UNRAVELING PREDNISOLONE RESISTANCE IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA: TOWARDS PERSONALIZED TARGETED THERAPY

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Ingrid Margaretha Antoon Anna Ariës

The coverdesign artistically represents an MTT-assay that was used in this thesis to determine prednisolone sensitivity of leukemic cells. Shown are living leukemic cells (and red blood cells) that convert a yellow dye in purple and dead leukemic cells that can not convert the yellow dye any more because they were killed by prednisolone. The red blood cells were only added for the design.

Het ontwerp op de kaft is een artistieke weergave van een MTT-assay gebruikt om prednisolon gevoeligheid van leukemiecellen te bepalen. Te zien zijn levende leukemiecellen (en rode bloedcellen) die een gele kleurstof omzetten in paars en dode leukemiecellen die niet meer de gele kleurstof omzetten daar ze gedood zijn door prednisolon. De omringende rode bloedcellen zijn louter toegevoegd voor het ontwerp.

Unraveling prednisolone resistance in pediatric acute lymphoblastic leukemia: towards personalized targeted therapy

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"Reach high, for stars lie hidden in your soul.
Dream deep, for every dream precedes the goal."
- Pamela Vaull Star

Voor alle kinderen met leukemie en hun ouders

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INTRODUCTION

Hematopoiesis

The concept that immature precursor cells (hematopoietic stem cells) continuously produce mature blood cells, a process termed hematopoiesis, was discovered 50 years ago. Mature blood cells have a finite lifespan and must be continuously replaced throughout life. The turnover of cells of the hematopoietic system in a man weighing 70 kg is close to 1 trillion cells per day [1]. This renewal process is supported by only a small population of hematopoietic stem cells in the bone marrow. Hematopoietic stem cells produce progenitor cells that are either dedicated to the myeloid or the lymphoid lineage (Figure 1). Common myeloid progenitors generate platelet producing megakaryocytes (thrombus formation), erythrocytes (oxygen transport), mast cells and myeloblasts that generate neutrophils, eosinophils, basophils and monocytes (primary immune response). The common lymphoid progenitors give rise to B-lymphocytes (humoral immunity) and different subsets of T-lymphocytes including natural killer cells (cell-mediated immunity) (Figure 1).

Acute Leukemia

In healthy individuals, a tight balance is maintained between proliferation, differentiation, and release of blood cells from the bone marrow. This balance is disturbed in leukemia, where malignant expansion of immature white blood cells, called blasts, in bone marrow and peripheral blood occurs. The first description of a patient with leukemia was published in 1827[2] , although it took until 1845 before doctors recognized it as a distinct disease called "white blood" [3,4]. Two years later the term leukemia was introduced by Virchow[5] and his cellular theory of the origin of leukemia is still the basic concept of the current understanding of the disease. Not much later a distinction between myeloid leukemia and lymphoblastic leukemia was discovered [6,7]. Currently, leukemia is classified as acute or chronic and lymphoid or myeloid. Acute leukemia develops rapidly and involves immature blood cells, whereas in contrast chronic leukemia is characterized by more mature blood cells taking years to progress. Acute lymphoid leukemia (ALL) is further subclassified in precursor-B and T-ALL [8]. ALL arising from B-cell precursor ALL (BCP-ALL) constitute 85% of all pediatric ALL cases and is the most common cancer in children below the age of 18 years. ALL is diagnosed in about 120 new cases in the Netherlands every year, with a peak incidence between 2-5 years of age [9]. Occassionaly a more mature B-ALL type of leukemia can be found.

The main focus of this thesis was on BCP-ALL and results were frequently related to T-ALL.

Risk factors in pediatric acute lymphoblastic leukemia

Several clinical and biological parameters are associated with prognosis of pediatric acute leukemia and include immunophenotype (pro-B and T-ALL are poor prognostic), genetic abnormalities, white blood cell count at diagnosis, age at diagnosis and therapy resistance. These parameters are used for risk stratification and accordingly for risk-adapted treatment strategies, that can be subdivided in low, intermediate and high risk treatment.

Genetic abnormalities in acute lymphoblastic leukemia and prognosis

A genetic predisposition for leukemia, like children with Down syndrome have, is rare. Leukemia is currently recognized as a sporadic disease caused by many different molecular genetic lesions. Recent studies suggest that childhood leukemia is initiated during fetal life, as rearrangements of leukemia-associated genes in childhood leukemia cells have been identified retrospectively in stored Guthrie cards made of neonatal heel blood spots [10,11]. Several genetic rear-

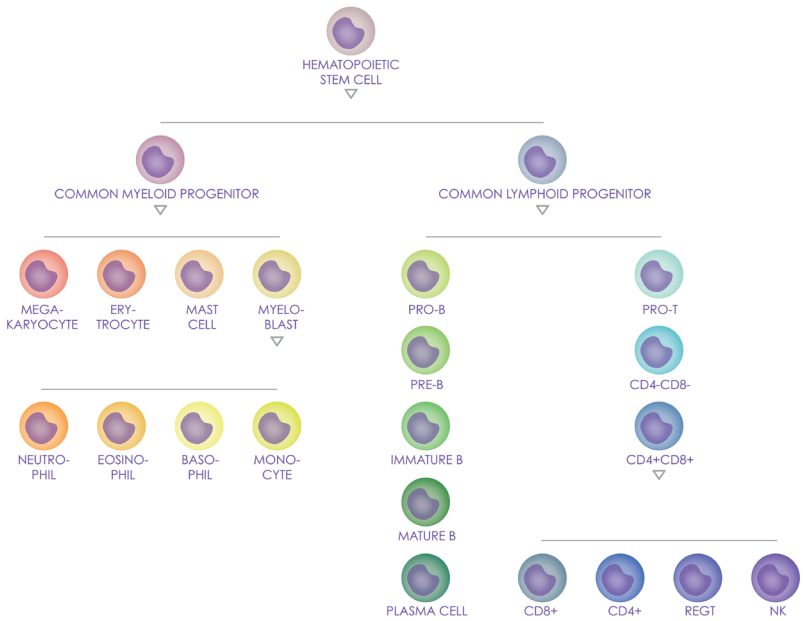

Figure 1. Schematic representation of the different lineages and stages during hematopoiesis.

rangements or abnormalities have been identified in BCP-ALL cells, as visualized in Figure 2A for Dutch children [12]. Hyperdiploid leukemic cells are found in 28% of pediatric BCP-ALL patients and are characterized by the presence of more than 50 chromosomes. The pathogenetic consequences of the chromosomal gains remain poorly understood. The gain of chromosomes X, 4, 6, 10, 14, 17, 18 and 21 is believed to comprise the primary genetic event in hyperdiploid ALL. Only recently, additional heterogenic small genetic anomalies have been identified as well, such as microdeletions of *ETV6*, *CDKN2A* and *PAX5*, and point mutations of FLT3, NRAS, KRAS, and PTPN11 [13]. Patient leukemic cells that harbour a *TEL-AML1* or *ETV6-RUNX1* caused by a (t12;21)(p13;q22) translocation comprise 28% of pediatric BCP-ALL patients (Figure 2A). A *TEL-AML1* translocation results in the fusion of TEL, a nuclear phosphoprotein of the ETS family of transcription factors, and the RUNX1 gene, a transcription factor regulating myeloid and lymphoid lineage specific genes [14]. Although *TEL-AML1* is believed to be an initiating event, it is not sufficient by itself to cause leukemia [15], as for instance the frequency of *TEL-AML1* found in surveys of blood spots far exceeds the incidence of childhood leukemia. Only recently it was discovered that STAT3 activity is necessary for *TEL-AML1* leukemia maintenance [16]. Both hyperdiploid patients and *TEL-AML1* patients have a good 5-year event-free survival of 85% or higher [12,17]. This good prognosis may be related to the sensitivity of these patients to L-asparaginase in both subtypes and sensitivity to antimetabolites methotrexate and 6-mercaptopurine in the hyperdiploid patients [18,19]. In contrast, *BCR-ABL1*-rearranged and *MLL*-rearranged patients, comprising each 3% of BCP-ALL, respectively (Figure 2A), merely reach a maximal overall survival of only 50% on previous treatment protocols [12]. *BCR-ABL1* positive ALL cells harbour the translocation t(9;22)(q34;q11) fusing BCR and ABL1 resulting into constitutively active Abl protein, a tyrosine kinase that drives proliferation and survival of the leukemic cells [17]. This fusion protein is considered the driving force in these leukemic cells, as treatment with BCR-ABL1 inhibitors dasatinib and imatinib in these patients led to impressive improvement of outcome [20,21]. *MLL*-rearranged ALL patients carry a rearrangement of the mixed lineage leukemia gene on chromosome 11q23. MLL is a highly promiscuous gene, as more than 50 fusion partner genes are identified to date. MLL-AF4 t(4;11)(q21;q23), MLL-ENL t(11;19)(q23;p13.3) and MLL-AF9 t(9;11)(p22;q23) are most frequently found in childhood ALL[22]. MLL is a histone methyltransferase which regulates gene transcription epigenetically. MLL-rearrangements produce a constitutively active MLL protein leading to an aggressive form of leukemia that is mainly found in infants [23]. Poor survival of these patients is linked to resistance of leukemic cells to prednisolone and L-asparaginase [12]. The intermediate prognostic group consists of

E2A-rearranged patients and B-other patients [12]. *E2A-PBX1* or *TCF3-PBX1* caused by t(1;19)(q23;p13.3) translocations are present in 8% of childhood ALL cases in the Netherlands [12] (Figure 2A). Translocations fuse the transactivation domain of the basic helix-loop-helix transcription factor E2A to the HOX DNA binding domain of PBX1. Whereas E2A is an important regulator in almost all stages of B-cell development, PBX1 is not expressed in the lymphoid compartment [24]. There is evidence that the oncogenic potential of the t(1;19) translocation may be due to the reduction of the levels of wild-type E2A or is caused by increased HOX activation by the E2A-PBX1 fusion protein [24]. B-other patients consist of patients that are negative for the other five genomic alterations, i.e. *TEL-AML1*, hyperdiploid, *BCR-ABL1*, *MLL* and *E2A-PBX1* rearrangements (Figure 2A). Of the abovementioned genotype, mainly *BCR-ABL1*, *TEL-AML1* and *MLL* are currently used for risk-adapted stratification of BCP-ALL in Dutch treatment protocols.

