quantified using $F_{Syn}$ calculations ($F_{Syn}$<1 indicates synergy) and plotted against fractional effect (i.e. inhibition of cell viability). In both SEM (red) and KOPN8 (blue), moderate to strong synergy was observed.




**Supplementary Figure S7: Drug combinations of MEK162 and Selumetinib with prednisolone**

(F) Dose-response curves of the SEM cell line exposed to either prednisolone alone (black), or in combination with Selumetinib (at the same concentrations used for MEK162). Low concentrations of Selumetinib can sensitize SEM cell towards prednisolone. (G) Effect of different single Selumetinib concentrations in SEM. (H) Combined exposure to prednisolone and low Selumetinib concentrations also sensitizes KOPN8 cells. (I) Response of KOPN8 towards single concentrations of Selumetinib. (J) Combinatorial effect of prednisolone and Selumetinib co-exposure (n=3) was quantified using $F_{Syn}$ calculations. Moderate to strong synergistic effects between prednisolone and Selumetinib are observed for both SEM (red) and KOPN8 (blue). (K) Western blot of SEM and KOPN8 exposed for 48 hours to DMSO (D), Selumetinib (S), MEK162 (M) or Trametinib (T), detecting glucocorticoid receptor (GR) and loading control (Actin).

# Chapter 6

## *CBL* mutations do not frequently occur in pediatric acute myeloid leukemia

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## ABSTRACT

RAS-pathway mutations, causing a proliferative advantage, occur in acute myeloid leukemia (AML) and *MLL*-rearranged leukemia. Recently, mutations in the *Casitas B lineage lymphoma* (*CBL*) gene were reported to be involved in RAS-pathway activation in various myeloid malignancies, but their role in pediatric AML is still unknown. We performed mutation analysis of 283 newly diagnosed and 33 relapsed pediatric AML cases. Only two mutant cases (0·7%) were identified in the newly diagnosed pediatric AML samples, of which one was *MLL*-rearranged. Both mutant cases showed *CBL* mRNA expression in the range of the non-mutated cases. Phosphorylated extracellular signal-regulated kinase (pERK) was not correlated with CBL protein expression ($n$=11). In conclusion, we report a very low *CBL* mutation frequency in pediatric AML, which, together with the lack of difference in protein and mRNA expression, illustrates the limited role of CBL in pediatric AML.

## INTRODUCTION

Enhanced proliferation and disrupted differentiation are known to constitute collaborative key events leading to the onset of leukemogenesis. In acute myeloid leukemia (AML), mutations in several members of the RAS-regulated signaling pathway result in proliferative advantage[1, 2]. Recently, Casitas B lineage lymphoma (CBL), a protein involved upstream in this pathway, was found to be mutated at high frequency (15%) in juvenile myelomonocytic leukemia (JMML), mutually exclusive from other RAS-pathway mutations[3]. The proto-oncogene *CBL* encodes an E3 ubiquitin ligase that negatively regulates receptor tyrosine kinases (like FLT3 and EGFR) and associated proteins including Grb2 and SOS, which are involved in RAS deactivation[4]. Mutations in the linker region and RING finger domain of CBL reduces its ubiquitin ligase potential, impairing the ability of Grb2 and SOS to suppress RAS signaling, and thereby over-activating downstream RAS targets[5].

*CBL* mutations have also been observed in 1–2% of adult AML, 8% of atypical chronic myeloid leukemia, and 13% of chronic myelomonocytic leukaemia[6-8]. RAS-pathway-associated mutations have been found in about 20% of pediatric AML cases, which mostly occur in specific subgroups, such as cytogenetically normal cases with *NPM1* mutations or *MLL*-partial tandem duplications, t(8;21), inv(16), and *MLL*-rearranged patients under the age of 2 years[9-11]. Furthermore, type I mutations in *MLL*-rearranged AML cases were almost always related with the RAS-pathway[9]. The collaboration of RAS-pathway mutations and *MLL*-rearrangements at a young age was also reported in pediatric *MLL*-rearranged acute lymphoblastic leukemia (ALL) cases (50%)[12]. The role of RAS-pathway mutations in leukemogenesis of *MLL*-rearranged ALL is further stressed by studies in MLL/ AFF1 (AF4)+ *Kras* mutation+ transgenic mice, which develop B-cell lymphoma and/ or leukemia with a relatively short latency (6 months), in contrast to transgenic mice models without *RAS* mutations[13-15]. One recent study reported the co-existence of *CBL* mutations and *MLL*-rearrangements in ~5% of infant ALL patients[16].

So far the frequency and clinical value of *CBL* mutations in pediatric AML is unknown. Therefore, we studied the frequency of *CBL* mutations and expression in a large cohort of pediatric AML.

## METHODS

### Patient samples

Viably frozen diagnostic bone marrow or peripheral blood from 319 AML samples, including 277 de novo, 9 secondary and 33 relapsed pediatric AML samples, were provided by the

Dutch Childhood Oncology Group (DCOG), the AML 'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG), the Czech Pediatric Hematology Group (CPH), and the St. Louis Hospital in Paris, France. Patient characteristics of the 277 de novo cases are shown in Table 1. In order to elucidate the role of *CBL* related to *MLL* in general, we used an *MLL*-rearranged enriched cohort [18 infant ALL patients, 100% t(4;11)(q21;q23), seven males] as controls. Informed consent was obtained from all patients after Institutional Review Board approval according to national law and regulations.

## Materials

Leukemic cells were isolated and enriched as previously described[17-19]. All resulting samples contained 80% or more leukemic cells, as determined morphologically by May-Grünwald-Giemsa (Merck, Darmstadt, Germany)–stained cytospins. A minimum of $5 \times 10^6$ leukemic cells was lysed in Trizol reagent (Gibco BRL/Life Technologies, Breda, the Netherlands) and stored at −80°C. Isolation of genomic DNA and total cellular RNA was performed as described before[20].

AML samples were routinely investigated for *MLL*-rearrangements as previously described[21]. The other common cytogenetic abnormalities in AML [such as t(8;21), inv(16), t(15;17)] were confirmed by conventional karyotyping, fluorescence *in situ* hybridization (FISH) or reverse transcription polymerase chain reaction (RT-PCR). For all infant ALL samples, karyotyping and analysis of possible *MLL*-rearrangements was performed with split-signal FISH, PCR or both (Primers are described in Table SI).

## *CBL* mutation analysis

PCR analysis for *CBL* mutations was performed on genomic DNA using a 2720 Thermal cycler (Applied Biosystems, Foster City, CA, USA). Primers and conditions used for *CBL* screening were identical to those used by Loh *et al*[3]. Briefly, these primers target exons 8 and 9, including the intron-exon boundaries of the *CBL* gene. Primer sequences are listed in Supplementary Table S1. Sequencing was done using a BigDye terminator v 1.1 Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 3130x/ Genetic Analyzer. Sequence analysis was done with CLC Workbench software (CLCbio, Aarhus, Denmark) with reference sequence ENST00000264033 (www.ensembl.org, release 59).

Of the mutated cases, germline material was obtained to investigate the *CBL* mutation status in healthy cells. We obtained DNA isolated from a cytospin at remission state from one case. From the other case, lymphocytes were isolated by magnetic-activated cell sorting (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's guidelines.

**Table 1: Patient characteristics *de novo* AML cohort consisting of 277 patients**

| | No. of samples (%) |
|---|---|
| **Sex (n=274)** | |
| Male | 159 (58) |
| Female | 115 (42) |
| **Age (median, range, years, n=277)** | 9,4 (0-18,5) |
| **WBC x $10^9$/l at Dx (median, range, n=247)** | 43,6 (1,2-483) |
| **FAB (n=270)** | |
| M0 | 14 (5) |
| M1 | 34 (13) |
| M2 | 52 (19) |
| M3 | 21 (8) |
| M4 | 77 (29) |
| M5 | 61 (23) |
| M6 | 4 (1) |
| M7 | 7 (3) |
| **Cytogenetics (n=261)** | |
| *MLL*-rearrangements | 64 (25) |
| t(8;21) | 25 (10) |
| inv(16) | 35 (13) |
| t(7;12) | 5 (2) |
| t(15;17) | 18 (7) |
| Cytogenetically Normal (CN)-AML | 53 (20) |
| AML-other | 61 (23) |
| **Molecular abnormalities (n samples)** | |
| *KIT* (261) | 21 (8) |
| *KRAS* or *NRAS* (261) | 54 (21) |
| *FLT3*-ITD (269) | 54 (20) |
| *FLT3*-D835/6 (43) | 2 (5) |
| *PTPN11* (261) | 3 (1) |
| *CEBPA* (238) | 17 (7) |
| *NPM1* (252) | 18 (7) |
| *MLL*-PTD (232) | 5 (2) |
| *WT1* (259) | 22 (12) |
| *TET2* (38) | 1 (3) |
| *DNMT3A* (142) | 3 (2) |
| *IDH1/2* (199) | 12 (6) |
| *NUP98/ NSD1* (261) | 12 (5) |
| *CBL* (277) | 2 (1) |

Patient characteristics of the *de novo* acute myeloid leukemia cohort. Numbers indicate frequency (%) unless specified otherwise. AML: acute myeloid leukemia, WBC: white blood cell count, Dx: diagnosis, FAB: French American British morphology classification.

### Gene expression profiling and RT-qPCR

Gene expression profiling (GEP) (Affymetrix HU133plus2.0; Affymetrix, Santa Clara, CA, USA) was performed on 272/ 277 of *de novo* pediatric AML patients as a subset of the recently published study (Balgobind *et al*, 2011c). GEP data were deposited in the GEO database under accession number GSE17855. To validate mRNA expression levels, quantitative real-time PCR(RT-qPCR) was performed on cDNA of 12 AML cell lines and 11 pediatric AML patient samples, selected on availability of cDNA, produced as previously described[22]. An ABI PRISM 7900HT sequence detector (Applied Biosystems) was used to validate the GEP results. Primers used for *CBL* are described in Supplementary Table S1. The average cycle threshold ($C_t$) value was used to calculate mRNA expression levels of *CBL* relative to the expression level of *GAPDH* using the comparative Cycle threshold ($\Delta C_t$) method[23]. For analysis of the *CBL* mutant transcripts we designed primers targeting exon 6 to 11 of *CBL* (Supplementary Table S1).

### Western Blot

For protein expression analysis 11 AML samples were selected based on *CBL* mutational status. Analysis was performed as previously described (Kuipers *et al*, 2011)[21]. Cell lysates containing 20 µg of protein were separated on 10% polyacrylamide gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Western Blots were probed with rabbit polyclonal IgG CBL (#2179; Cell Signaling, Danvers, MA, USA), rabbit polyclonal IgG phospho ERK1/2 (#4377; Cell Signaling) and mouse anti-beta-actin (ab6276; Abcam, Cambridge, MA, USA) antisera. Subsequently, the blots were probed with IRDye 800CW Goat-anti-Rabbit antibody (#926-32211; LI-COR, Lincoln, NE, USA) and IRDye 680 Goat-anti-Mouse antibody (#926-32220; LI-COR). Fluorescence was detected by the LI-COR Odyssey system (LI-COR).

### Functional analysis of *CBL* in AML

To study the influence of *CBL* expression on RAS pathway activation in pediatric AML cells we used the Kasumi-1 cell line [Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Braunschweig, Germany], originally derived from a pediatric AML patient, as a model. Cells were cultured in RPMI-1640 medium supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, the Netherlands) and penicillin 100 u/ml, streptomycin 100 µg/ml, and fungizone 0·125 µg/ml (PSF; Invitrogen, Breda, the Netherlands) and grown as suspension cultures at 37°C in humidified air containing 5% $CO_2$.

Cells ($10\times10^6$) were transfected by electroporation in 400 µl of RPMI 1640 with l-alanyl-l-glutamine (Invitrogen) containing 250 nM of either a mix of equal amounts of *CBL* siRNAs (Dharmacon ON-TARGETplus L-003003; Thermo Fisher Scientific, Etten-Leur, the Netherlands) or Non-targeting siRNA (Dharmacon ON-TARGETplus D-001810-01-05; Thermo

Fisher Scientific), in 4-mm electroporation cuvettes (BioRad, Veenendaal, the Netherlands; target sequences are described in Supplementary Table S1). Electroporation was performed by the use of a BioRad Genepulser MXcell (BioRad) by applying a rectangle pulse of 400 V for 10 milliseconds. To compensate for the amount of cell death induced merely as a consequence of the electroporation procedure, control cells were electroporated in the absence of siRNA. After a 15-min incubation at room temperature, the cells were diluted in 15 ml RPMI 1640 supplemented with 10% FCS and PSF and incubated at 37°C and 5% $CO_2$. They were maintained in culture for 168h. Cell counts were determined daily ($t$=6, 24, 48, 72, 96 and 168h). Cell samples of both experimental and control conditions were collected from the medium at every time point. They were washed with phosphate-buffered saline and stored as dry pellet or lysed in Trizol reagent and stored at −80°C. RT-qPCR and Western Blot were used as described above to validate CBL knockdown and consecutive phosphorylated extracellular signal-regulated kinase (pERK) up-regulation as a marker for RAS-pathway activity. Results from three repetitive experiments were analyzed together.

### Statistical analysis

Statistical analysis of GEP data was performed as previously reported[24, 25]. For comparison of *CBL* expression in different cytogenetic AML groups the Kruskal-Wallis test was used. For paired analyses of non-parametric variables, the Wilcoxon Signed Ranks Test was used. All analyses were performed with the Statistical Package for the Social Sciences (SPSS) software version 17.0 (SPSS Inc. Chicago, IL, USA). All tests were two-tailed and a $p$-value <0·05 was considered significant.

## RESULTS

### Mutation screening

*CBL* mutation screening was performed in 277 newly diagnosed *de novo* AML samples, 9 newly diagnosed secondary AML samples and 33 AML samples at relapse, including 22 paired diagnosis-relapse samples, and in the enriched MLL/ AF4 + ALL cohort ($n$=18).

In 2/277 of the *de novo* AML patients a heterozygous mutation was found (0.7%, confidence interval 0.2-2.6%); case 1 had a 91 base pair deletion combined with a nine base pair insertion of the intron 7-exon 8 boundary; case 2 had a single nucleotide exchange of the exon 8-intron 8 splice site (Figure 1). Patient characteristics of both mutants are listed in Table 2. Interestingly, the two patients with *CBL* mutations did not carry any other AML related molecular aberrations (*NPM1, WT1, NRAS, KRAS, CEBPA, PTPN11, KIT, FLT3, IDH1/2, DNMT3A, NUP98/NSD1*). All other AML cases, including all relapses and secondary AML

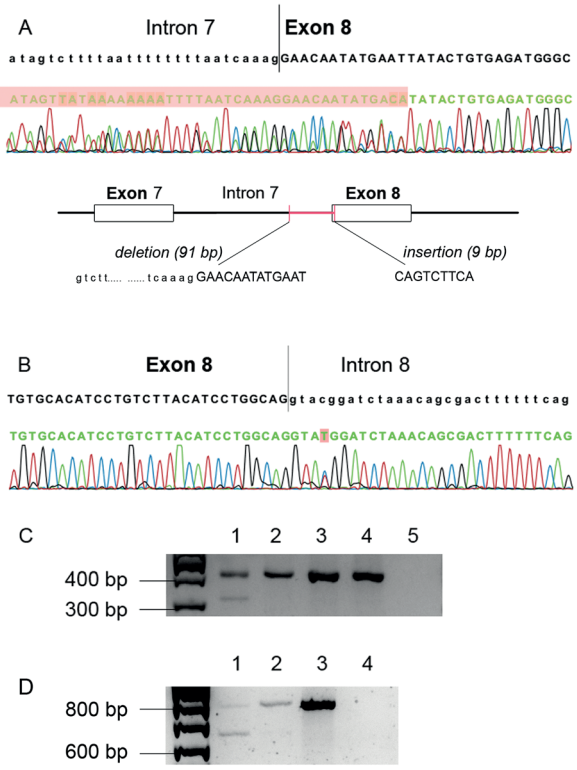


**Figure 1: Mutation analysis *CBL* exon 8 of mutant cases**

Case 1 has a large deletion at the intron 7-exon 8 boundary, shown in pink (A). A schematic overview is shown as well (A). Case 2 has a point mutation at the exon 8-intron 8 splice site, exon 8 +4C>T (B). Figure 1C shows a PCR electrophoresis on genomic DNA. Lane 1 corresponds to case1, lane 2 corresponds to case 2, lane 3 and 4 correspond to wild-type controls, and lane 5 corresponds to a negative control. Figure 1D shows a PCR electrophoresis on cDNA. Lane 1 corresponds to case 1, lane 2 corresponds to case 2, lane 3 corresponds to a wild-type control, and lane 4 corresponds to a negative control.