The availability of new genome-wide screening techniques led to the discovery of new genetic abnormalities in pediatric BCP-ALL. *BCR-ABL1*-like patients comprise the largest group identified among B-other cases of Dutch leukemic patients (Figure 2A). These patients have a similar gene expression profile as *BCR-ABL1* positive patients, but lack a *BCR-ABL1* translocation [25]. Characterization of *BCR-ABL1*-like patients revealed that more than 70% of these patients have abnormalities in B-cell differentiation genes, including IKZF1, PAX5, and EBF1 [25]. *BCR-ABL1*-like patients have a poor prognosis and merely reach an event-free survival of 50% [25,27,28].

Recent investigations have also identified B-other patients with an intrachromosomal amplification of chromosome 21, iAMP21 patients (Figure 2B) [29]. These patients benefit from receiving more intensive therapy in the UKALL2003 protocol (United Kingdom)[30]. Recent studies also showed CRLF2 overexpression in 7% of BCP-ALL patients, predominantly resulting from P2RY8-CRLF2 fusion or IGH@-CRLF2 rearrangement (Figure 2B) [31–33]. Increased CRLF2 expression in the absence of a rearrangement is also an adverse prognostic feature in some but not all treatment protocols (Figure 2B) [28,31]. t(8;14)(q23;q32.3) leading to a gene rearrangement of the oncogene c-Myc are also found in children with BCP-ALL [34]. A subset of B-other patients have alterations of the ETS-family transcription factor *ERG* (Figure 2B). The *ERG* deletions result in the expression of an aberrant C-terminal ERG fragment that functions as a competitive inhibitor of wildtype ERG. These patients generally have a favorable outcome [26]. Poor prognostic hypodiploid patients with less than 44 chromosomes is another distinct subgroup (Figure 2B) [26]. Few patients have a dic(9;12)(p11-13;p11-12) and represent a good prognostic group (Figure 2B) [35].

Several genetic abnormalitaties are also known for T-ALL (Figure 2B) of which ETP-ALL, a recently defined subset of thymocytes that retain stem-cell-like features, have the poorest prognosis [36].

These recently identified new genetic and prognostic abnormalities may be implemented in the risk stratification of future protocols, leading to improved treatment outcome and reduced therapy-induced cytotoxicity and mortality.

White blood cell count, age and prognosis

Discriminative and clinical predictive factors of outcome in ALL are white blood cell count and age at initial diagnosis. Children with a white blood cell count below 50,000 cells/µl tend to have a more favourable prognosis [37,38]. The association of high white blood cell count and prognosis is mainly driven by the fact that a higher tumor load is indicative of a more aggressive type of ALL. Furthermore, children between 1-10 years of age have better cure rates than children below 1 year or above 10 years of age [37,38]. The prognostic value between ages is most likely linked to peak incidences of specific genetic aberrations in different age categories. Hyperdiploidy is mainly found between 1 to 10 year of age, *TEL-AML1* in 2 to 5 year old patients, while MLL-rearrangements are primarily found in infants [37,38]. Moreover, age has been linked to *in vitro* resistance to chemotherapeutic drugs and *in vivo* response to induction treatment [39].

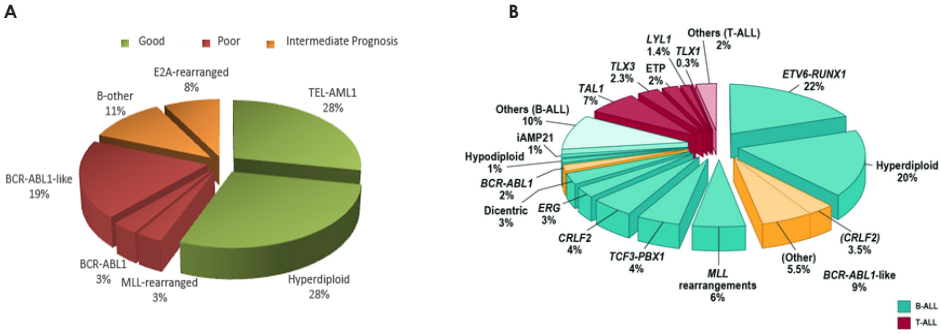

Figure 2. Frequency of genomic abnormalities in children with BCP-ALL and T-ALL.
A. Frequency of genomic abnormalities in Dutch pediatric BCP-ALL patients above 1 year. B. Frequency of all genomic abnormalities in BCP-ALL and T-ALL, including recently discovered novel genomic abnormalities [26].

Therapy resistance and cellular drug resistance

Therapy resistance is one of the most important risk factors for relapse. Around 1980 it was discovered that the percentage of marrow blasts on day 7 of induction therapy including vincristine, predniso(lo)ne, l-asparaginase, and daunorubicin had significant prognostic value [40–42]. Only a few years later PCR based methods were developed to detect residual leukemic cells that are below the limits of detection using conventional morphological assessment.

Minimal residual disease proved to be one of the most powerful predictors of outcome and was soon used in risk-stratification of childhood ALL [43–45]. In 1983, the Berlin-Frankfurt-Münster (BFM) study group demonstrated that peripheral blast count after a 7-day predniso(lo)ne pretreatment window was a predictive factor for treatment outcome [46]. Since then, *in vivo* predniso(lo)ne response has consistently been found to be one of the strongest independent prognostic factors for the prediction of treatment outcome in ALL-BFM studies [47]. In addition to the *in vivo* response to predniso(lo)ne also *in vitro* drug resistance of leukemic cells was predictive for an adverse clinical outcome in BCP-ALL [48]. Especially *in vitro* prednisolone resistance, but also to a lesser extent l-asparaginase and vincristine correlated significantly to disease free survival (Figure 3) [49,50].

In vitro prednisolone resistance and *in vivo* predniso(lo)ne response are correlated, although *in vitro* prednisolone resistance has a higher predictive value [51]. The presence of minimal residual disease after 2 to 4 weeks of therapy also correlates with *in vitro* resistance to prednisolone, whereas this was not observed for vincristine, L-asparaginase and doxorubicin [52]. Children with T-ALL are more *in vitro* resistant to prednisolone compared to children with the more favorable prognostic BCP-ALL immunophenotype [39]. Also children with acute myeloid leukemia, with survival rates reaching only 60-70%, are highly resistant to prednisolone, but also to all other chemotherapeutic drugs used in current ALL therapy regimens [53]. Furthermore, the poor prognostic subtypes in BCP-ALL, *BCR-ABL1* and *MLL*-rearranged patients (Figure 2A) are all highly resistant to

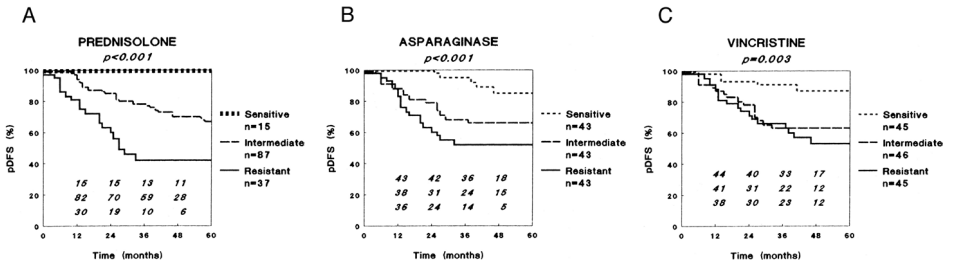

Figure 3. Relation between *in vitro* drug resistance and probability of disease free survival (pDFS) in children with newly diagnosed ALL[49].

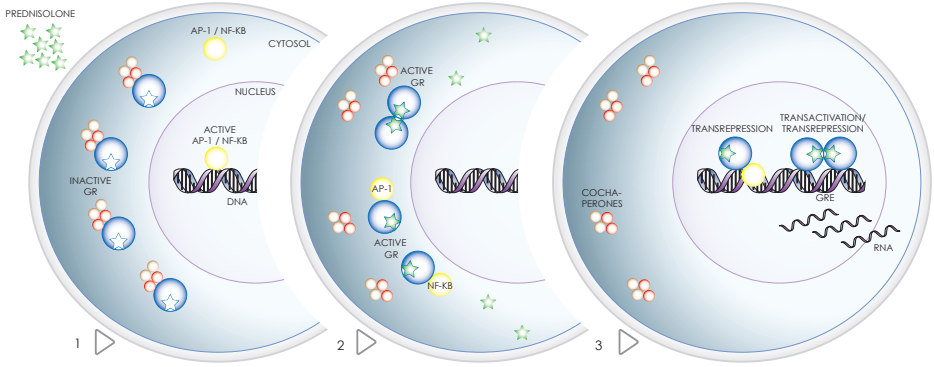

Figure 4. Prednisolone: mechanisms of action.

prednisolone [54–56]. Children with ALL who are older than 10 years or younger than 1, are more resistant to prednisolone compared to children who are between 1-10 years of age [39,56]. Resistance to drugs increases with age, as adults are more resistant to predniso(lo)ne than children suffering from the same leukemic type [57]. Furthermore, relapsed ALL cells acquire prednisolone resistance [58]. Altogether these results signify an important role for predniso(lo)ne resistance in treatment failure and relapse rate.

Prednisolone, mechanisms of action

In 1949 adrenocorticotrophic hormone (ACTH) was the second drug, after aminopterin, that produced prompt although brief remissions of ALL [59]. Predniso(lo)ne had similar activity and soon replaced ACTH. Another corticosteroid, dexamethasone was additionally added to treatment protocols. Predniso(lo)ne and dexamethasone are synthetic analogues of cortisone, a glucocorticoid that is naturally produced in the adrenal cortex and regulates several crucial processes, such as stress response, immune response and inflammation, but also carbohydrate and protein metabolism. Sixty years after the first discovery of its anti-leukemic capabilities, predniso(lo)ne is still the primary drug used in the treatment of BCP-ALL and T-ALL. It is also widely used to treat immunologic based diseases, such as asthma, chronic obstructive pulmonary disease, Crohn's disease, pericarditis, myasthenia gravis and others. To date, the exact mechanism how predniso(lo)ne targets immune cells or leukemic cells is not completely understood. It is known that prednisolone can passively diffuse pass the cell membrane due to its lipophilic structure, where it binds

the cytosolic glucocorticoid receptor (GR/NR3C1). Binding of prednisolone conformationally changes the GR dispersing inactivating cochaperones such as Hsp90, Hsp70, Hsp56 and Hsp40, leading to homodimerization (Figure 4). This receptor complex is then translocated into the nucleus within 20 min, where it binds to specific palindromic negative or positive glucocorticoid responsive elements leading to gene repression or activation, respectively [60,61]. Active GR can also sequester other transcription factors, such as AP-1 and NF-kB, thereby diminishing the transcriptional activation of AP-1 and NF-kB responsive genes [62]. Eight hours of prednisolone exposure of sensitive BCP-ALL cells already results in differential expression of 51 genes [63]. Eventually down- or upregulation of these numerous genes by prednisolone leads to the induction of apoptosis in particularly lymphoid cells.

Several prednisolone responsive genes are described that might be responsible for the observed induction in apoptosis, such as pro-apoptotic Bcl-2 family protein Bim [64], the redox-regulating thioredoxininteracting protein (Txnip) [63], the cell cycle inhibitors p19INK4d [65] and p57(Kip2) [66], several cMYC inhibitors [65], the anti-proliferative genes BTG1 and BTG2 [65] and many others. Support for the presence of a transcription independent mechanism originates from the discovery that a cytosolic DNA-binding defective variant of GR still induces apoptosis [67]. It has been shown that prednisolone rapidly increases cytosolic calcium levels in thymocytes and inhibition of this prevents prednisolone induced apoptosis [68]. In addition, steroids may diminish cellular antioxidant defenses, unabling cells to eradicate reactive oxygen species that are produced during normal metabolism, eventually inducing apoptosis [69]. T lymphoma cells overexpressing Thioredoxin, an important redox protein, become resistant to glucocorticoid induced apoptosis [69, 70]. In addition, hypoxic culture conditions prevented glucocorticoid induced apoptosis of thymocytes. A mitochondrial localization signal has been identified within the ligand binding domain of the glucocorticoid receptor [71]. Glucocorticoids can induce translocation of the GR to the mitochondria, where it actively induces apoptosis. The exact mechanisms behind mitochondrial GR provoked apoptosis remains poorly understood. It is thought to be triggered by either production of reactive oxygen species, rapid calcium mobilization, or by direct activation of mitochondrial gene transcription (reviewed in [72]). Another non-genomic mechanism is the release of not only the GR, but also Src kinase from cochaperones after prednisolone binding [73]. Src inhibition prevents glucocorticoid induced apoptosis. However, the exact contribution of Src kinases to glucocorticoid induced apoptosis is unclear, as others have also found that inhibition of Src kinases overcomes glucocorticoid resistance [74].

Modulating prednisolone resistance

Over the years, many investigations tried to elucidate and modulate predniso(lo)ne resistance. Evidently, first studies looked at clearance of predniso(lo)ne or defective expression of the glucocorticoid receptor itself. Up to five different splice variants of the GR have been described, the α, β, γ, GR-P and GR-A isoform. The expression level of the 5 splice variants both at base-line and after eight hours of prednisolone exposure was not associated with *in vitro* prednisolone resistance in leukemic samples of children with ALL [75]. In addition, polymorphisms or mutations in the GR gene are no major contributors to glucocorticoid resistance in childhood ALL [76]. Also the mRNA expression levels of chochaperone molecules are not related to *in vitro* prednisolone resistance [77]. Inhibition of the prednisolone interconverter 11β-HSD did sensitize T-ALL cell lines to prednisolone [78]. Overall these data suggest that resistance to prednisolone more likely is caused by mechanisms affecting the downstream intracellular signaling than by mechanisms affecting the effective prednisolone or GR receptor levels. For instance, core subunits of the SWI/SNF complex glucocorticoid-dependent transcription regulators, SMARCA4, ARID1A, and SMARCB1 associate with resistance to predniso(lo)ne and dexamethasone. Knockdown of SMARCA4 made a T-ALL cell line more resistant to prednisolone [79]. Prednisolone resistant cells also have defects in drug-induced apoptosis mechanisms, such as caspase-3 activation and PARP [80]. Overexpression of anti-apoptotic MCL1 and DAPK1 significantly associated with predniso(lo)ne resistance [81]. Indeed, down-regulation of MCL1 in prednisolone resistant leukemic cells sensitized to prednisolone [82]. In addition, rapamycin, a mTOR inhibitor, overcomes prednisolone resistance in leukemic cell lines by downregulating MCL1 [83].

Another apoptotic molecule involved in prednisolone sensitivity is the pro-apoptotic BCL-2-interacting mediator of cell death (BIM). BIM expression increases upon prednisolone treatment in sensitive, but not in resistant cells [84,85]. Overexpression of BIM converted a prednisolone resistant cell line into a sensitive cell line [86]. Calcium fluxes incited by prednisolone are necessary to induce apoptosis [68]. Inhibiting S100A8/S100A9, both calcium scavengers, sensitized MLL-rearranged ALL cells to prednisolone [87]. Also several pro-survival mechanisms are associated with the failure of prednisolone to induce apoptosis. Inhibition of important survival proteins JNK and ERK sensitizes ALL cell lines to prednisolone [88], as well as inhibition of PI3 kinase, AKT [89,90] and Src kinase [74]. Prednisolone resistance is also associated with increased glucose consumption, as inhibition of glycolysis with 2-deoxyglucose sensitized primary patients´ BCP-ALL cells to prednisolone [51].

Outline of this thesis

Predniso(lo)ne is one of the principal chemotherapeutic drugs used to treat children with ALL. Resistance to predniso(lo)ne, both *in vitro* and *in vivo*, is an unfavorable prognostic factor and has been used as a risk parameter in a.o. German COALL and BFM protocols as well as Dutch DCOG treatment protocols [50,92]. To date our knowledge about clinically relevant mechanisms of predniso(lo)ne resistance is limited. In this thesis we aimed to unravel predniso(lo)ne resistance and find new druggable targets that may point to new therapeutic interventions overcoming predniso(lo)ne resistance in children with ALL.

In Chapter 2 we examined the role of apoptotic proteins in prednisolone resistance. We hypothesized that prednisolone-mediated effects on key apoptotic proteins differ between *in vitro* prednisolone sensitive and resistant ALL patients' samples. We discovered that MCL1 plays an important role in resistance and is associated with altered glucose consumption of leukemic cells. Our research showed that inhibition of glycolysis and MCL1 synergistically reduced survival and concomitantly reversed prednisolone resistance in leukemic cells.

In Chapter 3 we investigated which other (non-apoptotic) genes may contribute to prednisolone resistance. We identified a significant increase in *EMP1* expression in *in vitro* prednisolone-resistant compared to -sensitive patients. Silencing of EMP1 inhibited leukemic survival, sensitized leukemic cells to prednisolone and abrogated migration and adhesion to mesenchymal stromal cells. EMP1 also contributed to microenvironment induced prednisolone resistance and was identified as an independent predictor for poor outcome in BCP-ALL.