**Table 2: Patient characteristics *CBL* mutant cases**

| | case 1 | case 2 |
|---|---|---|
| **Sex** | female | male |
| **Age at diagnosis (years)** | 0.8 | 15.0 |
| **WBC at diagnosis (x $10^9$/L)** | 475 | 1.4 |
| **FAB** | M4 | M6 |
| **Cytogenetic subgroup** | *MLL (FNBP1*, 9q34.11) | CN |
| **Molecular abnormalities** | None | None |
| **Follow-up data** | CCR, 27 months | LFU, day 0 |

Patient characteristics of both mutant cases. WBC: white blood cell count, FAB: French American British morphology classification, CN: cytogenetically normal, CCR: continuous complete remission, LFU: loss to follow-up.

cases, were homozygous for the wild type allele. No *CBL* mutations were found in the *MLL*-rearranged infant ALL samples.

Germline material of both mutated cases was analyzed. DNA derived from remission material of case 1 was wild type for *CBL* (data not shown), while the sorted lymphocytes of case 2 showed the same heterozygous point mutation in the exon 8-intron 8 splice site as the matched diagnosis sample (Supplementary Figure S1).

## *CBL* mRNA expression

Figure 2 shows *CBL* mRNA expression of pediatric AML patients as measured by gene expression array (probe 225231_at). RT-qPCR correlated moderately with the results obtained from GEP (Spearman $r$=0.41) (Supplementary Figure S2). *CBL* mRNA expression was not different between the cytogenetic groups in pediatric AML (Kruskal–Wallis test, $p$=0.55) (Figure 2). The two mutated cases expressed *CBL* mRNA levels within the range of all other cases.

Given that alternative splicing can occur for splice site mutations of *CBL*, we analyzed the

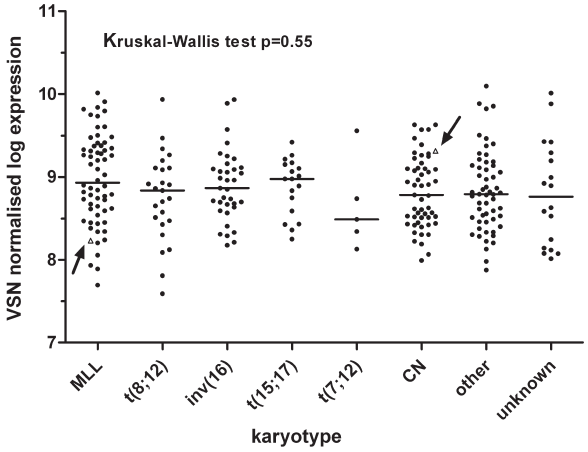


**Figure 2: *CBL* mRNA expression of 272 initial pediatric AML patients**
Graph showing the expression of probe set 225231_at, representing the *CBL* gene, after log transformation. Bars represent the median expression in each group. The arrows point to mutant cases, given as a triangle rather than a dot. CN: cytogenetically normal.

transcripts of cases 1 and 2. Case 1 expressed alternative splicing with a full-length transcript and one lacking exon 8 (Figure 1D). Case 2 only expressed the full-length transcript (Figure 1D).

### Western blotting

Western blotting showed no significant differences in protein expression of the two *CBL* mutants versus nine *CBL* wild type AML cases ($p$=0.8) (Figure 3). There was no evidence of truncated protein in both mutant cases (Figure 3). We could not detect pERK overexpression in patients with low CBL protein expression (Figure 3). Correlation of CBL protein expression with *CBL* mRNA expression on gene expression array was very poor (Spearman $r$=0.0) (Supplementary Figure S2).

### *CBL* knock-down by siRNA transfection

To study whether *CBL* mRNA down-regulation affects RAS-pathway activation in pediatric AML, *CBL* mRNA knock-down experiments were performed in a pediatric AML cell line. A reduction of *CBL* mRNA and CBL protein expression of 50–60% did not significantly affect cell counts and proliferation compared to the non-targeting siRNA control condition (Figure 4), whereas CBL protein knock-down did result in pERK up-regulation compared to the non-targeting siRNA control ($p$=0.03), with a maximum threefold increase at $t$=48h (Figure 4).

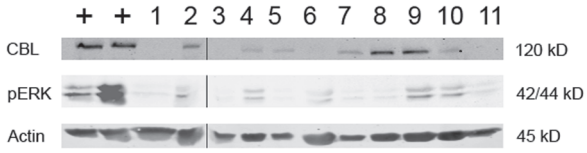


**Figure 3: Western Blot analysis of CBL from 11 AML patients**
Figure displaying three western blot sections. The 120 kD product represents CBL protein, the bands on 42/44 kD represent pERK. Actin was used as loading control (45 kD). Both lanes marked as + represent positive controls (cell lines HL60 and ME1 respectively), lanes 1 to 9 correspond to protein lysates of patients from diverse cytogenetic subgroups of pediatric AML, lane 10 corresponds to mutant case 1, lane 11 corresponds to mutant case 2. At the thin line one lane was spliced out. Differences in the protein levels of CBL do not correspond with *CBL* mutation status. There is no strict correlation between CBL protein levels and pERK protein levels in pediatric AML samples.

## DISCUSSION

This study aimed to elucidate the role of *CBL* in pediatric AML, triggered by reports showing high frequencies of inactivating *CBL* mutations in various myeloid neoplasms[3, 5-8, 26, 27]. Our results show that *CBL* mutations occurred only at a very low frequency (0.7%) in our well-documented and representative cohort of pediatric AML ($n$=319)[9]. This report showed similar low frequencies of *CBL* mutations as in adult AML (1–2%)[6, 7]. In adults *CBL* mutations were associated with core-binding factor leukaemia[6], which we could not confirm in this pediatric AML series, i.e., one of the mutated patients carried an *MLL*-rearrangement

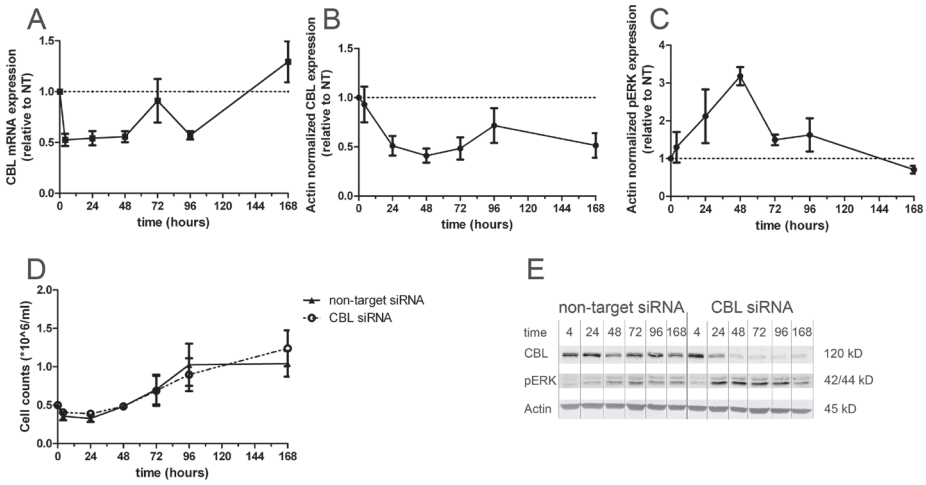


**Figure 4: *CBL* knockdown by siRNA transfection**
Figure showing results from *CBL* knockdown by siRNA transfection by electroporation in Kasumi cell line. *CBL* mRNA expression is shown relative to the non-target siRNA over time (A); mean and standard error of 3 consecutive experiments are shown. CBL protein expression normalized to the loading control actin is shown relative to non-target siRNA (B); mean and standard error of 3 consecutive experiments are shown. pERK protein expression normalized to the loading control actin is shown relative to non-target siRNA (C); mean and standard error of 3 consecutive experiments are shown. Cell counts during culture of both the experimental (*CBL* siRNA) and control (non-target siRNA) condition (D); mean and standard error of 3 consecutive experiments are shown. Western blot of both control (non-target siRNA) and experimental (*CBL* siRNA) condition (E); CBL, pERK and actin levels were determined. Time is shown in hours after electroporation in all panels. NT: non-target siRNA.

and the karyotype of the second mutated patient was normal. Like in the adult AML cohorts, in our two patients the identified mutations were heterozygous, which is in contrast to findings reported in JMML and CMML[3, 6, 7, 28-32].

Given that *CBL* mutations occur in high frequency in the RAS-pathway mediated disease JMML, the study design enabled us to test our hypothesis, that *CBL* mutations may reveal a new, more upstream mechanism for RAS-pathway activation in pediatric AML. For that reason, we anticipated that *CBL* mutations could be related to *MLL*-rearrangements, given the previously observed association between *MLL*-rearrangements and RAS-pathway mutations in pediatric acute leukaemias[9, 11, 12]. Surprisingly, only 1/64 (1.6%) of our *MLL*-rearranged AML samples was mutated and no mutations were found in the *MLL*-rearranged enriched cohort of infant ALL samples. Thus, we could not confirm the previously suggested relationship between *MLL*-rearrangements and *CBL* mutations[16].

The splice site *CBL* mutation of case 2 was previously reported in JMML and CMML[3, 8]. RT-PCR confirmed that this specific mutation does not affect the *CBL* splicing, as recently

reported in a study on adult myeloproliferative neoplasms[8]. Analysis of germline material showed that the point mutation was also present in sorted lymphocytes. In the previous reports, the patients that harbored this specific nucleotide change were not analyzed for the presence of the mutation in their germline material[3, 8]. Therefore, the relevance of this specific germline mutation for leukemogenesis is not clear.

Even though several studies have reported on *CBL* mutations in myeloid neoplasms, we are the first to study *CBL* mRNA and CBL protein expression concomitantly in diagnostic patient AML material. We found that *CBL* mRNA expression of the two mutated cases was not different from other pediatric AML cases, that there was no differential expression between the cytogenetic subgroups, and that mRNA levels were highly variable. The CBL protein expression of *CBL* mutants was low, and the expression in non-mutants showed a wide range. No evidence of truncated protein was found, and protein expression did not correlate with mRNA expression, suggesting post-transcriptional mechanisms for CBL protein regulation.

Nevertheless, in JMML where mRNA and protein levels were not compared, *CBL* mutated cases clearly demonstrated aberrant RAS-pathway activation[3]. Others have demonstrated interaction between CBL and FLT3 or KIT, and have shown cytokine independent growth for co-expression of these receptor tyrosine kinases with mutant CBL in murine myeloid cells[5, 33]. Co-expression of wild-type CBL, which is the case in our patients, significantly reduced this potential[34]. So far, it has not been reported whether CBL protein inactivation in pediatric AML leads to RAS-pathway activation like in JMML. We found that CBL protein knock-down results in RAS-activation by means of pERK protein up-regulation in an AML cell line Kasumi-1. This may suggest that low levels of CBL protein or dysfunctional CBL protein may activate the RAS-pathway in AML. This is important, as RAS-pathway activation is known to play an important proliferative role in pediatric AML, which can be targeted by currently available therapy, such as MEK-inhibitors[9, 11, 35-37]. Nevertheless, pERK activation was not uniformly apparent in our patient samples, or well correlated with CBL protein levels.

In conclusion, this study shows that *CBL* mutations are very rare in pediatric AML. Nevertheless, we showed that decreased CBL protein expression may be a mechanism for RAS-pathway activation in pediatric AML, like in JMML[3]. Therefore other factors besides *CBL* mutations, such as post-translational processing or enhanced degradation, may be involved in this process, in at least a subset of pediatric AML patients.

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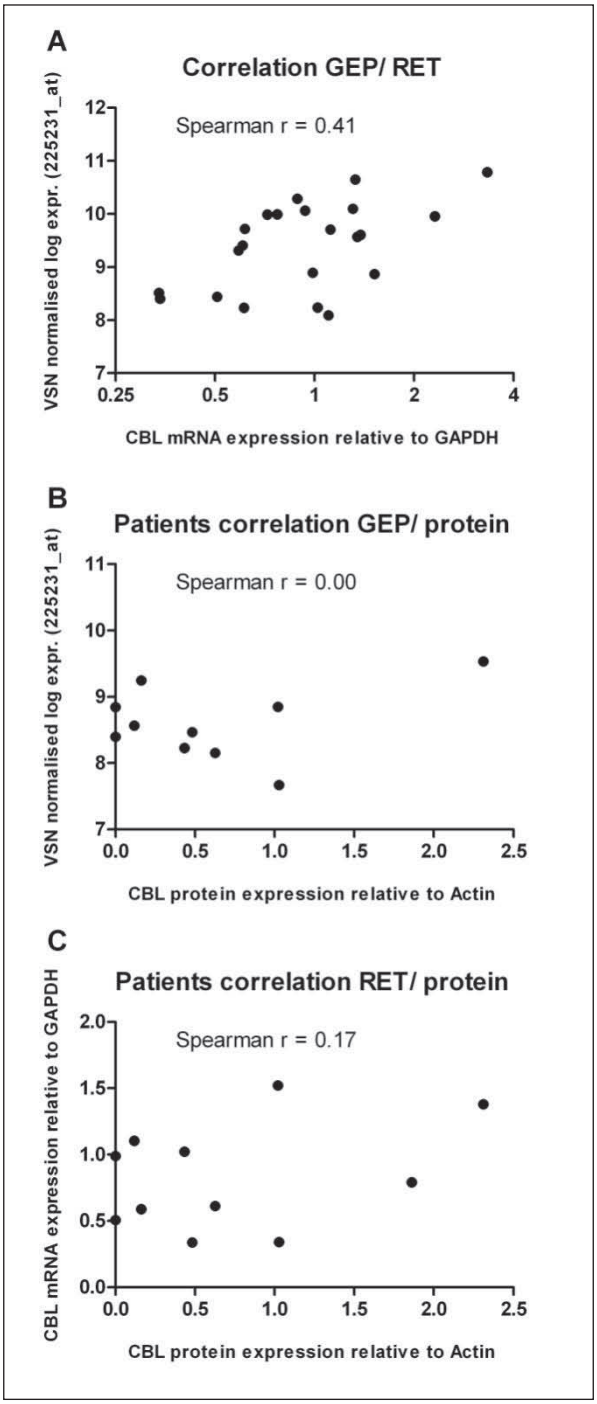
**Supplementary Table S1: Sequences of primers and probes**

| Primer/Probe | Sequence (5'-3') |
|---|---|
| *MLL* Forward | CGT CGA GGA AAA GAG TGA |
| *AF6* Reverse | TCC CGA TCA TCT TTG TTC |
| *AF10* Reverse | CTG GAA ATT TGC ATT TGT AA |
| *AF9* Reverse | ATG TTT CCA GGT AAC TCT GTA GT |
| *ENL* Reverse | TAC CCC GAC TCC TCT ACT T |
| *ELL* Reverse | CCC ATG ACT GGA GAC ATA CT |
| *AF4* Reverse | CTG-GGG-TTT-GTT-CAC |
| *CBL*-8F | ACC CAG ACT AGA TGC TTT CTG |
| *CBL*-8R | AGG CCA CCC CTT GTA TCA GT |
| *CBL*-9F | TTC AGA TGC ATC TGT TAC TAT CT |
| *CBL*-9R | AGT GTT TTA CGG CTT TAG AAG ACA |
| *CBL* Forward (RT-qPCR) | GCC GCC TTC TCC ATT CT |
| *CBL* Reverse (RT-qPCR) | CAG GGG GCA GTT TGT CTC |
| *GAPDH* Forward | GTC GGA GTC AAC GGA TT |
| *GAPDH* Reverse | AAG CTT CCC GTT CTC AG |
| *GAPDH* Probe | (FAM)-TCA ACT ACA TGG TTT ACA TGT TCC AA (TAMRA) |
| *CBL* Forward (RT-PCR) | TCC GGC TGA GCT GTA CTC |
| *CBL* Reverse (RT-PCR) | GTG GTG GTG GAA GAT CTC |
| *CBL* siRNA J-003003-09 | AAUCAACUCUGAACGGAAA |
| *CBL* siRNA J-003003-10 | GACAAUCCCUCACAAUAAA |
| *CBL* siRNA J-003003-11 | UAGCCCACCUUAUAUCUUA |
| *CBL* siRNA J-003003-12 | GGAGACACAUUUCGGAUUA |

Note: for siRNAs described sequences are target sequences. *CBL* PCR conditions for genomic DNA: 95°C for 2 minutes, 35 cycles of 94°C for 40 seconds, 58°C for 30 seconds, and 72°C for 40 seconds, one cycle of 72°C for 5 minutes. *CBL* and *GAPDH* RT-qPCR conditions: 50 °C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. *CBL* RT-PCR conditions: 95°C for 5 minutes, 35 cycles of 95°C for 15 seconds, 58°C for 45 seconds, and 72°C for 2 minutes, one cycle of 72°C for 10 minutes.


**Supplementary Figure S1: Mutation analysis *CBL* exon 8 of case 2 germline material**
The electropherogram is shown of sorted lymphocytes and myeloid blasts at diagnosis. An identical heterozygous point mutation is found: exon 8 + 4 C>T. Dx: diagnosis.




**Supplementary Figure S2: Correlation of CBL protein and *CBL* mRNA expression**

Graph showing *CBL* mRNA expression from GEP (probe set 225231_at) in correlation with *CBL* mRNA expression measured by RT-qPCR (A); *CBL* mRNA expression from GEP (probe set 225231_at) in correlation with CBL protein expression from western blot (B); and *CBL* mRNA expression measured by RT-qPCR in correlation with protein expression from western blot (C). Panel A shows data from 10 patient samples and 12 cell lines, panel B shows data from 10 patient samples and panel C from 11 patient samples. GEP: gene expression profiling, RET: relative expression calculation from RT-qPCR relative to *GAPDH* levels.

# Chapter 7

## *Versican* expression is an adverse prognostic factor in *MLL*-rearranged infant acute lymphoblastic leukemia

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## INTRODUCTION

Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) represents an aggressive type of leukemia, associated with a poor clinical outcome due to early relapses[1]. This poor prognosis is associated with a high incidence (~80% of the cases) of *MLL* translocations, which are independent predictors of an adverse outcome[1, 2]. In an attempt to identify genes that improve risk classification for *MLL*-rearranged infant ALL patients, Kang *et al.* recently build a prediction model for outcome based on gene expression profiling, which separated *MLL-AF4* cases in a low and a high risk-group. This model was based on the expression of 29 Affymetrix microarray probesets, including all probesets (n=5) corresponding to *VCAN*.[3] The human *VCAN* gene encoding for the hyaluronan-binding proteoglycan protein Versican, has four different splice variants designated V0, V1, V2 and V3[4]. Versican is known to be an important component of the extracellular matrix and plays a role in various cellular processes, including cell adhesion, proliferation, apoptosis, angiogenesis, migration and invasion[5]. Moreover, elevated *VCAN* expression has been observed in several types of human cancer, including leukemia, and is associated with clinical outcome in prostate and breast cancer[6-8].

Because *VCAN* expression was shown to contribute in the prediction model of 29 probes for outcome in *MLL*-rearranged infant ALL[3] and the prognostic value of *VCAN* expression alone has never been studied in *MLL*-rearranged infant ALL, we here studied the prognostic value of *VCAN* expression in a relatively large cohort of infant ALL patients.

## METHODS

Patient samples and characteristics, RNA, and cDNA were obtained as previously described[9]. We studied the *VCAN* mRNA expression in 68 infant ALL cases, including both patients carrying any *MLL* translocation (t(4;11), t(11;19), t(9;11), or other), and patients with germline *MLL*. We extracted the data of the probesets corresponding to *VCAN* (204619_s_at,204620_s_at, 211571_s_at, 215646_s_at) from our previously generated gene expression profiling data (Affymetrix HU133plus2.0 GeneChip microarray)[9]. Microarray data was normalized according to the Variance Stabilization and Normalization (VSN) method, as previously described[9]. VSN expression values of <4 typically represent expression levels that do not exceed the background noise levels, and are considered to correspond to expression levels that are very low or absent. The levels of mRNA expression were validated by quantitative RT- PCR analysis, as previously described [9]. The following primer combinations used for transcript amplification were used 5'to 3'; 'CCACGCTTCCTATGTGA'

and 'TTTCCCACTTTGACTTTATGT' for *VCAN* and 'GTCGGAGTCAACGGATT' and 'AAGCTTCCCGTTCTCAG' for reference gene *GAPDH*.

The *in vivo* prednisone response was assessed as part of the Interfant protocols[2]. *In vitro* prednisolone cytotoxicity data was available for a subset of patients and was determined by 4-day MTT assays as previously described[10]. Outcome was evaluated in terms of event-free survival (EFS) estimated by Kaplan-Meier and compared by Log-rank test in univariate and subgroup analyses, while multivariable analysis used the Cox regression model. Patient characteristics were analyzed using Fisher's Exact and Mann Whitney U-tests. Analysis was performed using SPSS Statistics version 20.0 (SPSS Inc. Chicago, IL, USA) and SAS version 9.2. *p*-values (two-tailed) less than 0.05 was considered statistically significant.

## RESULTS

As shown in Figure 1A, in most of the 68 patient samples analyzed, *VCAN* is not expressed, or expressed at a very low level. Patients carrying *MLL* translocation t(4;11) express *VCAN* in a broad range, including patients showing high expression levels (Figure 1A). To validate the results from our Affymetrix microarray data we also performed quantitative RT-PCR analysis on the majority (n=58) of the samples used in microarray analysis. We found a strong correlation between the microarray and RT-PCR expression data (Spearman r=0.83, $p<0.0001$) (Figure 1B).

Data on outcome was available on 60 patients. Overall, we found that *VCAN* expression at diagnosis is associated with poor outcome in *MLL*-rearranged infant ALL patients, with a 5-year EFS of 15.2% (SE 11.2%) for patients showing *VCAN* expression vs. 47.1% (SE 8.2%) for patients lacking *VCAN* expression ($p=0.002$, Figure 1C). Young age and high WBC counts at diagnosis, as well as a poor response to prednisone *in vivo*, are known risk factors in infant ALL[2, 11] and are used in the Interfant protocol to stratify *MLL*-rearranged cases into a high-risk group (HR, with age <6 months and either WBC counts $\leq 300x10^{-9}$/L or PPR) and a medium-risk group (MR, including all others). Therefore, we studied the relation of these prognostic factors with *VCAN* expression and their joint impact on outcome. Patients showing *VCAN* expression at diagnosis presented at a younger age compared with patients without *VCAN* expression: 86.4% (19/22) of patients expressing *VCAN* at diagnosis presented with ALL before 6 months of age, in contrast to 44.7% (17/38) of patients lacking *VCAN* expression ($p=0.002$). Furthermore, we found a significant negative correlation between age and *VCAN* mRNA expression at diagnosis among our *MLL*-rearranged infants (Spearman r= -0.498, $p<0.0001$). *VCAN* expression was significantly associated with WBC count at diagnosis ($p=0.012$): infants with *VCAN* expression were more likely to have high

WBC counts, ≥300x10$^{-9}$/L (59.1%), compared to those without *VCAN* expression (42.1%). None of the *VCAN* expressing cases had WBC counts below 100x10$^{-9}$/L, compared to 28.9% of the cases lacking *VCAN* expression. For patients with WBC counts ≥ 300x10$^{-9}$ there was no difference in outcome between those with and without *VCAN* expression (Table 1). However for patients with WBC counts < 300x10$^{-9}$/L *VCAN* expression was associated with a had worse outcome ($p$=0.0004, Table1). In addition, we analysed *VCAN* expression by *MLL*-rearrangement and observed that *VCAN* expression mostly occurs in t(4;11)-rearranged as compared to other *MLL*-rearranged cells (51.9% vs 32.0%, $p$=0.034). We found that t(4;11)-

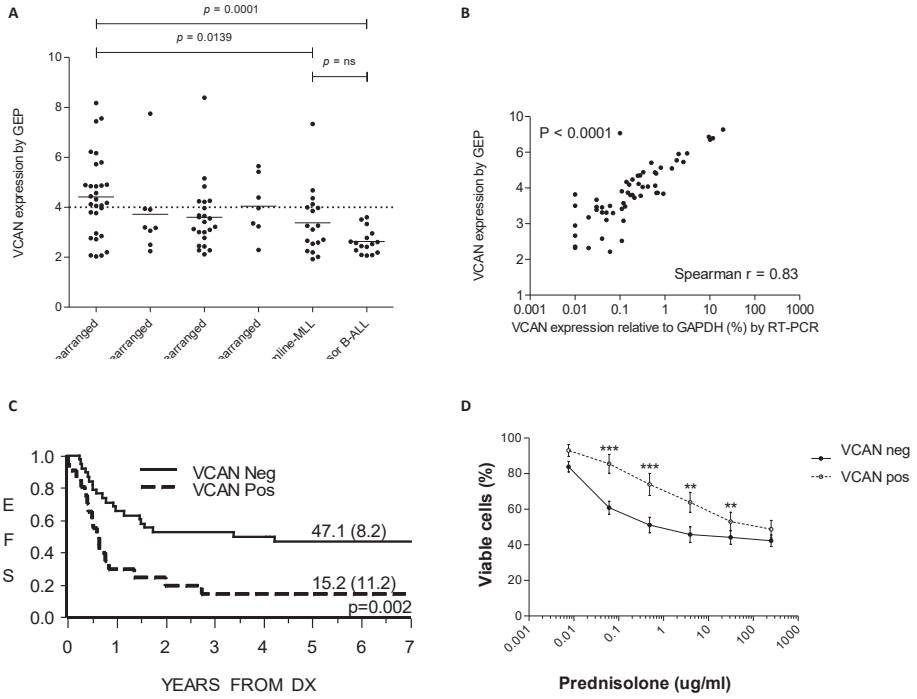


**Figure 1: *VCAN* mRNA expression in diagnostic *MLL*-rearranged infant ALL samples**
(A) VSN normalized microarray data (Affymetrix HU133plus2.0 GeneChips) showing the average expression of the five probesets (204619_s_at, 204620_s_at, 211571_s_at and, 215646_s_at.) corresponding to *VCAN*, in infant ALL patients carrying MLL translocation t(4;11) (n=30), t(9;11) (n=8), t(11;19) (n=23), other MLL translocations (i.e. 11q23; n=7), infant ALL patients without *MLL* translocations (i.e. germline *MLL*; n=18 ) and childhood ALL patients older than 1 year of age without *MLL* translocations (i.e. B-ALL; n=16) (B) Correlation between *VCAN* expression as determined by microarray analysis and quantitativeRT-PCR analysis for n=58 primary *MLL*-rearranged infant ALL samples. (C) 5-year EFS for *VCAN* expression-positive and negative *MLL*-rearranged. Survival data was available for 60 *MLL*-rearranged infant ALL cases. (D) *In vitro* prednisolone cytotoxicity in *MLL*-rearranged infant ALL patients, with (n=35) and without (n=25) *VCAN* expression at diagnosis, as determined by MTT-assays. Mean *in vitro* cytotoxicity responses were statistically analyzed using two-tailed Mann-Whitney U-test. Error bars represent standard error of the mean. (*0.05> p >0.01, **0.01> p >0.001, ***p<0.001).

**Table 1: Univariate analysis of *VCAN* expression and prognostic factors in *MLL*-rearranged infant ALL**

| | *VCAN negative* | | | *VCAN positive* | | | |
|---|---|---|---|---|---|---|---|
| | **Patients** | **Events** | **5-year EFS (SE)** | **Patients** | **Events** | **5-year EFS (SE)** | ***P-value*** |
| **Age at diagnosis (months)** | | | | | | | |
| 0-6 | 17 | 10 | 41.2% (12.1) | 19 | 15 | 16.8% (11.8) | 0.1604 |
| 6-12 | 21 | 10 | 51.4% (11.1) | 3 | 2 | - | - |
| **WBC count (x $10^9$/ L)** | | | | | | | |
| < 300 | 22 | 8 | 62.9% (10.5) | 9 | 7 | - | 0.0004 |
| ≥ 300 | 16 | 12 | 25.0% (12.5) | 13 | 10 | 16.7% (14.4) | 0.5164 |
| **11q23 abnormality** | | | | | | | |
| t(4;11) | 13 | 4 | 69.2% (12.8) | 14 | 11 | 15.5% (13.8) | 0.0032 |
| other positive | 25 | 16 | 36.0% (10.0) | 8 | 6 | - | 0.0415 |
| **Risk Stratification** | | | | | | | |
| MR | 29 | 12 | 58.2% (9.2) | 9 | 7 | - | 0.0006 |
| HR | 9 | 8 | 11.1% (16.6) | 13 | 10 | 16.7% (14.4) | 0.4650 |

Univariate survival analysis of the prognostic factors, including age at diagnosis, white blood cell (WBC) count at diagnosis, 11q23 abnormality (t;411 vs. other), prednisone response and risk stratification in *MLL*-rearranged infant ALL patients for *VCAN* expression-positive and negative cases. WBC= white blood cell count, PGR=prednisone good responder, PPR=prednisone poor responder, MR=medium risk, HR=high risk.

rearranged infant ALL patients positive for *VCAN* expression had a 5-year EFS of 15.5% compared to 69.2% in t(4;11)-rearranged infants without *VCAN* expression ($p$=0.0032, table 1). No significant association between *VCAN* expression at diagnosis and *in vivo* prednisone response was found ($p$=0.766), while a highly significant correlation was observed between *in vitro* resistance to prednisolone and *VCAN* expression (Figure 1D). As shown in Table 1, within the subgroup of prednisone good responders (PGR, i.e. patients with <1000 cells/mL in peripheral blood after 7 days of treatment with prednisone and a single intrathecal dose of methotrexate) there was a significant difference in EFS according to *VCAN* expression ($p$=0.013), while this was not observed in prednisone poor responders (PPR). Infants with *VCAN* expression (59.1%) were more likely at HR, compared to patients without *VCAN* expression (42.1%, $p$=0.011), but this did not translate into a poorer outcome ($p$=0.4650). On the contrary, MR patients with *VCAN* expression showed a significantly worse outcome than MR lacking *VCAN* expression ($p$=0.0006, Table 1).