In Chapter 4 we investigated the role of NR4A-family receptors in prednisolone resistance. NR4A-family gene and protein levels were increased in *in vitro* prednisolone-resistant compared to sensitive cases. Knockdown of the NR4A-family in primary patient samples did not sensitize to prednisolone, suggesting that resistance to prednisolone is not caused by altered regulation of the glucocorticoid receptor.

In Chapter 5 we addressed the role of survival proteins associated with prednisolone resistance. We identified an impaired regulation of RAS-RAF-MEK, STAT6 and c-MET protein phosphorylation levels in prednisolone resistant leukemic cells. In addition, a high frequency of RAS-pathway activating mutations was found. Prednisolone combined with a MEK inhibitor (Trametinib) eradicated almost all prednisolone resistant RAS-mutated primary patients' leukemic cells.

The work presented in this thesis is discussed supplemented with perspectives for future studies in **Chapter 6** and summarized in **Chapter 7** (in English and in Dutch).

REFERENCES

1. Dancey JT, Deubelbeiss KA, Harker LA, Finch CA. Neutrophil kinetics in man. The Journal of clinical investigation. 1976 Sep;58(3):705–15.
2. Velpeau A. Sur la resorption du pusaet sur l'alteration du sang dans les maladies clinique de persection nenemant. Premier observation. Rev Med. 1827;2:216.
3. Bennett J. Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood. Edinburgh Med Surg J. 1845;64:413–23.
4. Craigie D. Case of disease of the spleen in which death took place in consequence of the presence of purulent matter in the blood. Edinburgh Med Surg J. 1845;64:400–13.
5. Virchow R. Die leukämie. In R Virchow, ed, Gesammelte abhandlungen zur wissenschaft lichen medizin. Frankfurt, Germany: Meidinger, 1856, pp 190-211.
6. Friedriech N. Ein neuer fall von leukämie. Virchow's Arch Pathol Anat. 1857;12:37–58.
7. Neumann E. Ueber myelogene leukämie. Berl Klin Wochenschr. 1878;15:69–72.
8. Pinkel D. Genotypic classification of childhood acute lymphoid leukemia. Leukemia. 1999 Apr;13 Suppl 1:S90–1.
9. Velde CJH va de, Graaf WTA van der, Krieken JHJM van, Marijnen CAM, Vermorken JB. Oncologie. Bohn Stafleu van Loghum; 2011. p. 717.
10. Wiemels JL, Xiao Z, Buffler PA, Maia AT, Ma X, Dicks BM, et al. In utero origin of t(8;21) AML1-ETO translocations in childhood acute myeloid leukemia. Blood. 2002 May 15;99(10):3801–5.
11. Gale KB, Ford AM, Repp R, Borkhardt A, Keller C, Eden OB, et al. Backtracking leukemia to birth: Identification of clonotypic gene fusion sequences in neonatal blood spots. Proceedings of the National Academy of Sciences. 1997 Dec 9;94(25):13950–4.
12. Meijerink JPP, den Boer ML, Pieters R. New genetic abnormalities and treatment response in acute lymphoblastic leukemia. Seminars in hematology. 2009 Jan;46(1):16–23.
13. Paulsson K, Forestier E, Lilljebjörn H, Heldrup J, Behrendtz M, Young BD, et al. Genetic landscape of high hyperdiploid childhood acute lymphoblastic leukemia. Proceedings of the National Academy of Sciences of the United States of America. 2010 Dec 14;107(50):21719–24.
14. Hong D, Gupta R, Ancliff P, Atzberger A, Brown J, Soneji S, et al. Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. Science. 2008 Jan 18;319(5861):336–9.
15. Zelent A, Greaves M, Enver T. Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia. Oncogene; 2004 May 24;23(24):4275–83.
16. Mangolini M, de Boer J, Walf-Vorderwülbecke V, Pieters R, den Boer ML, Williams O. STAT3 mediates oncogenic addiction to TEL-AML1 in t(12;21) acute lymphoblastic leukemia. Blood. 2013 Jul 25;122(4):542–9.
17. Pui C-H, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. Journal of clinical oncology 2011 Feb 10;29(5):551–65.

18. Kaspers GJ, Smets LA, Pieters R, Van Zantwijk CH, Van Wering ER, Veerman AJ. Favorable prognosis of hyperdiploid common acute lymphoblastic leukemia may be explained by sensitivity to antimetabolites and other drugs: results of an in vitro study. Blood. 1995 Feb 1;85(3):751–6.
19. Ramakers-van Woerden NL, Pieters R, Loonen AH, Hubeek I, van Drunen E, Beverloo HB, et al. TEL/AML1 gene fusion is related to in vitro drug sensitivity for L-asparaginase in childhood acute lymphoblastic leukemia. Blood. 2000 Aug 1;96(3):1094–9.
20. Zwaan CM, Rizzari C, Mechinaud F, Lancaster DL, Lehrnbecher T, van der Velden VHJ, et al. Dasatinib in Children and Adolescents With Relapsed or Refractory Leukemia: Results of the CA180-018 Phase I Dose-Escalation Study of the Innovative Therapies for Children With Cancer Consortium. Journal of clinical oncology. 2013 May 28;31(19):2460–8.
21. Schultz KR, Bowman WP, Aledo A, Slayton WB, Sather H, Devidas M, et al. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. Journal of clinical oncology. 2009 Nov 1;27(31):5175–81.
22. Jansen MWJC, Corral L, van der Velden VHJ, Panzer-Grümayer R, Schrappe M, Schrauder A, et al. Immunobiological diversity in infant acute lymphoblastic leukemia is related to the occurrence and type of MLL gene rearrangement. Leukemia. 2007 Apr;21(4):633–41.
23. Pieters R, Schrappe M, De Lorenzo P, Hann I, De Rossi G, Felice M, et al. A treatment protocol for infants younger than 1 year with acute lymphoblastic leukaemia (Interfant-99): an observational study and a multicentre randomised trial. Lancet. 2007 Jul 21;370(9583):240–50.
24. Aspland SE, Bendall HH, Murre C. The role of E2A-PBX1 in leukemogenesis. Oncogene; 2001 Sep 10;20(40):5708–17.
25. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JGCAM, Peters STCJM, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. The lancet oncology. 2009 Feb;10(2):125–34.
26. Mullighan CG. The molecular genetic makeup of acute lymphoblastic leukemia. Hematology 2012 Jan 1;2012(1):389–96.
27. Loh ML, Zhang J, Harvey RC, Roberts K, Payne-Turner D, Kang H, et al. Tyrosine kinome sequencing of pediatric acute lymphoblastic leukemia: a report from the Children's Oncology Group TARGET Project. Blood. 2013 Jan 17;121(3):485–8.
28. Van der Veer A, Waanders E, Pieters R, Willemse ME, Van Reijmersdal S V, Russell LJ, et al. Independent prognostic value of BCR-ABL1-like signature and IKZF1 deletion, but not high CRLF2 expression, in children with B-cell precursor ALL. Blood. 2013 Aug 23;
29. Rand V, Parker H, Russell LJ, Schwab C, Ensor H, Irving J, et al. Genomic characterization implicates iAMP21 as a likely primary genetic event in childhood B-cell precursor acute lymphoblastic leukemia. Blood. 2011 Jun 23;117(25):6848–55.
30. Moorman A V, Robinson H, Schwab C, Richards SM, Hancock J, Mitchell CD, et al. Risk-Directed Treatment Intensification Significantly Reduces the Risk of Relapse Among Chil-

dren and Adolescents With Acute Lymphoblastic Leukemia and Intrachromosomal Amplification of Chromosome 21: A Comparison of the MRC ALL97/99 and UKALL2003 Trials. Journal of clinical oncology 2013 Sep; 31(27):3389-96.