In order to evaluate the independent prognostic impact of *VCAN* expression, we applied a Cox model including the risk group stratification described above. Interestingly, there was a significant interaction between *VCAN* expression and risk stratification, indicating

that *VCAN* expression had a different impact in HR vs. MR patients ($p$=0.01). This model showed that the outcome of MR patients with *VCAN* expression was significantly worse than that of MR lacking *VCAN* expression (Hazard ratio=4.43, 95%CI=1.72–11.36, $p$=0.002). On the contrary, *VCAN* expression in HR patients did not have any impact (Hazard ratio=0.67, 95% CI=0.26-1.70, $p$=0.399).

## DISCUSSION

Inhibiting VCAN synthesis could be a potential therapy-strategy in *MLL*-rearranged infant ALL cells. Genistein, a tyrosine kinase inhibitor, has been reported to inhibit VCAN synthesis in vascular smooth muscle cells and malignant mesothelioma cell lines[12, 13]. Besides, it was shown recently that Genistein induces apoptotic cell death, inhibits cell growth and a G2/M arrest in adult T-cell leukemia (ATL) cells [14]. Pre-clinical studies showed that prostate, breast, and colon cancer cells were sensitized by Genistein to other chemotherapeutics[15, 16]. However, Genistein has also been reported to make childhood ALL cells more resistant to daunorubicin[17].

In conclusion, *VCAN* expression in *MLL*-rearranged infant ALL patients is associated with a poor prognosis and could be used in combination with Interfant-06 stratification criteria to identify MR patients at high risk of treatment failure. Therefore, VCAN inhibition may represent an attractive therapeutic strategy that should be explored in pre-clinical studies.

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# Chapter 8

## Minimal mesenchymal stromal cell contaminations compromise mRNA profiling in acute lymphoblastic leukemia cells derived from co-culture experiments

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Submitted

## ABSTRACT

Acute lymphoblastic leukemia (ALL) in infants is a high-risk malignancy characterized by a high rates of early relapses originating from the bone marrow. To decipher the role of the bone marrow microenvironment (BMME) in this type of childhood leukemia, we initially performed microarray-based gene expression profiling of leukemic cells cultured *in vitro* on supportive mesenchymal stromal cell (MSC) layers. As such we intended to identify transcriptional changes involved in the BMME-leukemic cell interactions. Unfortunately, in the course of these experiments, we became aware that contaminating MSCs in the leukemia samples were compromising our results. In order to characterize to what extent contaminating MSCs influenced our data, we set out to perform a series of comprehensive experiments which led us to conclude that as little as 1% MSC contamination is sufficient to establish a significant MSC expression profile within our leukemic samples. As co-culturing of malignant hematological cells on MSC layers represents a widely used method, we decided to report and share all of our findings. With this, we would like to emphasize that transcriptional data obtained from *in vitro* co-culture experiments should be interpreted with great caution.

## INTRODUCTION

In recent years the relevance of the bone marrow microenvironment (BMME) in leukemia maintenance and progression has become evident, revealing that reciprocal interactions between leukemic and bone marrow (BM) stromal cells favor leukemic cells survival [1-3]. Acute lymphoblastic leukemia (ALL) in infants (<1 year of age) represents an aggressive type of leukemia that is associated with a high incidence (~80% of the cases) of translocations of the *MLL* (*Mixed Lineage Leukemia*) gene [4, 5]. Despite the fact that *MLL*-rearranged infant ALL cells typically are resistant to important chemotherapeutics [5-7], morphological complete remission (CR) is achieved in the majority of patients. Yet, favorable treatment results are hampered by exceedingly high relapse rates during treatment [5, 7-9]. As the vast majority of relapses originate from the BM, it is plausible to assume that the BMME provides a protective sanctuary for small subsets of leukemic cells that evade initial chemotherapy and give rise to the re-emergence of the leukemia. Hence, the BMME is likely to play a pivotal role in *MLL*-rearranged infant ALL.

A commonly used method to study BMME-leukemia interactions *in vitro* involves culturing of leukemic cells on top of layers of mesenchymal stromal cells (MSCs) [10-16]. Such co-culture studies revealed that MSCs support hematopoietic and leukemic cell maintenance *in vitro* through cell-cell contact and/or via the secretion of propitious soluble factors [10, 11]. Moreover, several studies demonstrated changes in miRNA, mRNA, and protein expression in various hematologic malignancies induced by supportive stromal layers [13-17]. Encouraged by these results, we studied the role of the BMME in *MLL*-rearranged infant ALL. We observed that, when cultured on mesenchymal stromal cell (MSC) layers, part of the *MLL*-rearranged ALL cells adhere to the MSCs, while another portion of the leukemic cells failed to adhere and remained in suspension. From this we reasoned that applying microarray-based gene expression profiling (GEP) on the separated adherent and non-adherent *MLL*-rearranged ALL cell fractions should potentially reveal important transcriptomic changes involved in the interactions between *MLL*-rearranged ALL cells and MSC layers. Obviously, interacting membrane receptors and/or adhesion molecules would represent attractive candidates for targeted inhibition in order to impede the ability of *MLL*-rearranged ALL cells to interact with the BMME.

However, based on our initial results and subsequent validation experiments we increasingly raised doubts regarding our seemingly promising GEP data, and felt that our results may have been compromised by contaminating MSCs (although minute) in the adherent leukemic cell fractions. On top of that, our concerns were significantly strengthened by Paggetti *et al.* who demonstrated that a contamination as small as 0.01% of MSC in leukemic chronic lymphocytic leukemia (CLL) samples derived from *in vitro* co-culture

experiments substantially influenced miRNA expression profiles, inevitably leading to false-positive results [18]. During the course of our study, we were forced to conclude that our mRNA profiles were indeed corrupted by minute contaminations of interfering MSCs. Yet, as the *in vitro* co-culturing of malignant hematological cells on MSC layers is a widely used method, we decided to report our results, including evidence of the profound consequences of contaminating MSCs. With this we strive to strengthen the warning from Paggetti *et al.*, and hope to alert researchers to interpret data obtained from *in vitro* co-culturing experiments with caution.

## METHODS

### *MLL*-rearranged ALL and mesenchymal cell lines

The t(4;11)-positive cell lines SEM and RS4;11 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), BEL-1 was a kind gift from Dr. Tang (University Paris, France). All cell lines were maintained as suspension cultures in RPMI 1640 with L-Alanyl-L-Glutamine (Invitrogen Life Technologies, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS) (Integro, Zaandam, the Netherlands)

The human embryonal mesenchymal cell line H92-1 was a kind gift from Dr. Dzierzak (Erasmus University Rotterdam, The Netherlands) and were cultured in Myelocult H5100 (Stem cell technologies, Grenoble, France) with 35% MEM Alpha (Invitrogen) supplemented with 10% FCS (Integro), 1% Glutamax (Invitrogen), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen), and 12.5 µM/ml β-mercaptoethanol. The murine OP9 stromal cells (ATCC, Wesel, Germany) were cultured in MEM Alpha (Invitrogen) supplemented with 10% FCS (Integro), 1% Glutamax (Invitrogen), 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.125 µg/ml fungizone (Invitrogen). All cell lines and patient cells were cultured at 37 °C in humidified air containing 5% $CO_2$.

### Patient samples and cell lines

Bone marrow or peripheral blood samples from untreated infants (below one year of age) diagnosed with ALL were collected at the institutes participating in the international collaborative Interfant protocol [5]. Informed consent was obtained according to the Helsinki declaration, and approved by the Institutional Review Board of the Erasmus University Medical Center. All samples were processed as described before[19].

### Co-culture of *MLL*-rearranged cell lines with mesenchymal stromal layers

Human and murine stromal cells were cultured on a 0.01% porc gelatin A (Sigma-Aldrich, Zwijndrecht, The Netherlands) coated culture dishes and cultured until 90% confluency, upon which the cells were irradiated with 30 Gray to prevent overgrowth. Medium was refreshed one day after irradiation. Four days after irradiation *MLL*-rearranged leukemic cells were resuspended in AIMV medium (Invitrogen) and were plated at a density of $3x10^4$ cells/$cm^2$ on the confluent irradiated stromal layers at 37°C in 5% $CO_2$.

### Harvesting cells from the co-culture

After four days of co-culture the leukemic and mesenchymal cells were collected by their distinctive phenotype, as previously described [11]. Briefly, the non-adherent (NA) leukemic cells were harvested by aspiration of the supernatant, the stromal layer was gently washed twice with PBS to remove the remaining NA cells. The adherent (AD) cells, which adhere to the mesenchymal stromal layer, were harvesting by extensive washing with PBS. The mesenchymal cells were collected by incubating the stromal layer with trypsin. To establish the contamination of the leukemic cell faction with stromal cells, the purity was determined morphologically on May-Grünwald-Giemsa (Merck)–stained cytospins. All used leukemic cell samples had a purity of >97% blasts, more specific the NA samples were 99-100% pure and the AD samples 97-99% pure.

### RNA extraction

Genomic RNA were extracted from ~$0.5x10^6$ leukemic cells using All prep DNA/RNA Microkit (Qiagen, Venlo, The Netherlands) according to the manufacturer's guidelines, and quantified on a Nanodrop ND-1000 spectrophotometer (Isogen). The integrity of RNA was assessed on standard 0.8% agarose gels and on an Agilent 2100 Bioanalyzer (Agilent).

### Gene expression profiles

Gene expression profiles were generated as previously described [20]. Briefly, high-quality RNA was reverse transcribed using T7-linked oligo-dT primers, and the obtained cDNA was used as a template to synthesize biotinylated cRNA. Labeled cRNA was then fragmented and hybridized to HU133plus2.0 GeneChips (Affymetrix) according to the manufacturer's guidelines. The infant ALL patient gene expression data presented in this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus [21] and is accessible via GEO Series accession number GSE19475.

### Quantitative real-time PCR analysis

cDNA was obtained by reverse transcriptase of the total RNA and used to validate mRNA expression by quantitative real-time PCR analysis using the DyNAmo SYBR Green qPCR kit (Finnzymes, Espoo, Finland), as previously described [22]. Primer combinations used for

transcript amplification of selected target genes as well as the housekeeping reference genes *glyceraldehyde-3-phosphate dehydrogenise* (*GAPDH*), *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta polypeptide* (*YWHAQ*), $\beta_2$ *microglobulin* (*B2M*), and *beta-actin* (ACTB) are listed in Supplementary Table S1. All samples were analyzed in duplicate.

### Statistical Analysis

Statistical analyses of gene expression profiles were performed in R using Bioconductor packages (R Development Core Team, 2011). A *p*-value, corrected for multiple testing by the false discovery rate step-up procedure of Benjamini & Hochberg [23], of <0.05 was considerate significant. GenePattern version 3.7.0 was used to generate heatmaps.[24] The other statistical analyses were performed with SPSS Statistics version 17.0 (SPSS Inc. Chicago, IL, USA). All tests were two-tailed and a *p*-value below 0.05 was considered significant.

## RESULTS

### Gene expression profiling in *MLL*-rearranged ALL cells obtained from MSC co-culture experiments

In order to study the role of the BMME in *MLL*-rearranged infant ALL, we co-cultured t(4;11)-positive ALL cell lines (n=3, in duplicate) and primary t(4;11)-positive infant ALL patient samples (n=2) on MSC layers and observed two distinct phenotypes. A portion of the leukemic cells adhered to the MSCs (i.e. adherent leukemic cells), while others remained in suspension (i.e. non-adherent leukemic cells). Upon observing these different phenotypes, we were interested if differential gene expression between adherent and non-adherent leukemic cells could reveal genes that play a role in cell-cell contact between *MLL*-rearranged ALL cells and MSCs. Therefore, we separately harvested non-adherent and adherent leukemic cell fractions and generated GEP data using Affymetrix HU133plus2.0 GeneChips. In addition we generated GEP data from the mentioned *MLL*-rearranged ALL cell line and patient samples cultured in the absence of MSCs, as well as from MSCs cultured in the absence of leukemic cells. Next, the obtained GEP data was subjected to Limma (Linear models for microarray data) analysis, identifying a core signature consisting of n=55 significantly differentially expressed probesets (corresponding to n=43 genes) between adherent and non-adherent *MLL*-rearranged ALL cells (Figure 1). Remarkably, all of these genes appeared to be up-regulated in the adherent cells. Most of these genes encode for proteins involved in cell-cell adhesion and the extracellular matrix (ECM). Up-regulation in the adherent fraction of the primary *MLL*-rearranged infant ALL patient samples could be confirmed for most of these genes (Figure 2). The up-regulation of the identified core signature could be validated by quantitative RT-PCR analysis in adherent and non-adherent

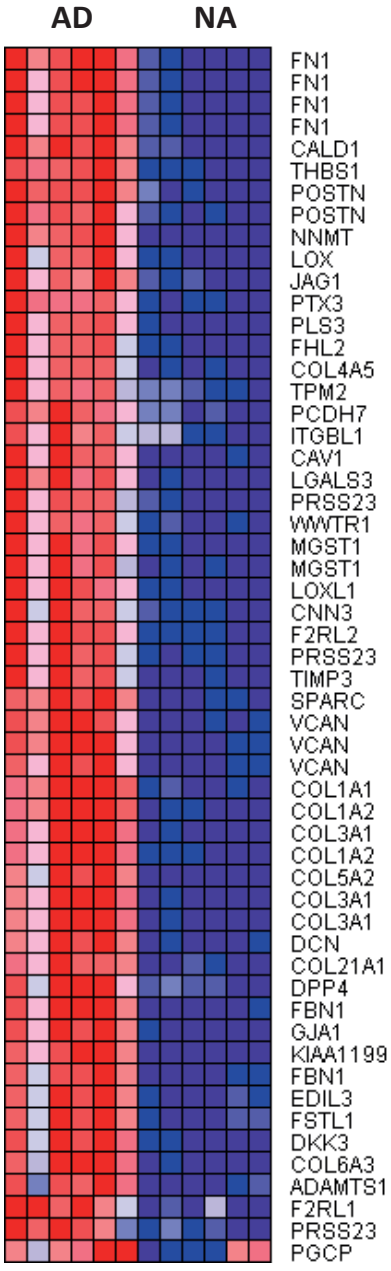


**Figure 1: Heatmap visualization of the 55 differentially up-regulated probesets**

Heatmap visualization (corresponding to 43 genes) in t(4;11)-rearranged ALL cells that were able to adhere (adherent; AD) to mesenchymal stromal cells and those that were not able to adhere (non-adherent; NA). Data was obtained from three different t(4;11)-rearranged ALL cell lines in duplicate experiments. Columns represent samples and rows represent genes. Relative high expression is shown in red and low expression in blue.

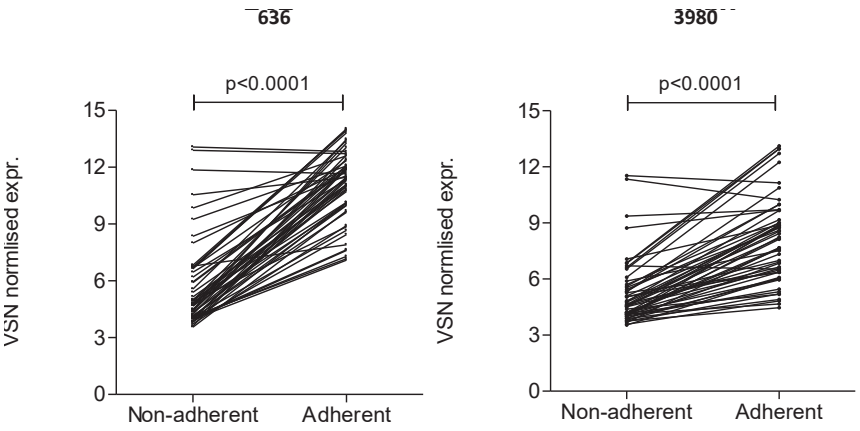


**Figure 2: mRNA expression of genes from t(4;11)-toplist**
mRNA expression of gens was determined by GEP in non-adherent and adherent co-culture samples in two different diagnostic t(4;11)-rearranged infant ALL patients samples. Each line represents one probeset. Wilcoxon matched pairs test was used to determine the upregulation of the toplist overall.

leukemic cell fractions obtained from independent experiments using the t(4;11)-positive ALL cell lines SEM and RS4;11 (Supplementary Figure S1). Based on our quantitative RT-PCR data, the genes in the identified core signature appeared to be up-regulated up to ~750-fold. Next we studied the expression of these genes in diagnostic infant ALL samples, from our recent published GEP dataset. [20] The majority of these genes were hardly expressed in diagnostic *MLL*-rearranged infant ALL samples (Figure 3).

## The cell-cell interaction-associated gene expression signature solely originates from contaminating MSCs

Initially our observations led us to assume that the observed up-regulation of genes involved in cell-cell interactions and ECM remodeling in *MLL*-rearranged ALL cells involved a specific gene signature that is rapidly induced upon adherence to MSCs, and which is readily down-regulated once the physical contact with the MSCs is lost. Yet, an alternative explanation could be that the observed gene expression signature was influenced by contaminating MSCs, despite the high purity (97-99.9% leukemic cells) of our extracted adherent *MLL*-rearranged ALL fractions. This alternative explanation became increasingly plausible with the publication of Paggetti *et al.*, showing that even a contamination of 0.01% of MSCs could lead to misinterpretations of miRNA expression data in the co-cultured leukemic cells [18].

Therefore we studied the expression of genes seemingly up-regulated in adherent *MLL*-rearranged ALL cells in our MSCs. We found that all of the genes were abundantly expressed in the MSCs, and at much higher levels than observed in the adherent leukemic cell fractions.

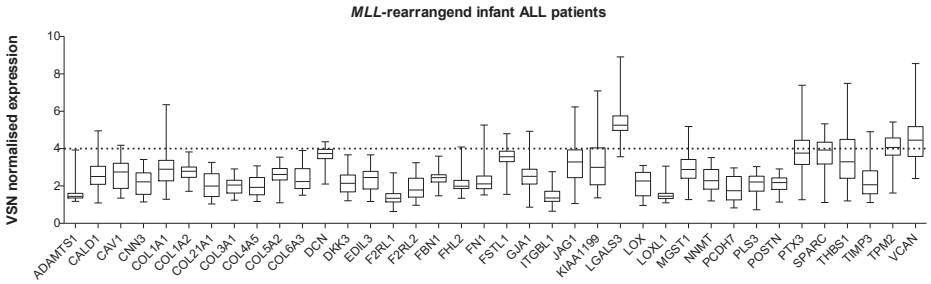


**Figure 3: mRNA expression in *MLL*-rearranged infant ALL samples**

VSN normalized mRNA expression based on GEP from diagnostic infant ALL samples. All plots are based on one probeset, except for the following genes, which the plot is based on the average of multiple probesets; *COL1A2* (n=2), *FBN1* (n=2), *MGST1* (n=2), *POSTN* (n=2), *COL3A1* (n=3), *PRSS23* (n=3), *VCAN* (n=4) and, *FN1* (n=4). Expression value of 4 is used as cutoff for expression, below 4 it is considerated that there is no expression (dashed line).

This is exemplified in Figure 4A, showing data for two Affymetrix HU133plus2.0 probesets corresponding to the *COL1A2* gene, encoding the human collagen I, alpha-2 polypeptide. The distribution of *COL1A2* expression as shown in Figure 4A is representative for all differentially expressed genes. The observation that all of the up-regulated genes appeared to be abundantly expressed in the MSCs advocated in favor of cell contamination. We wondered if such small numbers of contaminating cells could cause the enormous up-regulation of genes in the adherent fraction. Therefore, we performed experiments in which we deliberately mixed varying percentages of MSCs into *MLL*-rearranged ALL samples that were cultured in the absence of MSC layers. Immediately after mixing the cells, the samples were subjected to RNA extraction to avoid possible transcriptomic changes as a result of cell-cell interactions between the leukemic and MSCs. In addition we added the supernatant derived from 4-day MSC cultures to leukemic cells in order to explore the possibility of extracellular vesicles or cells debris was responsible for the observed up-regulation of gene expression. Next we performed quantitative RT-PCR analysis on the following genes: *COL1A2*, *FN1*, *LGALS3*, *LOX*, *LOXL1*, *SPARC*, *THBS1* and *VCAN*. We found that the mRNA expression of all of these genes increased with an increasing percentage of MSC contamination (Figure 4B, Supplementary Figure S2). Next, we compared the levels of expression in these deliberately contaminated samples to that of our adherent and non-adherent leukemic cell fractions derived from our co-culture experiments. As demonstrated in Figure 4C-D, the level of *COL1A2* expression in the non-adherent leukemic cells was comparable to that of 0.1% contamination, whereas in adherent leukemic cells the level of expression correlated with 1-10% contamination. Again, the example of *COL1A2* was representative for the other genes studied (data not shown). We also observed up-regulation of gene expression in SEM cells to which we only added the supernatant derived from MSCs, suggesting that even cell debris in the supernatant is potentially influencing gene expression.

We studied the correlation of the expression levels of eight randomly picked genes in our core signature between leukemic samples with increasing percentages of contaminating MSCs with that of pure MSC samples. We found that with an increasing contamination of MSCs in the leukemic cell samples, the correlation with pure MSC samples improved. In fact, at levels of contamination between 1-10% MSC in the leukemic cell samples, the

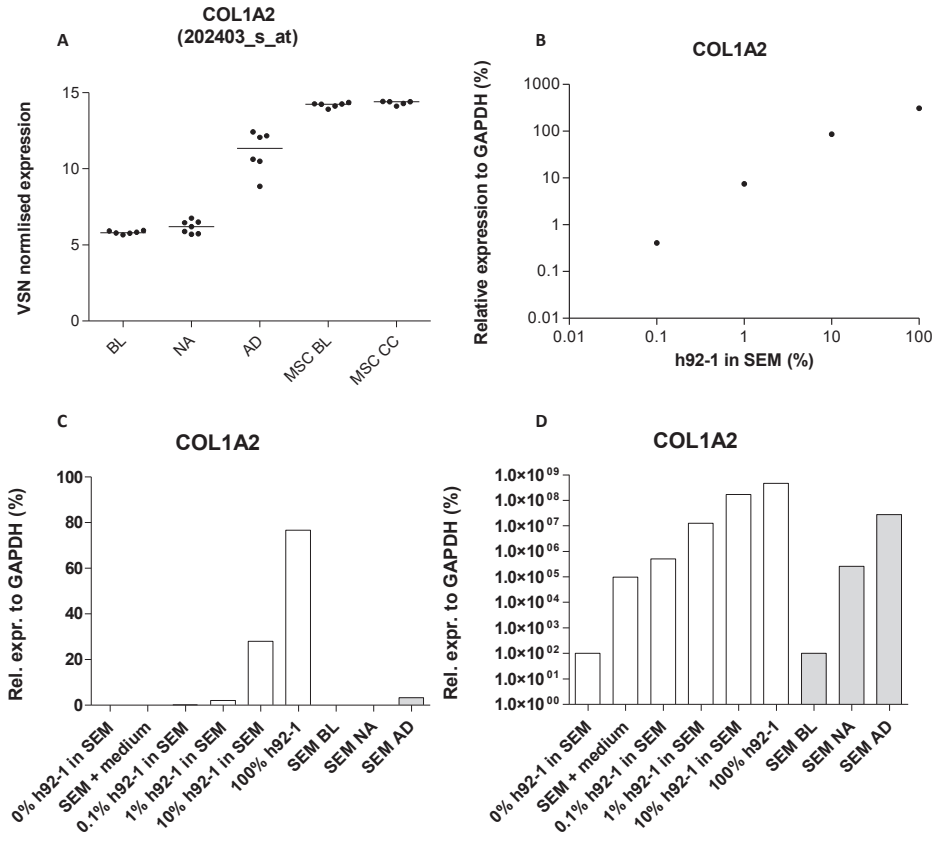


**Figure 4: VSN normalized GEP in *MLL*-rearranged ALL cell lines**

(A) SN normalized GEP expression of the *COL1A2* probesets, in cells *MLL*-rearranged ALL cell lines (before co-culture (BL), non-adherent (NA), adherent (AD)) and in stromal cells (no co-culture(MSC BL), from co-culture (MSC CC)) The trend in expression is representive for all the probesets from the t(4;11)-toplist. (B) mRNA expression of *COL1A2* relative to *GAPDH* housekeeping gene in SEM samples contaminated with increasing percentages of stromal cells (H92-1). The expression for *FN1*, *LGALS3*, *LOX*, *LOXL1*, *SPARC*, *THBS1* and, *VCAN* demonstrate the same trend as for *COL1A2*, all genes are depicted in supplementary figure 2.(C) *COL1A2* mRNA expression relative to *GAPDH* housekeeping gene in co-culture samples and contamination samples (D) and corrected for the baseline expression of SEM cells. The white bars represent the contamination samples and the grey bars represent the co-culture samples. mRNA expression of *FN1*, *LGALS3*, *LOX*, *LOXL1*, *SPARC*, *THBS1* and, *VCAN* demonstrate the same trend as for *COL1A2*, all genes are depicted in supplementary figure 3.

correlation of gene expression with that of pure MSC samples was nearly perfect (spearman R=1.000, $p<0.0001$) (Figure 5). Hence, 1-10% contamination of MSCs in a leukemic cell population apparently is sufficient to alter the mRNA expression levels entirely towards a MSC profile.

One alternative possibility that could explain the observed up-regulation of genes in our adherent leukemic cell samples apart from contaminating MSCs would be the transfer of mRNAs from MSCs to leukemic cells via secreted extracellular vesicles. The fact that we found up-regulation of gene expression in leukemic cells to which we only added the supernatant of cultured MSCs seem to be in favor of such a mechanism. To explore this possibility, we also performed additional co-culture experiments in which the leukemic cells were separated from the MSCs by inserted Transwell membrames with pore sizes of 1.0 µm or 0.4 µm. In case of a 1.0 µm pore size, extracellular vesicles like microvesicles secreted by the MSCs should still be able to reach the leukemic cells. In contrast, the membrames

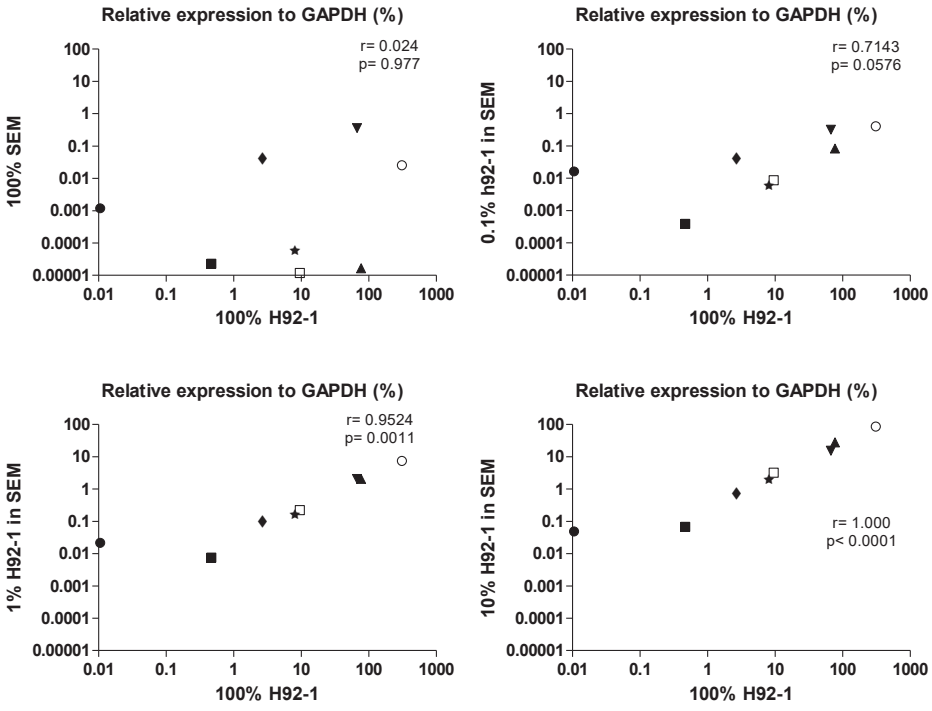


**Figure 5: Stromal mRNA expression in contaminated leukemic samples**
Correlation of stromal mRNA expression and stromal contaminated leukemic samples, based on eight of the t(4;11)-toplist genes (▲*COL1A2*, ○*FN1*, ■*LGALS3*, □*LOX*, ●*LOXL1*, ▼*SPARC*, ★*THBS1*, ♦*VCAN*). On the x-axis the mRNA expression of 100% stromal cells (H92-1) and on the y-axis the expression in of stromal (H92-1) contaminated leukemic samples, in varies percentages (0-10%) is displayed.