31. Chen I-M, Harvey RC, Mullighan CG, Gastier-Foster J, Wharton W, Kang H, et al. Outcome modeling with CRLF2, IKZF1, JAK, and minimal residual disease in pediatric acute lymphoblastic leukemia: a Children's Oncology Group study. Blood. 2012 Apr 12;119(15):3512–22.
32. Cario G, Zimmermann M, Romey R, Gesk S, Vater I, Harbott J, et al. Presence of the P2RY8-CRLF2 rearrangement is associated with a poor prognosis in non-high-risk precursor B-cell acute lymphoblastic leukemia in children treated according to the ALL-BFM 2000 protocol. Blood. 2010 Jul 1;115(26):5393–7.
33. Harvey RC, Mullighan CG, Chen I-M, Wharton W, Mikhail FM, Carroll AJ, et al. Rearrangement of CRLF2 is associated with mutation of JAK kinases, alteration of IKZF1, Hispanic/Latino ethnicity, and a poor outcome in pediatric B-progenitor acute lymphoblastic leukemia. Blood. 2010 Jul 1;115(26):5312–21.
34. Moore S, Suttle J, Bain S, Story C, Rice M. Acute lymphoblastic leukemia characterized by t(8;14)(q11.2;q32). Cancer genetics and cytogenetics. 2003 Feb;141(1):1–4.
35. Behrendt H, Charrin C, Gibbons B, Harrison CJ, Hawkins JM, Heerema NA, et al. Dicentric (9;12) in acute lymphocytic leukemia and other hematological malignancies: report from a dic(9;12) study group. Leukemia. 1995 Jan;9(1):102–6.
36. Coustan-Smith E, Mullighan C, Onciu M, Behm FG, Raimondi SC, Pei D, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. Lancet Oncology 2009 Feb;10(2):147-56.
37. Friedmann AM. The Role of Prognostic Features in the Treatment of Childhood Acute Lymphoblastic Leukemia. The Oncologist; 2000 Aug 1;5(4):321–8.
38. Möricke A, Zimmermann M, Reiter A, Gadner H, Odenwald E, Harbott J, et al. Prognostic impact of age in children and adolescents with acute lymphoblastic leukemia: data from the trials ALL-BFM 86, 90, and 95. Klinische Pädiatrie. 217(6):310–20.
39. Pieters R, den Boer ML, Durian M, Janka G, Schmiegelow K, Kaspers GJ, et al. Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. Leukemia : 1998 Sep;12(9):1344–8.
40. Gaynon PS, Bleyer WA, Steinherz PG, Finklestein JZ, Littman P, Miller DR, et al. Day 7 marrow response and outcome for children with acute lymphoblastic leukemia and unfavorable presenting features. Medical and Pediatric Oncology. 1990;18(4):273–9.
41. Gajjar A, Ribeiro R, Hancock M, Rivera G, Mahmoud H, Sandlund J, et al. Persistence of circulating blasts after 1 week of multiagent chemotherapy confers a poor prognosis in childhood acute lymphoblastic leukemia. Blood. 1995 Aug 15;86(4):1292–5.
42. Miller DR, Leikin S, Albo V, Vitale L, Sather H, Coccia P, et al. Use of prognostic factors in improving the design and efficiency of clinical trials in childhood leukemia: Children's

Cancer Study Group Report. Cancer treatment reports. 64(2-3):381–92.
43. Wasserman R, Galili N, Ito Y, Silber JH, Reichard BA, Shane S, et al. Residual disease at the end of induction therapy as a predictor of relapse during therapy in childhood B-lineage acute lymphoblastic leukemia. Journal of clinical oncology 1992 Dec;10(12):1879–88.
44. Brisco MJ, Condon J, Hughes E, Neoh SH, Sykes PJ, Seshadri R, et al. Outcome prediction in childhood acute lymphoblastic leukaemia by molecular quantification of residual disease at the end of induction. Lancet. 1994 Jan 22;343(8891):196–200.
45. Van Dongen JJ, Seriu T, Panzer-Grümayer ER, Biondi A, Pongers-Willemse MJ, Corral L, et al. Prognostic value of minimal residual disease in acute lymphoblastic leukaemia in childhood. Lancet. 1998 Nov 28;352(9142):1731–8.
46. Riehm H, Reiter A, Schrappe M, Berthold F, Dopfer R, Gerein V, et al. [Corticosteroid-dependent reduction of leukocyte count in blood as a prognostic factor in acute lymphoblastic leukemia in childhood (therapy study ALL-BFM 83)]. Klinische Pädiatrie. 199(3):151–60.
47. Schrappe M, Reiter A, Zimmermann M, Harbott J, Ludwig WD, Henze G, et al. Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Münster. Leukemia. 2000 Dec;14(12):2205–22.
48. Pieters R, Huismans DR, Loonen AH, Hählen K, van der Does-van den Berg A, van Wering ER, et al. Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet. 1991 Aug 17;338(8764):399–403.
49. Kaspers GJL, Veerman AJP, Pieters R, Van Zantwijk CH, Smets LA, Van Wering ER, et al. In Vitro Cellular Drug Resistance and Prognosis in Newly Diagnosed Childhood Acute Lymphoblastic Leukemia. Blood. 1997 Oct;90(7):2723–9.
50. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Körholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. Journal of clinical oncology. 2003 Sep 1;21(17):3262–8.
51. Kaspers GJL, Pieters R, Van Zantwijk CH, VanWering ER, Van Der Does-Van Den Berg A, Veerman AJP. Prednisolone Resistance in Childhood Acute Lymphoblastic Leukemia: Vitro-Vivo Correlations and Cross-Resistance to Other Drugs. Blood. 1998;92(1):259–66.
52. De Haas V, Kaspers GJL, Oosten L, Bresters D, Pieters R, Van Der Velden VHJ, et al. Is there a relationship between in vitro drug resistance and level of minimal residual disease as detected by polymerase chain reaction at the end of induction therapy in childhood acute lymphoblastic leukaemia? British journal of haematology. 2002 Sep;118(4):1190–1.
53. Zwaan CM, Kaspers GJL, Pieters R, Hählen K, Huismans DR, Zimmermann M, et al. Cellular drug resistance in childhood acute myeloid leukemia is related to chromosomal abnormalities. Blood. 2002 Nov 1;100(9):3352–60.
54. Hongo T, Okada S, Inoue N, Yamada S, Yajima S, Watanabe C, et al. Two groups of Philadelphia chromosome-positive childhood acute

lymphoblastic leukemia classified by pretreatment multidrug sensitivity or resistance in in vitro testing. International journal of hematology. 2002 Oct;76(3):251–9.

55. Gupta M, Kumar A, Dabadghao S. In vitro resistance of leukaemic blasts to prednisolone in bcr-abl positive childhood acute lymphoblastic leukaemia. The Indian journal of medical research. 2002 Dec;116:268–72.
56. Dordelmann M, Reiter A, Borkhardt A, Ludwig W-D, Gotz N, Viehmann S, et al. Prednisone Response Is the Strongest Predictor of Treatment Outcome in Infant Acute Lymphoblastic Leukemia. Blood. 1999 Aug 15;94(4):1209–17.
57. Maung ZT, Reid MM, Matheson E, Taylor PR, Proctor SJ, Hall AG. Corticosteroid resistance is increased in lymphoblasts from adults compared with children: preliminary results of in vitro drug sensitivity study in adults with acute lymphoblastic leukaemia. British journal of haematology. 1995 Sep;91(1):93–100.
58. Klumper E, Pieters R, Veerman AJ, Huismans DR, Loonen AH, Hählen K, et al. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. Blood. 1995 Nov 15;86(10):3861–8.
59. Farber S. The effect of ACTH in acute leukemia in childhood. In JR Mote, ed, First Clinical ACTH conference (New York, Blakiston, 1950).
60. Malkoski SP, Dorin RI. Composite glucocorticoid regulation at a functionally defined negative glucocorticoid response element of the human corticotropin-releasing hormone gene. Molecular endocrinology (Baltimore, Md). 1999 Oct;13(10):1629–44.
61. Almawi WY, Melemedjian OK. Molecular mechanisms of glucocorticoid antiproliferative effects: antagonism of transcription factor activity by glucocorticoid receptor. Journal of leukocyte biology. 2002 Jan;71(1):9–15.
62. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocrine reviews. 2003 Aug;24(4):488–522.
63. Tissing WJE, den Boer ML, Meijerink JPP, Menezes RX, Swagemakers S, van der Spek PJ, et al. Genomewide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. Blood. 2007 May 1;109(9):3929–35.
64. Wang Z, Malone MH, He H, McColl KS, Distelhorst CW. Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. The Journal of biological chemistry. 2003 Jun 27;278(26):23861–7.
65. Yoshida NL, Miyashita T, U M, Yamada M, Reed JC, Sugita Y, et al. Analysis of gene expression patterns during glucocorticoid-induced apoptosis using oligonucleotide arrays. Biochemical and biophysical research communications. 2002 May 17;293(4):1254–61.
66. Alheim K, Corness J, Samuelsson MKR, Bladh L-G, Murata T, Nilsson T, et al. Identification of a functional glucocorticoid response element in the promoter of the cyclin-dependent kinase inhibitor p57Kip2. Journal of molecular endocrinology. 2003 Jun;30(3):359–68.
67. El-Naghy M, Johnson BH, Chen H, Ansari NH, Zhang W, Moller P, et al. The pathway of leukemic cell death