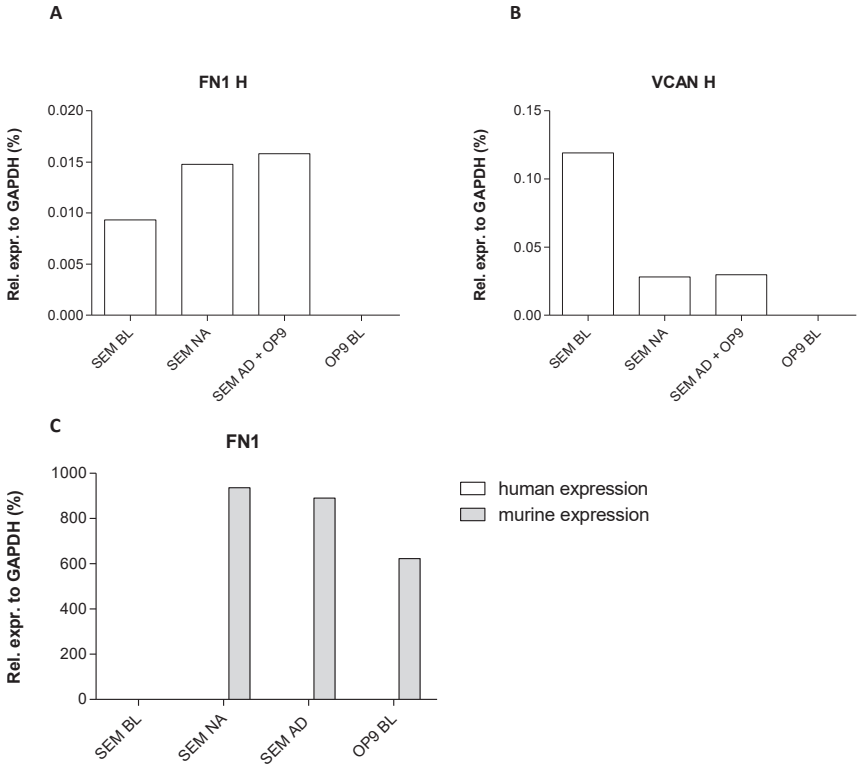


**Figure 6: mRNA expression in co-culture samples**
(A) mRNA expression relative to *GAPDH* housekeeping gene, as determined by RT-PCR, in human leukemic SEM cells (SEM BL), non-adherent human leukemic cells (SEM NA) and adherent human leukemic cells (SEM AD), and blank murine stromal OP9 cells (OP9 BL). SEM NA and SEM AD samples were derived from a co-culture of 4 days. (B) Human specific *FN1* expression, human specific *VCAN* expression, (C) human and murine specific *FN1* expression.

with a 0.4 μm pore size should largely block vesicle transfer. The results from these experiments demonstrated that the up-regulation of gene expression in the leukemic cells was completely lost by using either of the membranes (data not shown). This excludes the option that extracellular vesicles secreted by MSCs were responsible for (part of) the observed up-regulation of the genes in our core signature.

## Co-culture murine stromal cells and leukemic SEM cell line

Another approach to study whether or not the observed increases in gene expression was due to contamination is to perform co-culture experiments using murine instead of human MSCs. Therefore we used the OP9 murine stromal cell line as an supportive layer for the human leukemic SEM cell line. After four days of co-culture we harvested the adherent and non-adherent leukemic cells. Next, we performed quantitative RT-PCR analysis using species

specific primers (as enlisted in Supplementary Table S1) for the following genes; *COL1A2*, *FN1*, *LOXL1*, *THBS1*, and *VCAN*. We found that the expression of human *COL1A2*, *THBS1*, and *LOXL1* was absent in all samples. Human *VCAN* and *FN1* were expressed, but we could not observe an up-regulation in the adherent leukemic cell fraction (Figure 6A-B). On the other hand, we did observe high expression of all murine genes, except for *VCAN*, in the both the non-adherent and adherent leukemic cell fractions, as well as in pure OP9 cells. Furthermore, when we visualize the human (leukemic) and murine (stromal) expression of these genes in one graph it becomes very clear that there is hardly any leukemic expression of these genes (Figure 6C). This indicates that the expression of these genes found in our human-human co-cultures was stromal expression, so very likely to be caused by the contaminating stromal cells.

## DISCUSSION

In the present study we used a co-culture approach to study the influence of stromal-leukemic interations in Initially we identified a strong core signature of genes differentially expressed, but as all genes were highly expressed in the MSCs and barely in leukemic cells. This, together with the recent publication of Paggetti *et al.*[18], raised the question whether our results were actually caused by leukemic-stromal cell interactions or perhaps by MSC contamination in our leukemic cell fractions. Subsequently we performed various analyses and experiments, all of which strongly suggested that the obtained results were solely due to contaminating MSCs in our leukemic samples in our co-culture system.

In conclusion, we demonstrate that contaminating MSCs have large effects on GEP of leukemic cells derived from co-culture systems. This is in concordance with the recent findings of compromised miRNA expression, as described by Paggetti *et al.*[18] We expect that similar problems will occur with protein analyses in direct co-culture experiments. Although we do not question the value of *in vitro* co-culture models, we would like to emphasize the potential danger of contaminating stromal cells in the cell population of interest. Therefore, to evaluate results derived from such co-culture experiments should be analyzed with great caution, and a proper experimental design using relevant controls, as well as critical data analysis, are warranted in order to avoid misinterpretation of obtained data.

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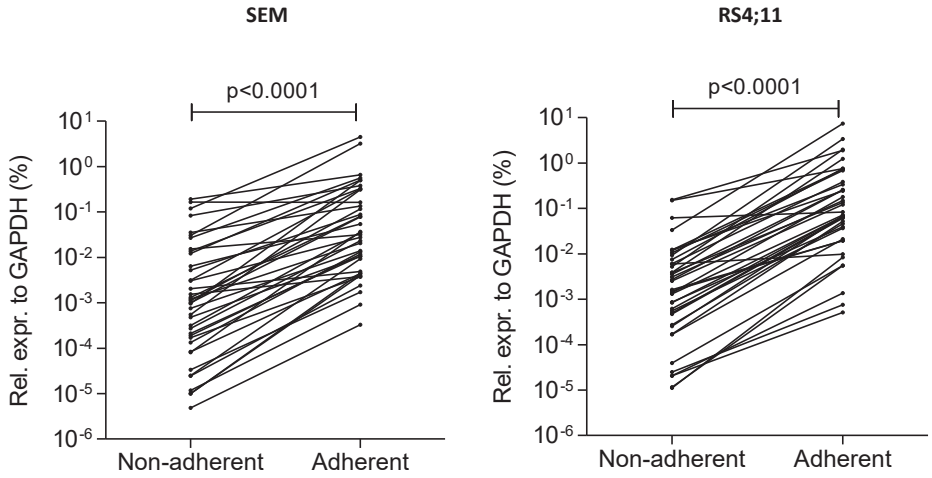
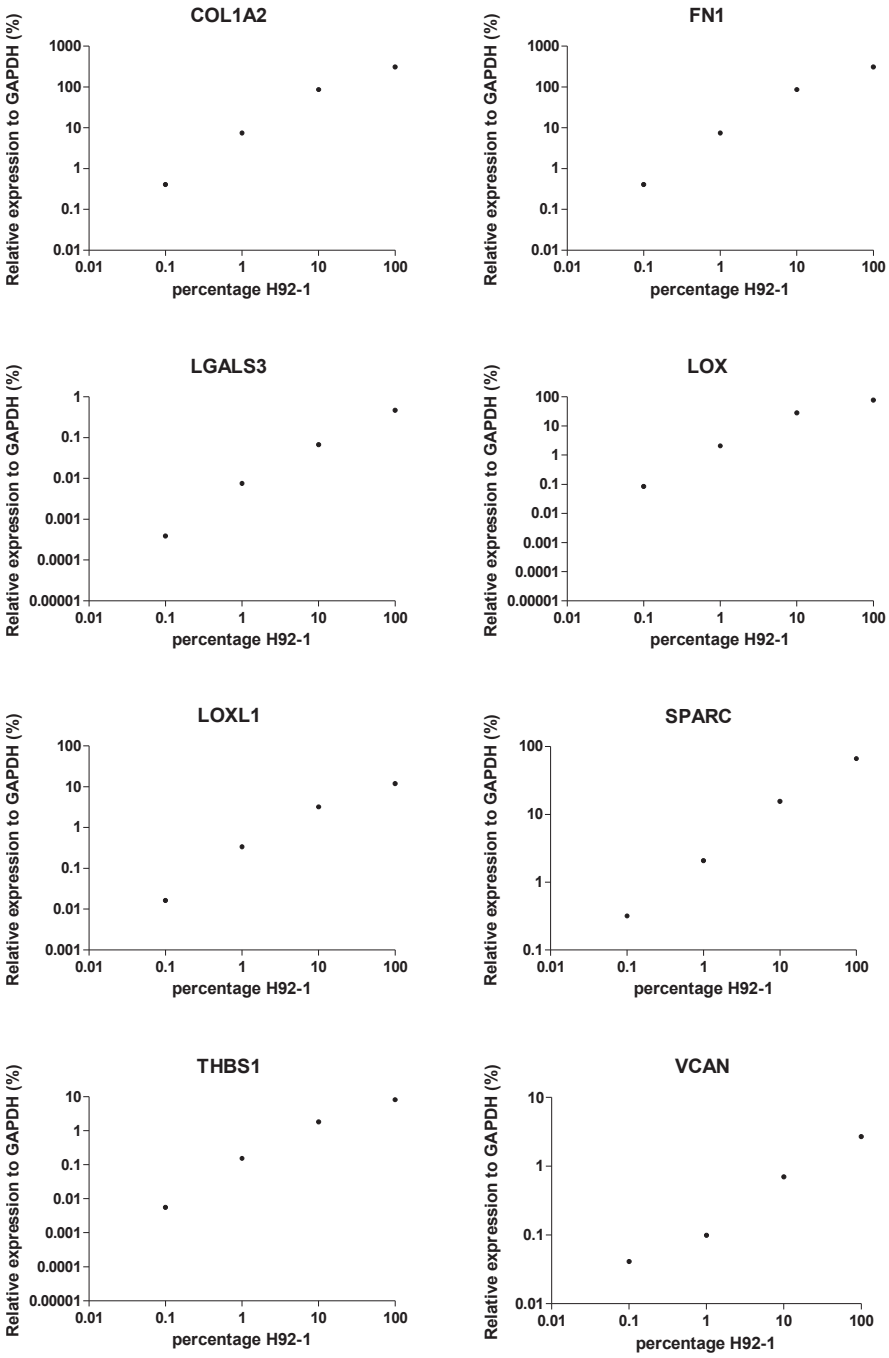



**Supplementary Figure S1:** mRNA expression of genes from t(4;11)-toplist as determined by GEP in non-adherent and adherent co-culture samples in two different t(4;11)-rearranged cell lines. Each line represents one probeset. Wilcoxon matched pairs test was used to determine the upregulation of the toplist overall.




**Supplementary Figure S2:** mRNA expression of t(4;11)-toplist genes (*COL1A2, FN1*, *LGALS3*, *LOX*, *LOXL1*, *SPARC*, *THBS1*, *VCAN)* relative to *GAPDH* housekeeping gene in SEM samples contaminated with increasing percentages of stromal cells (H92-1).

**Supplementary Table S1: Sequences of primers**

| Primer | Sequence (5'-3') |
|---|---|
| *ADAMTS1* Forward | AAGCTGCTCCGTCATAGA |
| *ADAMTS1* Reverse | GTCCTCCCCAAATGTAAAC |
| *CALD1* Forward | CTCCCAAACCTTCTGACTT |
| *CALD1* Reverse | CAGCATGGGTTTCTTTAGA |
| *CAV1* Forward | CGGAGGGACATCTCTACA |
| *CAV1* Reverse | CAAGCGGTAAAACCAGTATT |
| *CNN3* Forward | GACCCAGGTTCAGACTACTC |
| *CNN3* Reverse | TGGCTGGCTCCTTTATT |
| *COL1A1* Forward | CCTCGGAGGAAACTTTG |
| *COL1A1* Reverse | CAGCTTCCCCATCATCT |
| *COL1A2* Forward | TCAGACCCAAGGACTATGA |
| *COL1A2* Reverse | CTGCCAGCATTGATAGTTT |
| *COL1A2* Forward (human specific) | ATGGTCGCACTGGACAT |
| *COL1A2* Reverse (human specific) | AGCCCTGTAGAAGTCTCCA |
| *COL1A2* Forward (murine specific) | CGGACCCAGTGGTATTG |
| *COL1A2* Reverse (murine specific) | CAGACCACGTTCACCTCTA |
| *COL21A1* Forward | TGACCCAGAACAGAACAAC |
| *COL21A1* Reverse | TCCCGGAGGACAAATAC |
| *COL3A1* Forward | CATGCCAAATATGTGTCTGT |
| *COL3A1* Reverse | CTGGGGAGAATAGTTCTGAG |
| *COL4A5* Forward | GAGCCTGGTGGAATTACTT |
| *COL4A5* Reverse | CCGGCTGGGTTATAGTC |
| *COL5A2* Forward | TTCCGCAAAGCAGAGT |
| *COL5A2* Reverse | AGGCGCAAAAAAGTCAT |
| *COL6A3* Forward | GGTGCTGGTCCTCATAAG |
| *COL6A3* Reverse | TTCCGGGACAGTAAACAC |
| *DCN* Forward | TCAGCCGGATTGTGTT |
| *DCN* Reverse | GTGGCCTTCATGATTTATCT |
| *DKK3* Forward | GCCACCCTCAATGAGAT |
| *DKK3* Reverse | TTTGCCAGGTTCACTTCT |
| *EDIL3* Forward | AGTTCGGCAAAGGTGATA |
| *EDIL3* Reverse | CCTCGGTATGCTTCACTTA |
| *F2RL1* Forward | CATCCAAGGAACCAGTAGAT |
| *F2RL1* Reverse | CAGGGTGCTTCTTCTTAGTT |
| *F2RL2* Forward | TGTGCTCCATGATTTTACAG |
| *F2RL2* Reverse | TTTGCCAAGTTGTTTGTATC |
| *FBN1* Forward | AACCGAGGATTTCAACAA |
| *FBN1* Reverse | ACAGGGGTTTTTCTCACA |
| *FHL2* Forward | AAGCGGCTCTCTGACTC |

**Supplementary Table S1: Sequences of primers (continued)**

| Primer | Sequence (5'-3') |
|---|---|
| *FHL2* Reverse | CTCCCGCAGGATGTACT |
| *FN1* Forward | GGAGGAGACCACATGAGA |
| *FN1* Reverse | TCCCAGGCAAGTACAATC |
| *FN1* Forward (human specific) | CCTGCACCTGGAGTCTAC |
| *FN1* Reverse (human specific) | CGTGCCCAGAATTGTT |
| *FN1* Forward (murine specific) | AAAGGGAACGACAGT |
| *FN1* Reverse (murine specific) | ACAATCGGTGCATCTCTC |
| *FSTL1* Forward | CCAGCCCAGTTGTTTG |
| *FSTL1* Reverse | TGGCAGTTTCATTCTGTTC |
| *GAPDH* Forward (human specific) | GTCGGAGTCAACGGATT |
| *GAPDH* Reverse (human specific) | AAGCTTCCCGTTCTCAG |
| *GAPDH* Forward (murine specific) | CTCCTGCGACTTCAACAG |
| *GAPDH* Reverse (murine specific) | TCTGGGATGGAAATTGTG |
| *GJA1* Forward | TGCCTTTTCATTTTACTTCA |
| *GJA1* Reverse | GATTGGGAAAGACTTGTCAT |
| *JAG1* Forward | CTGGCCGAGGTCCTATA |
| *JAG1* Reverse | GGGCGGCAGAACTTAT |
| *KIAA1199* Forward | AGGGGAAGGAGTTTTGAG |
| *KIAA1199* Reverse | GGAAGCAGGTCAGAGTGA |
| *LGALS3* Forward | CCTGCACCTGGAGTCTAC |
| *LGALS3* Reverse | CGTGCCCAGAATTGTTAT |
| *LOX* Forward | CAGCCGACCAAGATATTC |
| *LOX* Reverse | GCTGGGGTTTACACTGAC |
| *LOXL1* Forward | CTGCGGAGGAGAAGTGT |
| *LOXL1* Reverse | GGTTGCCGAAGTCACA |
| *LOXL1* Forward (human specific) | CTGCGGAGGAGAAGTGT |
| *LOXL1* Reverse (human specific) | GGTTGGGGAGGAAGTCT |
| *LOXL1* Forward (murine specific) | GGGCAGGTGTTCAGTC |
| *LOXL1* Reverse (murine specific) | CACGCAGCAGAAGAATG |
| *MGST1* Forward | TTGCCAATCCAGAAGACT |
| *MGST1* Reverse | CGTGCTCCGACAAATAGT |
| *NNMT* Forward | AGAAGGGCTGAACTGATG |
| *NNMT* Reverse | AATCCCGAGGGTTAAAAT |
| *PCDH7* Forward | TCCCAGAGGACAACTATGA |
| *PCDH7* Reverse | AGCTGCACTGAAGGTCTC |
| *PGCP* Forward | TGGGAAGCACTCTCACTT |
| *PGCP* Reverse | CCCTGGCCTTTTCACT |
| *PLS3* Forward | TGGGTGGAACTTCAGAGT |
| *PLS3* Reverse | CCAGCCCTCAAATCTTC |

**Supplementary Table S1: Sequences of primers (continued)**

| Primer | Sequence (5'-3') |
|---|---|
| *POSTN* Forward | TTGCCCTGGTTATATGAGA |
| *POSTN* Reverse | ACTCGGTGCAAAGTAAGTG |
| *PTX3* Forward | TTGCGATTCTGTTTTGTG |
| *PTX3* Reverse | TTCCGAGTGCTCCTGA |
| *SPARC* Forward | CTGCGGGTGAAGAAGAT |
| *SPARC* Reverse | TCCAGGGCGATGTACTT |
| *THBS1* Forward | TTCACCGCCTACAGATG |
| *THBS1* Reverse | CTGGGGGTTTTCTCAAG |
| *THBS1* Forward (human specific) | TCCCCTAGTCTATCACAAC |
| *THBS1* Reverse (human specific) | TGCCACAGCTCGTAGAA |
| *THBS1* Forward (murine specific) | ATCCGCACCACCTACATC |
| *THBS1* Reverse (murine specific) | GTTCTGGCAGTGACACTCT |
| *TIMP3* Forward | GCCCCATGTGCAGTAC |
| *TIMP3* Reverse | TGTCGGTCCAGAGACACT |
| *TPM2* Forward | ACCAGGCCCTCAAGTC |
| *TPM2* Reverse | TCTGCTCGGGTCTCAG |
| *VCAN* Forward | CCACGCTTCCTATGTGA |
| *VCAN* Reverse | TTTCCCACTTTGACTTTATGT |
| *VCAN* Forward (human specific) | GTCCGGGATTGAAGACACA |
| *VCAN* Reverse (human specific) | GGATGGGATATCTGACAGTC |
| *VCAN* Forward (murine specific) | ACGCAAACACATGAATCA |
| *VCAN* Reverse (murine specific) | CAAGGTGAATCCATAGTGACT |
| *WWTR1* Forward | TGACCCCAGACATGAGAT |
| *WWTR1* Reverse | CTGCGTTTTCTCCTGTATC |

RT-PCR thermocycling conditions: 95°C for 5 minutes, 35 cycles of 95°C for 15 seconds, 58°C for 45 seconds, and 72°C for 2 minutes, one cycle of 72°C for 10 minutes.

# Chapter 9

Summary and
general discussion

In this final chapter we summarize and discuss our main findings from this thesis. First we will recapitulate main characteristics of infant acute lymphoblastic leukemia (ALL). Next we will discuss our most important results, relevant prognostic factors in infant ALL, the potential clinical impact, and suggestions for treatment strategies.

## INFANT ACUTE LYMPHOBLASTIC LEUKEMIA

Over de past decades therapeutic strategies and outcome for pediatric acute ALL improved enormously, leading to current survival-rates approaching 90%[1]. Although treatment of infant (<1 year of age) ALL patients usually involves more aggressive chemotherapeutic regimes, clinical outcome for these young patients remains poor, with event free survival (EFS) rates of ~50%[2, 3]. A strong independent prognostic factor of poor outcome in infant ALL is the presence of an *MLL* translocation[2]. *MLL* translocation occur in ~80% of all infant ALL cases[4]. The most recurrent *MLL* translocations are t(4;11), t(11;19) and t(9;11), which generate the chimeric fusion proteins MLL-AF4, MLL-ENL and MLL-AF9, respectively. In approximately 95% of *MLL*-rearranged infant ALL cases morphologically complete remission is achieved [5, 6]. Despite the initial good response on chemotherapeutics, good prognoses in infant ALL patients are hampered by high relapse-rates (30-50%). Most relapses occur within the first year after diagnosis, while still on treatment[5-7]. Relapsed infant ALL is generally assumed to be inevitably fatal.

## RELAPSED INFANT ACUTE LYMPHOBLASTIC LEUKEMIA

In **chapter 2** we describe the clinical outcome of a large cohort of relapsed infant ALL patients treated with the interfant-99 treatment protocol[2]. Overall we found a 20.9% survival for all relapsed infant ALL patients and 24.9% for those treated with curative intent. This is in concordance with the 25.6% 5-year overall survival (OS) in recurrent or refractory infant ALL reported by Tomizawa *et al.*[8]. We found that young age (< 6 months of age) and high white blood cell (WBC) counts (>300x$10^9$/L) at initial diagnosis were associated with inferior outcome after the first relapse, but response to a prednisone prophase prior to first line of treatment did not. Furthermore, patients who relapsed earlier (<24 months after diagnosis) and patients with bone marrow (BM) involvement (either isolated or combined) had a worse outcome. In concordance with previous studies we found that the majority of relapses (56.9%) in our cohort occured within the first year after diagnosis. We demonstrate that time of relapse is of prognostic relevance. Patients who relapsed within 24 months after diagnosis had a 1.5-fold increase in the risk of death. Most of the relapses originated from the BM (71.8%), which was associated with a dismal outcome compared with patients with

isolated extramedullary relapses. We have demonstrated that relapsed infant ALL is not invariably lethal, and with that underlined the relevance of offering treatments with curative intents. However, the majority of relapsed infant ALL patients remain to be challenged with a very poor prognosis. Hence, the development of therapeutic strategies specifically designed to increase the prognosis for relapsed infant ALL patients is warranted. Yet, to accomplish this, better insights into the biology and underlying mechanisms of relapse are required.

In this thesis we discuss different factors that contribute to infant ALL disease outcome, disease progression and relapse. As we discuss in **chapter 3** we found that *in vitro* resistance to standard chemotherapeutic agents as currently used in the treatment for infant ALL, is associated with a high risk of therapy failure and consequently poor event-free survival (EFS) rates, with the majority of events being relapses. These data confirm that relapsed infant ALL cells are indeed more resistant to chemotherapy as compared with the leukemic cells at diagnosis. In other words, in most of the cases the current treatment protocol for infant ALL patients fails to eliminate all leukemic cells, and selects for the most resistant cells that subsequently are able to give rise to disease relapses that are even more difficult to treat.

As the vast majority of relapses originate from the BM (see **chapter 2**), we hypothesized that the bone marrow microenvironment (BMME) plays an important supportive role in therapy-resistance and relapses in infant ALL patients. Furthermore, we describe in **chapter 4** that almost all infants harboring a *RAS* mutation suffer from a relapse within the first 3 years after achieving complete remission. We hypothesize that relapses in infant ALL are caused by a inter-play between biological factors of the leukemic clone as well as the biological supportive environmental factors. Unraveling the biological mechanism causing leukemic relapse could lead to new targets for therapy strategies. Treatment options reducing the risk of relapse as well as more effective relapse-treatment for infant ALL patients.

## BONE MARROW MICROENVIRONMENT

In recent years the relevance of the BMME in leukemia maintenance and disease progression has become evident, revealing that reciprocal interactions between leukemic and BM stromal cells favor leukemic cells survival[9-11]. As the majority of infant ALL relapses originate from the BM, it is plausible to assume that the BMME plays a role in relapsed infant ALL, by providing a protective sanctuary for small subsets of leukemic cells that evade initial chemotherapy and give rise to the re-emergence of the leukemia. A commonly used method to study BMME-leukemia interactions involves *in vitro* co-culturing of leukemic

cells with supportive mesenchymal stromal cell (MSC) layers[12-17] . To decipher the role of the BMME in infant ALL, we initially performed microarray-based gene expression profiling (GEP) of leukemic cells co-cultured *in vitro* with MSC layers, as described in **chapter 8**. Unfortunately, in the course of these experiments, we became aware that contaminating MSCs in the leukemic samples were compromising our results. In order to characterize to what extent contaminating MSCs influenced our data, we set out to perform a series of comprehensive experiments.

We concluded that minute contaminating MSCs have large effects on GEP data of leukemic cells derived from co-cultures, which appeared in line with recent findings of Paggetti *et al.*[18]. As co-culturing of malignant hematological cells on MSC layers represents a widely used method, we would like to emphasize the potential danger of contaminating MSCs in order to avoid misinterpretation of obtained data. Therefore based on these results we could not demonstrate the role of the BMME in (relapsed) infant ALL within the duration of this research project. Admittedly, MSC contamination may have been prevented by placing fine-grained filters between the MSC layers and the leukemic cells during co-culture experiments. However, this would also prevent direct contact between the leukemic cells and MSCs, limiting communication between the two cell types to cytokine and chemokine exchange via the culture medium only. However, as demonstrated by Polak *et al.*, ALL cells depend on direct interactions with MSCs via tunneling nanotubes (TNTs) for intercellular communication[19]. Hence, performing co-culture experiments in which the MSC layers are separated from the leukemic cells by fine-grained filters would likely not have resulted in relevant data.

With current advanced technologies, we would re-design our original experiments in which infant ALL cells are co-cultured in direct contact with MSC layers, following a cell sorting strategy to eliminate contaminating cells in either cell fraction. To further exclude the risk of contaminating cells, we would suggest to replace GEP of bulk cell populations with a currently available single-cell sequencing approach.

## PROGNOSTIC FACTORS

The presence of an *MLL*-rearrangement is known as one of the most predictive factors of poor clinical outcome in infant ALL patients[2]. In turn, among *MLL*-positive infant ALL patients other important risk factors have been identified, including young age (<6 months), WBC at diagnosis, a pro- B-cell immunophenotype characterized by the absence of the CD10 cell surface marker, and a poor *in vivo* response to a 7-day prophase of prednisone treatment [2, 20, 21]. In the Interfant treatment protocols for infant ALL patients, age, WBC

counts, and *in vivo* prednisone response are used to stratify *MLL*-rearranged cases into risk groups and allocate cases to corresponding treatment strategies. Infants younger than 6 months at diagnosis, displaying high WBC counts (≥300x$10^{-9}$/L) or a poor prednisone response are allocated to the high-risk (HR) arm of the treatment protocol, whereas all other cases are allocated to the medium-risk (MR) treatment arm. Yet, to further improve patient stratification and better prediction which of the patients are at high risk of developing disease relapses, additional risk factors and/or prognostic markers are required. In this thesis we identified the presence of (sub)clonal *RAS* mutations (**chapter 4**) and high *VCAN* expression (**chapter 7**) to represent two novel prognostic markers for *MLL*-rearranged infant ALL.

## *RAS*-PATHWAY

In search of new therapeutic targets in *MLL*-rearranged acute leukemia, recent studies focused on the *RAS*-pathway[22-27]. In **chapter 4** we indentify the presence of *RAS* mutations as an independent predictor of an very poor outcome in infant ALL. In a substantial subset (14-24%) of our cohort of infant ALL samples we detected *RAS* mutations. The highest frequency of *RAS* mutations was found in patients carrying the t(4;11)-translocation. In fact, none of the t(4;11)-positive infant ALL patients, carrying a *RAS* mutation, survived their malignancy and deceased within 4 years from diagnosis. Interestingly, patients harboring a *RAS* mutation appeared to have higher WBC counts at diagnosis ($p$=0.013), and were found to be more resistant to glucocorticoids *in vitro* ($p$<0.05). Thus, the presence of a *RAS* mutation seemed to coincide with already known risk factors. Yet, in a multivariate analyses including known risk factors in infant ALL, the presence of a *RAS* mutation represented an independent predictor of a poor clinical outcome.

About half of our detected *RAS* mutations appeared to be present at a subclonal level. This observation was recently confirmed by studies demonstrating high frequencies 47-63.8% of (sub)clonal RAS/PI3K pathway mutations in pediatric *MLL*-rearranged ALL samples[27, 28]. Trentin *et al.* demonstrate that the mutational clones present at relapse were in almost all cases also detectable at diagnosis[28]. Combined these data suggest that the *RAS-mutated* clone may represent the chemoresistant cell fraction that evades initial chemotherapy to give rise to disease relapses. Hence, targeting the *RAS* pathway, for instance using MEK inhibitors such as Trametinib or Selumetinib, could be beneficial for *MLL*-rearranged infant ALL patients carrying (sub)clonal *RAS* mutations, especially since Trametinib exposure appeared to sensitize *MLL*-rearranged ALL cell to prednisolone *in vitro* (**chapter 5**).

However, our research group recently published a validation study in which *in vivo* efficacy of the MEK inhibitor Trametinib was tested against an *MLL*-rearranged infant ALL cell line carrying a clonal *RAS* mutation in a xenograft mouse model[29]. This study demonstrated that Trametinib mono-therapy was not sufficient to significantly inhibit overall leukemia progression in mice[29], despite similar studies showing Selumetinib and Trametinib to effectively inhibit leukemia progression in xenograft mouse models of childhood ALL and AML [30, 31] . Nonetheless, Trametinib did seem to specifically target *MLL*-rearranged ALL cells in the BM of the mice, showing reduced leukemic cell numbers present in the BM in ~50% of the mice upon Trametinib treatment[29]. Thus, the implementation of a MEK inhibitor within the current treatment protocol for infant ALL patients may well reduce the frequency of disease relapses of the *RAS* mutated clone, and at the same time could potentiate the effects of prednisone which already is part of the standard treatments of childhood ALL. Such an implementation may well be feasible in the near future as promising results with MEK inhibitors are being reported in clinical trials for various adult malignancies [32-36].

In **chapter 6** we set out experiments to identify mutations in the *Casitas B lineage lymphoma* (*CBL*) gene, another important gene from the *RAS*-pathway. Recently, mutations in *CBL* were found in other types of hematological malignancies and appeared to exist mutually exclusive from other *RAS*-pathway mutations[37-39]. However, we were not able to detect mutations in *CBL* among our cohort of infant ALL samples.

## *VERSICAN* EXPRESSION

Recently high *Versican* (*VCAN*) expression was identified to be of prognostic value in a prediction model based on Affymetrix microarray probesets associated with a poor outcome in *MLL*-rearranged infant ALL patients. Elevated *VCAN* expression has been observed in various types of human cancer, including leukemia, and is associated with poor clinical outcome[40-42]. In **chapter 7** we describe the relation of clinical outcome and *VCAN* expression in a large cohort of infant ALL patients. *VCAN* expression at diagnosis was associated with a poor outcome in *MLL*-rearranged infant ALL patients. Furthermore we found a different impact of *VCAN* expression in the risk-stratification groups. Infant ALL patients who display *VCAN* expression were more likely to be allocated to the HR arm of the treatment protocol, without an effect on outcome. On the contrary, patients allocated to the MR group who displayed *VCAN* expression showed a significantly worse outcome compared as compared to patients lacking *VCAN* expression. *VCAN* expression could therefore be used to identify patients within the MR group, with high risk of therapy-resistance or relapse. We hypothesized that indentifying these "high-risk" patients within the MR group could lead to

re-allocation of these patients to the HR arm of the treatment protocol, which seems more appropriate for these patients.