caused by glucocorticoid receptor fragment 465*. Experimental cell research. 2001 Nov 1;270(2):166–75.
68. McConkey DJ, Hartzell P, Amador-Pérez JF, Orrenius S, Jondal M. Calcium-dependent killing of immature thymocytes by stimulation via the CD3/T cell receptor complex. Journal of immunology (Baltimore, Md : 1950). 1989 Sep 15;143(6):1801–6.
69. Tome ME, Baker AF, Powis G, Payne CM, Briehl MM. Catalase-overexpressing Thymocytes Are Resistant to Glucocorticoid-induced Apoptosis and Exhibit Increased Net Tumor Growth. Cancer Res. 2001 Mar 1;61(6):2766–73.
70. Torres-Roca JF, Tung JW, Greenwald DR, Brown JM, Herzenberg LA, Katsikis PD. An early oxygen-dependent step is required for dexamethasone-induced apoptosis of immature mouse thymocytes. Journal of immunology. 2000 Nov 1;165(9):4822–30.
71. Sionov RV, Cohen O, Kfir S, Zilberman Y, Yefenof E. Role of mitochondrial glucocorticoid receptor in glucocorticoid-induced apoptosis. The Journal of experimental medicine. 2006 Jan 23;203(1):189–201.
72. Gavrilova-Jordan LP, Price TM. Actions of steroids in mitochondria. Seminars in reproductive medicine. 2007 May;25(3):154–64.
73. Marchetti MC, Di Marco B, Cifone G, Migliorati G, Riccardi C. Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. Blood. 2003 Jan 15;101(2):585–93.
74. Spijkers-Hagelstein JAP, Mimoso Pinhanços S, Schneider P, Pieters R, Stam RW. Src kinase-induced phosphorylation of annexin A2 mediates glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. Leukemia. 2013 Apr;27(5):1063–71.
75. Tissing WJE, Lauten M, Meijerink JPP, den Boer ML, Koper JW, Sonneveld P, et al. Expression of the glucocorticoid receptor and its isoforms in relation to glucocorticoid resistance in childhood acute lymphocytic leukemia. Haematologica. 2005 Sep;90(9):1279–81.
76. Tissing WJE, Meijerink JPP, den Boer ML, Brinkhof B, van Rossum EFC, van Wering ER, et al. Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. Clinical cancer research. 2005 Aug 15;11(16):6050–6.
77. Tissing WJE, Meijerink JPP, den Boer ML, Brinkhof B, Pieters R. mRNA expression levels of (co)chaperone molecules of the glucocorticoid receptor are not involved in glucocorticoid resistance in pediatric ALL. Leukemia. 2005 May;19(5):727–33.
78. Sai S, Nakagawa Y, Yamaguchi R, Suzuki M, Sakaguchi K, Okada S, et al. Expression of 11beta-hydroxysteroid dehydrogenase 2 contributes to glucocorticoid resistance in lymphoblastic leukemia cells. Leukemia. 2011 Jul 25;35(12):1644–8.
79. Pottier N, Yang W, Assem M, Panetta JC, Pei D, Paugh SW, et al. The SWI/SNF chromatin-remodeling complex and glucocorticoid resistance in acute lymphoblastic leukemia. Journal of the National Cancer Institute. 2008 Dec 17;100(24):1792–803.
80. Holleman A, den Boer ML, Kazemier KM, Janka-Schaub GE, Pieters R. Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lym-

phoblastic leukemia. Blood. 2003 Dec 15;102(13):4541–6.
81. Holleman A, den Boer ML, de Menezes RX, Cheok MH, Cheng C, Kazemier KM, et al. The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia. Blood. 2006 Jan 15;107(2):769–76.
82. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, et al. Association of high-level MCL-1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia. Blood. 2010 Feb 4;115(5):1018–25.
83. Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, et al. Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. Cancer cell. 2006 Oct;10(4):331–42.
84. Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. J Biol Chem. 2004 Dec 31;279(53):55809–17.
85. Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. FEBS letters. 2006 Jun 12;580(14):3539–44.
86. Ploner C, Rainer J, Niederegger H, Eduardoff M, Villunger A, Geley S, et al. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. Leukemia. 2008 Feb;22(2):370–7.
87. Spijkers-Hagelstein JAP, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, et al. Elevated S100A8/S100A9 expression causes glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. Leukemia. 2012 Jun;26(6):1255–65.
88. Miller AL, Garza AS, Johnson BH, Thompson EB. Pathway interactions between MAPKs, mTOR, PKA, and the glucocorticoid receptor in lymphoid cells. Cancer cell international. 2007 Jan;7:3.
89. Spijkers-Hagelstein JAP, Pinhanços SM, Schneider P, Pieters R, Stam RW. Chemical genomic screening identifies LY294002 as a modulator of glucocorticoid resistance in MLL-rearranged infant all. Leukemia. 2013 Aug 20;
90. Fuka G, Kantner H-P, Grausenburger R, Inthal A, Bauer E, Krapf G, et al. Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts. Leukemia. 2012 May;26(5):927–33.
91. Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJC, et al. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood. 2009 Feb 26;113(9):2014–21.
92. Reiter A, Schrappe M, Ludwig WD, Hiddemann W, Sauter S, Henze G, et al. Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. Blood. 1994 Nov 1;84(9):3122–33.

THE SYNERGISM OF MCL1 AND GLYCOLYSIS ON PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA CELL SURVIVAL AND PREDNISOLONE RESISTANCE

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ABSTRACT

In vitro and *in vivo* resistance to prednisolone are predictive for an adverse prognosis in pediatric precursor-B acute lymphoblastic leukemia. Causes of resistance are still poorly understood. In this study, we observed that prednisolone exposure of prednisolone-sensitive patients' leukemic cells decreased anti-apoptotic MCL1 protein levels by 2.9-fold, while MCL1 protein expression in prednisolone-resistant leukemic patients' cells was unaffected ($p<0.01$). Locked nucleic acid oligonucleotides directed against MCL1 reduced MCL1 protein levels by 82±16% ($p<0.05$) in leukemic cells, decreased proliferation by 9-fold and sensitized to prednisolone up to 80.8-fold, compared to a non-silencing-control locked nucleic acid ($p<0.05$). Remarkably, we discovered that MCL1-silencing upregulated the glucose consumption of leukemic cells by 2.5-fold ($p<0.05$), suggesting a potential rescue mechanism mediated by glycolysis. Targeting glycolysis by 2-deoxyglucose synergistically inhibited leukemic survival by 23.2-fold in MCL1-silenced cells ($p<0.05$). Moreover, 2-deoxyglucose and MCL1 locked nucleic acid concomitantly sensitized leukemic cells to prednisolone compared to MCL1 locked nucleic acid or 2-deoxyglucose alone ($p<0.05$). In conclusion, these results indicate the need to target both MCL1 and glycolysis simultaneously to inhibit leukemic survival and sensitize acute leukemia patients towards prednisolone.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common cancer in children. Although cure rates have greatly improved over recent years, treatment is still ineffective in 20% of patients. Unsuccessful treatment can be ascribed to resistance of primary leukemic cells to antileukemic drugs [1]. Poor prognosis is particularly associated with resistance to prednisolone in childhood ALL, both *in vivo* and *in vitro* [2]. To increase current survival rates it is therefore necessary to overcome prednisolone resistance.

To identify therapeutic targets to overcome prednisolone resistance, we previously performed microarray analysis on primary precursor-B acute lymphoblastic leukemia (BCP-ALL) cells of pediatric patients [3]. This indicated a role in prednisolone resistance for MCL1, an anti-apoptotic member of the BCL2 family that is frequently overexpressed in a variety of cancers and that contributes to cancer cell survival and apoptosis resistance [4,5]. Functional studies revealed that silencing of MCL1 sensitized leukemic cells to prednisolone [6,7].

In addition, we and others discovered that glycolysis is increased in prednisolone-resistant leukemic cells [8,9]. Microarray analysis on primary BCP-ALL cells of pediatric patients indicated an increased expression of several glycolytic enzymes and glucose transporters in prednisolone resistant patients [3]. Furthermore, 2-deoxyglucose (2-DG), an inhibitor of glycolysis, sensitized both leukemic cell lines and patients' ALL cells to prednisolone [8,9].

For three reasons, we now hypothesize that anti-apoptosis sustained by MCL1, and glycolysis are linked processes and concomitantly induce drug resistance in leukemia.1) Cellular respiration and apoptosis are closely related survival pathways both associated with prednisolone resistance [6–9], and other targeted molecular leukemia therapies, such as imatinib [10]. 2) Increased glucose metabolism has been directly linked to MCL1 stabilization and attenuation of apoptosis [11,12], and 3) BCL2 family members can, besides their apoptotic function, adjust oxidative phosphorylation [13,14].

In the present study, we show that silencing of MCL1 by specifically designed locked nucleid acid antisense oligonucleotides against *MCL1* mRNA (MCL1 LNA) inhibited cell survival and sensitized to prednisolone in both BCP-ALL and T-ALL leukemic cells. Moreover, we discovered higher glucose consumption in ALL cells after MCL1 silencing by both shMCL1 and MCL1 LNAs. Most importantly, we demonstrate that 2-DG treatment of MCL1-silenced cells decreased glucose consumption and synergistically reduced leukemic survival. Moreover, MCL1 LNA and 2-DG concomitantly reversed prednisolone resistance in leukemic cells. These data provide evidence that MCL1 and glycolysis should be targeted

simultaneously to effectively inhibit leukemic survival and to reverse prednisolone resistance in ALL.

METHODS

Cell culture and primary cells

Leukemic cells from children with newly diagnosed ALL were isolated from bone marrow aspirates and prednisolone resistance was assessed, as previously described [1,3]. Informed consent was given by patients as approved by the local institutional review board. Only samples with ≥ 90% leukemic cells upon processing were used in the present study. Reh, 697, Sem, Jurkat, Loucy and HEK293T cells were obtained from DMSZ. The leukemic cell lines were cultured in RPMI+Glutamax (Gibco) and HEK293T cells in DMEM+Glutamax (Gibco) at 37°C in humidified air containing 5% CO_2. Medium was supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco), 0.125 µg/ml fungizone (Gibco), and 10% Fetal Calf Serum (Integro). Reh, 697 and Sem are BCP-ALL cell lines with an *ETV6-RUNX1+, E2A-PBX1+, MLL-AF4+* translocation respectively. Jurkat is a mature tetraploid T-ALL cell line and Loucy is an immature early T-cell precursor (ETP-ALL) cell line. All cell lines were tested for their resistance to prednisolone. HEK293T, a human embryonal kidney cell line, was used for the production of viral particles. Cell viability and cell count were determined by a trypan blue exclusion staining assay and analyzed by MACSQuant.

LNA transfection and 2-deoxyglucose treatment

Cell lines were cultured in the presence of either 10 µM locked nucleotide acid oligonucleotides directed against *MCL1* (MCL1 LNA), i.e. SPC4120 (MCL1 LNA-a), SPC4342 (MCL1 LNA-b), SPC4343 (MCL1 LNA-c), or a non-silencing control oligonucleotide LNA, i.e. SPC3088. Twenty-four hours after LNA transfection, cells were cultured with and without 0.5 mM 2-deoxyglucose (Sigma). Supplemental Figure 1A-B illustrates that 0.5 mM 2-deoxyglucose hampers cell count only modestly, while it has a quantifiable effect on glucose consumption. After 96 hours, culture medium was replaced by fresh medium containing fresh LNA +/- 2-DG.

Apoptosis measurement

AnnexinV/PI double positive and AnnexinV single positive cells were measured on the BD FACS Calibur flow cytometer. $0.2*10^6$ cells were incubated for 15 minutes in 200µl AnnexinV binding buffer (Molecular probes) containing 2 µg/

ml propidium iodide (Molecular probes) and 1:1000 AnnexinV Alexa Fluor® 633 (Molecular probes).

Quantitative RT-PCR

MCL1 mRNA levels were quantified by incorporation of SYBR Green (Thermo Scientific) by quantitative real-time PCR (Applied Biosystems 7900HT). Primers for *MCL1* were; 5'-GGAGGAGGACGAGTTGTAC-3' (forward) and 5'-AAG GCA CCA AAA GAA ATG-3' (reverse). RNA was extracted using a Rneasy minikit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 1 µg RNA by 8 IU/µl MMLV (Promega), 20nM oligodT primers, 1µM random hexamer primers (Invitrogen), 200 µM dNTPs and 1 IU/µl RNAsin in MMLV-buffer (Promega). Primers used for the reference gene RPS20, were 5'-AAGGGCTGAGGATTTTTG-3' (forward) and 5'-CGTTGCGGCTTGTTAG-3' (reverse).

Reverse Phase Protein Array

Primary leukemic cells were cultured for 48h with 0 µg/ml, 1 µg/ml or 250 µg/ml prednisolone. Protein was isolated and lysates were spotted twice in triplicate on glass-backed nitrocellulose-coated array slides by the facility of Dr. E. F. Petricoin, George Mason University-Manassas USA. Slides were stained with specific antibodies and incubated with a biotinylated secondary antibody. Slides were scanned using the NovaRay scanner and protein levels were calculated relative to the total amount of protein per sample using MicroVigene Software. Antibodies used were: MCL1 antibody (Sigma HPA008455), Bcl-XL (Cell Signaling 2762), BCL-2 (Cell Signaling 2872) and p53 (Cell Signaling 9282).

Western Blot and Immunoblotting

Cell pellets were lysed in lysis buffer supplemented with protease inhibitors, and protein concentration was quantified according to the BCA assay (Pierce). Twenty micrograms of protein were used as input for western blot analysis using anti-MCL1 (Sigma HPA008455), anti-BCL-2 (Cell Signaling 2872), anti-β-actin (Abcam ab6276), anti-Clathrin (Santa Cruz Sc12734), IRDye 800CW-labeled anti-rabbit and IRDye 680CW-labeled anti-mouse (Li-Cor IRDye). Protein levels were quantified using the Oddysey system.

In vitro MTT drug-resistance assay

Cytotoxicity of cells towards prednisolone (Bufa Pharmaceutical Products) were determined by the *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) drug-resistance assay, as described previously [1].

Glucose-consumption assay

Glucose levels were measured with the glucose assay kit (Sigma, GAGO-20). Briefly, the supernatant of cultured cells was diluted 25 times in milliQ, supplemented with assay solvent containing Glucose Oxidase, Peroxidase and o-Dianisidine and incubated for 30 minutes at 37°C. Hereafter, 12N sulfuric acid was added and levels of the spectrophotometric end-product oxidized o-Dianisidine were measured at 540nm using the Versamax (Molecular Devices). To calculate glucose consumption, values were compared with plain RPMI glucose levels and corrected for cell growth.

Synergistic effect

Synergistic effects were calculated from equi-effective drug concentrations by the equitation postulated by Berenbaum[15] [Drug A in combination with B] / [Drug A alone] + [Drug B in combination with A]/[Drug B alone]. A synergy factor (Fsyn) <1 indicates synergy, whereas a Fsyn of 1 indicates additivity and a Fsyn >1 points to antagonism between two drugs.

Statistical analyses

Prednisolone exposure effects within either prednisolone resistant or sensitive patients was analyzed with a Kruskall-Wallis test. A Mann-Whitney U test was used to compare resistant to sensitive patients. The Mann-Whitney U test was also applied to analyze the effects of shMCL1. MCL1 LNA experiments were compared with a *T* test. $p<0.05$ was considered statistically significant.

Lentiviral production and infection

Lentiviral helper vectors pRSV-Rev (Addgene plasmid 12253), pMDLg/pRRE (Addgene plasmid 12251), pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) were provided by Prof. D. Trono (Geneva, Switzerland). pLKO.1 Mission short hairpin RNA (shRNA) vectors were purchased from Sigma-Aldrich, i.e. SHC005 against eGFP, and TRCN0000005518 and TRCN0000197024 against MCL1. Infections were performed as follow; 70-80% confluent HEK293T cells were transfected with shMCL1, pMD2.G and psPAX2 complemented with $CaCl_2$ and HEPES-buffered saline in the presence of 25 µM chloroquine (Sigma). Virus-containing supernatant was collected, filtered 0.45µm, and concentrated by ultracentrifugation at 32,000rpm, 1hr, 4°C. Viral titers were determined with a HIV-1 p24 Antigen ELISA kit according to the manufacturer's protocol (ZeptoMetrix). Infection occurred during 45' 1800 rpm spin-oculation of 0.5*106 cells/ml with 2.5 TU/cell viral particles and 5 µg/ml polybrene (Sigma-Aldrich).

After 24h, infected cells were selected in 0.5 µg/ml in the case of 697 and Loucy, 1 µg/ml for Reh and Sem, and 2 µg/ml puromycin for Jurkat.

RESULTS

Downregulation of MCL1 by prednisolone is impaired in prednisolone resistant leukemic cells of patients.

Glucocorticoids are known to induce apoptosis by downregulation of anti-apoptotic BCL-2 family members independent of p53 [16,17]. We analyzed protein expression of BCL-2 family members and p53 in three prednisolone sensitive and three resistant primary patient samples after exposure to prednisolone for 48h (Figure 1 and Supplemental Figure 2A-D). After *in vitro* prednisolone exposure, the expression of MCL1 in leukemic cells of *in vitro* prednisolone sensitive pediatric ALL patients significantly decreased by 2.9-fold ($p<0.01$). Whereas, in prednisolone resistant ALL patient cells, these levels did not change upon prednisolone exposure. Prednisolone did not affect the expression levels of BCL-XL , BCL2 nor p53 (Figure 1 and Supplemental Figure 2A-D). A similar decrease in MCL1 but not in BCL2 was seen using an extended dilution series of prednisolone (Supplemental Figure 2E).

MCL1 is a potent target to inhibit leukemic survival and to sensitize to prednisolone in pediatric ALL.

Three different newly-developed LNA oligonucleotides directed against *MCL1* were up to 90% effective in silencing MCL1 in five distinct leukemic BCP-ALL and T-ALL cell lines, i.e. *MLL-AF4*$^+$ BCP-ALL (SEM), *ETV6-RUNX1*$^+$ BCP-ALL (REH), *E2A-PBX1*$^+$ BCP-ALL (697), ETP-ALL (Loucy) and tetraploid T-ALL (Jurkat) cells ($p<0.01$ Supplemental Figure 3 and 4). MCL1 LNA-b provided the most potent and reproducible knockdown of these three MCL1 LNAs. Knockdown achieved by the three MCL1 LNAs was comparable to the knockdown obtained after stable lentiviral transduction of two short hairpin RNA directed against *MCL1*, i.e. shMCL1a and -b ($p<0.05$; Supplemental Figure 5). The MCL1 LNAs inhibited leukemic survival up to 90%, increased apoptosis up to 60% and sensitized to prednisolone up to 80.8-fold in five distinct leukemic cell lines, all compared to a non-silencing LNA control ($p<0.05$; Supplemental Figure 6, 7 and 8). These MCL1 LNA results were comparable to shMCL1 results (Supplemental Figure 9) and indicates that targeting MCL1 may be clinically important.