Moreover, investigating *VCAN* expression as a potential target for novel therapeutic strategies could be of interest. For instance, Genistein, a tyrosine kinase inhibitor, has been reported to inhibit VCAN synthesis in various malignancies[43-45], and pre-clinical studies showed that Genistein sensitizes various types of malignant cells, including childhood ALL cells, to other chemotherapeutic agents[46-48]. Therefore we hypothesize that Genistein, because of its chemotherapeutic-sensitizing effects, could especially be beneficial for therapy-resistant and relapsed infant ALL patients.

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# Chapter 10

Nederlandse samenvatting
Lijst van publicaties
Curriculum Vitae
PhD portfolio
Dankwoord

## NEDERLANDSE SAMENVATTING

In de afgelopen decennia zijn de overlevingskansen voor kinderen met acute lymfatische leukemie (ALL) sterk verbeterd tot ongeveer 90%. De overlevingskansen voor zuigelingen (kinderen jonger dan 1 jaar) met ALL blijven helaas nog steken op 50%. Dit ondanks de meer agressieve chemotherapie waar deze jonge kinderen mee behandeld worden. ALL bij zuigelingen is in verschillende opzichten anders dan bij kinderen ouder dan 1 jaar. Bij ongeveer 80% van de zuigelingen is er een genetisch defect aanwezig, wat bij oudere kinderen slechts voorkomt bij 1% van de patiënten. Dit genetische defect is een herschikking van het zogenaamde *MLL* gen. Dit betekent dat een deel van het *MLL* gen gekoppeld wordt aan een deel van een ander gen. Hierdoor wijzigt de functie van het *MLL* gen, welke normaliter een belangrijke rol speelt in de ontwikkeling van witte bloedcellen. Een verandering van de functie van het *MLL* gen kan leiden tot het ontstaan van ALL. De aanwezigheid van deze *MLL*-herschikking is bij zuigelingen met ALL een belangrijke voorspeller voor een slechte kans op overleving. Ongeveer 95% van de zuigelingen met een *MLL*-herschikking in de leukemiecellen komt na de initiële chemotherapie in remissie. Dat houdt in dat de leukemiecellen niet meer aantoonbaar zijn in het bloed en beenmerg. Ondanks dat de meeste zuigelingen in remissie komen, hebben ze een grote kans (~50%) dat de ziekte weer terug komt, oftewel een recidief. De meeste recidieven ontstaan tijdens het eerste jaar na de diagnose en dus daarmee nog tijdens de behandeling met chemotherapie.

Er werd over het algemeen verondersteld dat een recidief bij zuigelingen met ALL altijd fataal is. Echter, in **hoofdstuk 2** beschrijven we de klinische uitkomsten van een grote groep zuigelingen met een ALL recidief. Uit ons onderzoek bleek dat na 5 jaar ongeveer 20% van deze zuigelingen nog in leven was. Verschillende factoren waren van invloed op de genezingskans na het recidief. Jonge leeftijd (<6 maanden) en een zeer hoog aantal witte bloedcellen in het bloed bij diagnose bleken geassocieerd met een slechte uitkomst na het eerste recidief. Hiernaast bleek dat zuigelingen, die binnen 2 jaar na diagnose een recidief kregen of waarbij het recidief in het beenmerg plaats vond een slechtere kans op overleving hebben. Ruim de helft van de recidieven in onze groep zuigelingen vond plaats in het eerste jaar na diagnose, dus nog tijdens behandeling. Het tijdstip van het recidief is een belangrijke voorspeller voor de overleving. Patiënten, die binnen 2 jaar na de diagnose een recidief kregen, hebben een 1.5 maal vergroot risico om te overlijden, dan kinderen die een later recidief kregen. De meeste recidieven ontstaan in het beenmerg, wat geassocieerd wordt met een slechtere uitkomst ten opzichte van patiënten waarbij het recidief op een andere plaats in het lichaam ontstaat. Ons onderzoek laat zien dat een recidief van zuigelingen ALL niet altijd fataal hoeft te zijn. Dit geeft voldoende reden om deze groep patiënten te behandelen met een curatieve intentie. Het gros van de patiënten met een recidief heeft echter nog steeds een erg slechte prognose. Daarom is het van belang om therapeutische

opties te ontwikkelen voor deze groep patiënten. Om dit te bereiken is meer inzicht nodig in de biologie van ALL bij zuigelingen en hoe een recidief ontstaat. We denken dat recidieven worden veroorzaakt door een samenspel tussen biologische factoren van de leukemiecellen en de biologische omgevingsfactoren in het beenmerg. Nader onderzoek naar deze biologische mechanismes, die een rol spelen bij het ontstaan van een recidief, kunnen bijdragen aan het ontwikkelen van nieuwe therapeutische mogelijkheden om het risico op recidief te verlagen, en het effectiever behandelen van recidieven.

In **hoofdstuk 3** testen we in het laboratorium de gevoeligheid van de leukemiecellen van patiënten voor de standaard chemotherapeutica, die gebruikt worden bij de behandeling. Het blijkt dat, wanneer leukemiecellen in het laboratorium minder gevoelig zijn voor chemotherapeutica, er een verhoogd risico is op een recidief ALL en overlijden. Met andere woorden, met het huidige behandelingsprotocol voor ALL bij zuigelingen lukt het niet altijd om alle leukemiecellen volledig te elimineren. De meest resistente leukemiecellen krijgen de kans om uit te groeien tot een recidief en een nog moeilijker te behandelen ALL.

Zoals we in **hoofdstuk 2** beschrijven ontstaat de meerderheid van de recidieven in het beenmerg. Omdat bloedcellen geproduceerd worden in het beenmerg, is dit de meest voorkomende locatie voor een leukemierecidief. We denken dat het beenmerg een belangrijke omgevingsrol speelt in therapieresistentie en het ontstaan van recidieven van ALL bij zuigelingen. Gedacht wordt dat leukemiecellen in staat zijn om interacties met de beenmergcellen aan te gaan en zich zo in het beenmerg kunnen verschuilen voor de chemotherapie. Zeer kleine aantallen leukemiecellen, welke met de huidige technieken niet meer te detecteren zijn in het beenmerg van de patiënt, overleven zo de chemotherapie en groeien later uit tot een recidief. In **hoofdstuk 8** beschrijven we hoe we de interacties tussen deze cellen onderzocht hebben en welke genen daarbij van belang waren. Helaas kwamen we tijdens deze experimenten tot de conclusie dat ons model, welke ook veel door anderen gebruikt wordt om dit soort interacties te bestuderen, de onderzoekresultaten onbetrouwbaar maakte. Hierdoor konden we geen conclusies verbinden aan de behaalde resultaten.

Zoals eerder genoemd is de aanwezigheid van een *MLL*-herschikking een van de belangrijkste voorspellers voor een lagere kans op overleving voor zuigelingen met ALL. Andere belangrijke voorspellers zijn jonge leeftijd (< 6 maanden) bij diagnose, zeer hoge aantallen witte bloedcellen bij diagnose en een slechte reactie op de eerste week van prednison behandeling. Deze verschillende voorspellers worden gebruikt om zuigelingen in te delen in verschillende risicogroepen, met meer en minder kans op overleving. De behandelingsprotocollen zijn aangepast op deze risicogroepen. Zuigelingen met hoog risico zullen een agressievere chemotherapie ontvangen en soms beenmergtransplantatie. Om nog beter te

kunnen voorspellen welke zuigelingen een groot risico op een slechte uitkomst hebben is het noodzakelijk meer risicofactoren te identificeren.

In **hoofdstuk 4** onderzoeken we of de aanwezigheid van een defect van het *RAS* gen een dergelijke risicofactor is. Het blijkt dat bij een significant aantal (14-25%) van de zuigelingen met ALL een mutatie in het *RAS* gen in de leukemiecellen aanwezig is. Hiernaast blijkt dat bijna alle zuigelingen met een mutatie van het *RAS* gen in de leukemiecellen binnen 3 jaar na complete remissie een recidief krijgen. Binnen 5 jaar na de diagnose is nog maar 11% van deze zuigelingen in leven in tegenstelling tot 45% bij de zuigelingen zonder deze *RAS* mutatie. De aanwezigheid van een *RAS* mutatie in de leukemiecellen blijkt dus een voorspeller voor een zeer slechte klinische uitkomst, onafhankelijk van andere bekende risicofactoren bij zuigelingen met ALL. Omdat deze uitkomsten gebaseerd zijn op een klein aantal onderzochte zuigelingen, zouden deze bevestigd moeten worden in een grotere groep zuigelingen met ALL. Naast het screenen van *RAS* mutaties hebben we in **hoofdstuk 6** een screening uitgevoerd voor mutaties in het *Casitas B lineage lymphoma* (*CBL*) gen. Het *CBL* gen is een ander belangrijk gen binnen het *RAS*-pathway. We hebben echter geen *CBL* mutaties gevonden in de onderzochte leukemiecellen van zuigelingen met ALL en dus geen relatie kunnen vaststellen.

Therapeutische middelen gericht op het *RAS*-pathway, zoals Trametinib of Selumetinib, zouden gunstig kunnen zijn voor patiënten met een *RAS* mutatie in de leukemiecellen. Zeker aangezien we in **hoofdstuk 5** laten zien dat blootstelling van leukemiecellen aan Trametinib in het laboratorium, deze gevoeliger maakt voor prednisolon, een van de belangrijkste middelen tegen leukemie. Prednison is een niet-werkzame stof welke in het lichaam wordt omgezet in het werkzame prednisolon. Een goede reactie op prednison bij de start van de chemothepeutische behandeling is een voorspeller voor een hogere overlevingskans. Recent heeft onze onderzoeksgroep in een *in vivo* muismodel niet kunnen aantonen dat Trametinib als monotherapie de leukemieprogressie van *RAS*-gemuteerde *MLL*-herschikte ALL cellen voldoende kon remmen. Wel werd aangetoond dat de behandeling van Trametinib het aantal leukemiecellen met 50% reduceerde in een deel van deze muizen. Trametinib zou dus mogelijk het effect van prednison, reeds aanwezig in de standaard behandeling, kunnen versterken. Er zijn veelbelovende resultaten met therapeutische middelen, die het *RAS*-pathway remmen, bij maligniteiten bij volwassenen beschreven. Het implementeren van zo'n middel in de behandeling voor zuigelingen met ALL zou in de nabije toekomst haalbaar kunnen zijn.

Een andere potentiële kandidaat om nieuwe therapeutische opties tegen te ontwikkelen is het zogenaamde *Versican* (*VCAN*) gen. Recent onderzoek heeft aangetoond dat een verhoogde activiteit (expressie) van het *VCAN* gen samen gaat met een lagere overlevingskans

bij verschillende maligniteiten, waaronder zuigelingen met een *MLL*-herschikte ALL. In **hoofdstuk 7** bevestigen we deze associatie tussen hoge *VCAN* expressie in de leukemiecellen tijdens diagnose en lagere overlevingskans in ons cohort van zuigelingen met ALL. We vonden ook een verschil van impact van *VCAN* expressie in de verschillende risicogroepen. Patiënten met een hogere *VCAN* expressie in de leukemiecellen waren vaker toegewezen tot het hoog-risico behandelprotocol, zonder effect op de klinische uitkomst. Patiënten uit de medium-risico behandelgroep en met hoge *VCAN* expressie hadden een significant lagere overlevingskans vergeleken met patiënten zonder *VCAN* expressie. *VCAN* expressie zou dus gebruikt kunnen worden om binnen de medium-risicogroep, patiënten te identificeren met een verhoogd risico op therapieresistentie of een recidief. We denken dat het identificeren van deze hoog-risico patiënten binnen de medium-risicogroep zou kunnen leiden tot herverdeling van deze patiënten naar de hoog-risico behandelgroep, met een meer passende behandeling voor deze patiënten. Hiernaast is het interessant om te onderzoeken of *VCAN* een potentieel aangrijpingspunt geeft voor nieuwe behandelingsopties. Van het middel Genistein is bijvoorbeeld beschreven dat het de synthese van het VCAN eiwit remt in verschillende maligniteiten. Preklinische studies lieten zien dat Genistein verschillende typen maligne cellen, waaronder leukemiecellen van kinderen, gevoeliger maakt voor andere chemotherapeutica. Door deze sensibiliserende eigenschappen zou Genistein met name geschikt kunnen zijn voor zuigelingen met een therapieresistente of recidief ALL.

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## CURRICULUM VITAE

Emma Margriet Cécile werd geboren op 19 november 1981 te Utrecht. Zij behaalde haar diploma aan het Christelijk Gymnasium te Utrecht in 2000.

Hierna startte zij met de opleiding Culturele Antropologie en sociologie der niet-westerse samenlevingen aan de Universiteit Utrecht, waar zij in 2001 haar propedeuse behaalde.

Ze werd in 2002 toegelaten tot de studie Geneeskunde aan de Erasmus Universiteit. In 2009 verrichtte ze haar onderzoekstage op de afdeling kinderoncologie van het Erasmus Medisch Centrum. Onder begeleiding van prof. dr. R. Pieters en dr. R.W. Stam deed ze onderzoek naar mitochondriale genetische variaties bij zuigelingen met acute lymfatische leukemie (ALL) . In 2010 behaalde ze haar artsdiploma.

In 2010 begon ze aan haar promotietraject op de afdeling kinderoncologie van Erasmus Medisch Centrum. Onder begeleiding van prof. dr. R. Pieters en dr. R.W. Stam deed ze onderzoek naar determinanten van klinische uitkomst bij zuigelingen met ALL, wat resulteerde in dit proefschrift.

Vanaf 2014 is ze als jeugdarts werkzaam binnen de jeugdgezondheidszorg bij het Centrum voor Jeugd en Gezin Rijnmond. In 2017 startte ze, onder begeleiding van drs. R. Gelauf-Visser en drs. I. Moorman-Wildevuur, de opleiding tot jeugdarts KNMG bij onderzoeksinstituut TNO te Leiden. In 2019 zal ze haar diploma behalen.

Emma woont in Rotterdam samen met haar echtgenoot Egbert van der Graaff en hun zoons Oscar (2012), Felix (2014) en Boris (2016).

# PHD PORTFOLIO

| | |
|---|---|
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| **General courses** | |
| Research management for PhD-students | 2011 |
| **Biomedical courses** | |
| Classical Methods for Data Analysis (NIHES) | 2010 |
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| Analysis of microarray gene expression data using R (Molmed) | 2011 |
| **Seminars and Workshops** | |
| Annual Molecular Medicine Day, Erasmus MC | 2011-2014 |
| Annual Pediatric Oncology Symposium, Erasmus MC | 2010-2013 |
| Annual PhD Day, Erasmus MC | 2010-2013 |
| KiKa Promovendi dag | 2011-2014 |
| **Oral presentations** | |
| 8 oral presentations at the weekly pediatric research meetings and pediatric oncology research meetings | 2010-2014 |
| 44th Congress of the International Society of Paediatric Oncology, Londen, UK | 2012 |
| 8th Bi-annual Childhood Leukemia Symposium, Santiago, Chili | 2012 |
| **Poster presentations** | |
| 17th European Hematology Association, Amsterdam, The Netherlands (2x) | 2012 |
| 8th Bi-annual Childhood Leukemia Symposium, Santiago, Chili | 2012 |
| 25th Annual Meeting of the international BFM Study Group,Praag, Tsjechië | 2014 |
| **Conferences** | |
| The Bone Marrow Niche, Stem Cells, and Leukemia: Impact of Drugs, Chemicals, and the Environment, The New York Academy of Sciences, New York, USA | 2013 |
| SKION-Máxima Research Retraite, De Bilt, the Netherlands | 2013 |
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