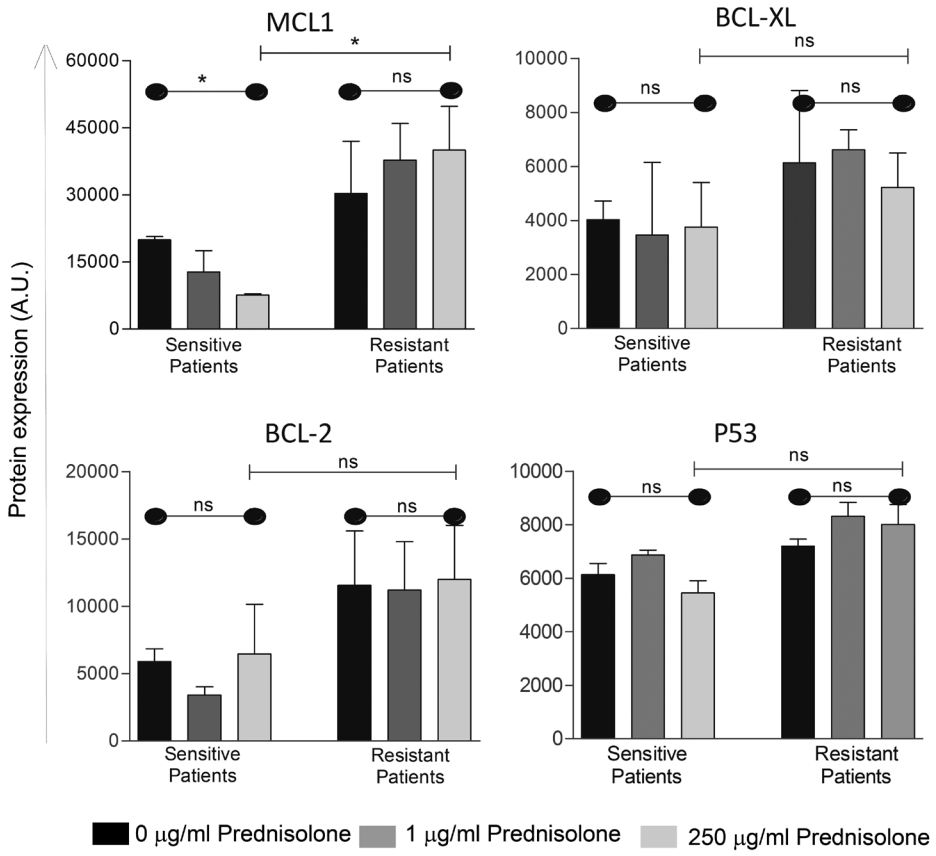


Figure 1. Downregulation of MCL1 by prednisolone is impaired in prednisolone resistant leukemic cells of patients.

Leukemic cells of three *in vitro* prednisolone sensitive and three *in vitro* prednisolone resistant patients, were exposed *in vitro* for 48 hours with 0 μg/ml, 1 μg/ml or 250 μg/ml prednisolone. Protein expression levels of MCL1, BCL-XL, BCL-2 and p53 were analyzed by reverse phase protein array. Bar indicates the mean plus SEM of three independent patient samples. (A Kruskal-Wallis test was used to compare 0, 1, 250 μg/ml data points indicated by 0--0 and a Mann-Whitney U test was used to compare data between sensitive and resistant patients indicated by |--| *p<0.05, **p<0.01). A.U. Arbitrary units.

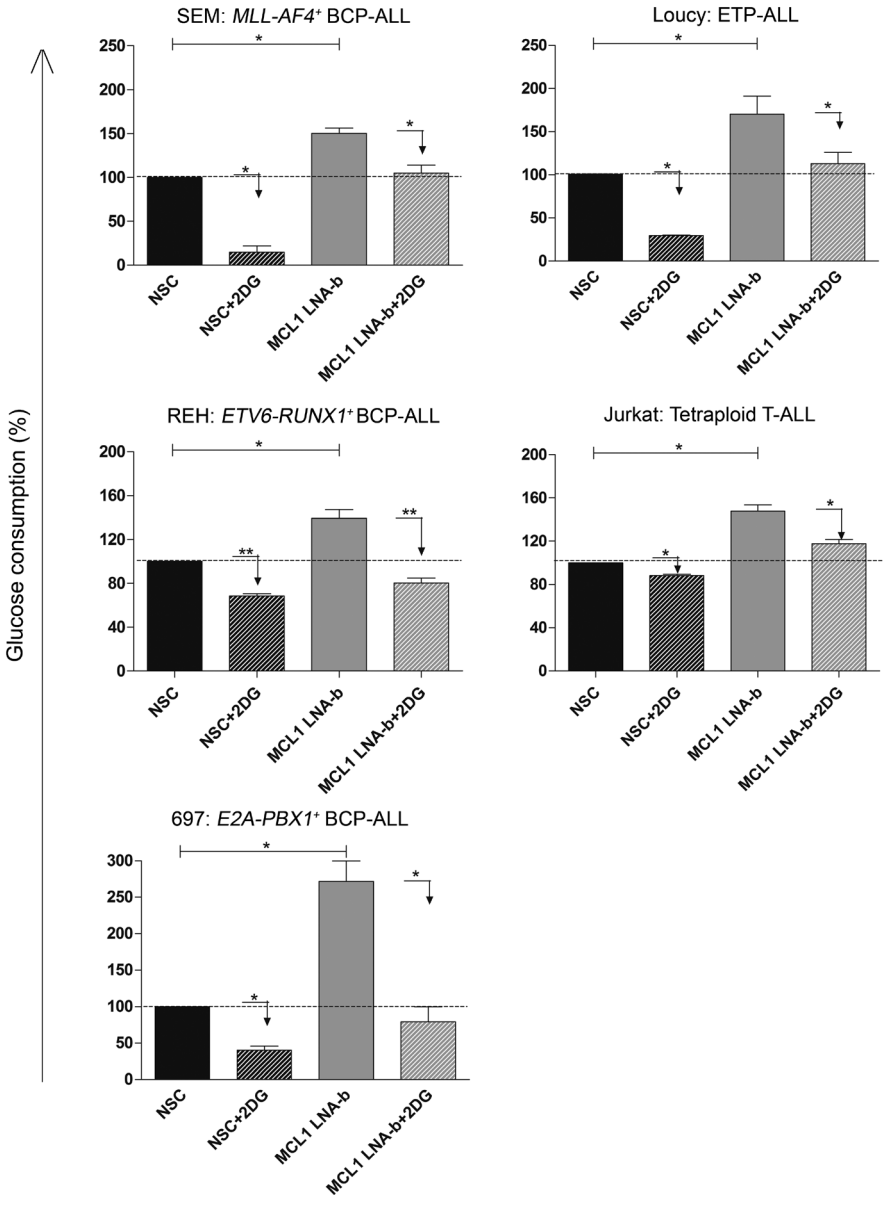


Figure 2. MCL1-silenced cells upregulate glycolysis, which can be reduced by 2-DG.
Glucose consumption of *MLL-AF4*+ BCP-ALL, *ETV6-RUNX1*+ BCP-ALL, *E2A-PBX1*+ BCP-ALL, ETP-ALL and tetraploid T-ALL cell line after treatment with MCL1 LNA-b and/or a non-silencing control LNA (NSC) with or without 0.5mM 2-DG was examined with a glucose consumption assay. To calculate glucose consumption, values were compared with glucose levels in plain RPMI medium and corrected for cell growth. Data are presented as means plus SEM of three (*ETV6-RUNX1*+ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01, ***p<0.001).

MCL1-silenced cells upregulate glycolysis. Targeting glycolysis in MCL1 silenced cells synergistically inhibits leukemic survival and concomitantly reverses prednisolone resistance.

To test our hypothesis that MCL1 and glycolysis cooperate in prednisolone resistance, we examined the consumption of glucose in five distinct MCL1 silenced cell lines. A significant increase in glucose consumption, on average 149%, was observed upon MCL1 silencing by MCL1 LNA ($p<0.01$ MCL1 LNA-b Figure 2 and MCL1 LNA-a and LNA-c Supplemental Figure 10) and by shMCL1 ($p<0.01$ Supplemental Figure 11), compared to non-silencing LNA controls.

This finding suggested that MCL1 silenced cells might increase their glycolysis to rescue from apoptosis. This prompted us to investigate the effect of targeting both MCL1 and glycolysis on leukemic cell survival and prednisolone cytotoxicity. Co-exposure of MCL1 LNA and 2-DG significantly reduced glucose consumption ($p<0.05$ MCL1 LNA-b Figure 2 , MCL1 LNA-a and MCL1 LNA-c Supplemental Figure 10). In all cell lines, cotreatment of cells with MCL1 LNA and 2-DG synergistically inhibited leukemic cell survival by 30%-75%, compared to MCL1 LNA or 2-DG alone, except in the tetraploid T-ALL ($p<0.05$, F*syn* <1, Figure 3 Panel A and Supplemental Figure 12). Furthermore, the addition of prednisolone decreased leukemic survival even more ($p<0.05$ Figure 3 Panel B and Supplemental Figure 13). Incubation of leukemic cells with both MCL1 LNA and 2-DG concomitantly, albeit moderately, sensitized to prednisolone up to 1.48-fold compared to MCL1 LNA or 2-DG alone ($p<0.05$; Figure 3 Panel C and Supplemental Figure 14).

The synergism of MCL1 LNAb, 2-DG and prednisolone was best visible in the intermediate responsive cells, i.e. *MLL-AF4*$^+$, *ETV6-RUNX1*$^+$ and ETP-ALL cell line, since the sensitive *E2A-PBX1*$^+$ cell line was already prone to die by monotherapy alone and the highly resistant tetraploid cell line will most likely need higher

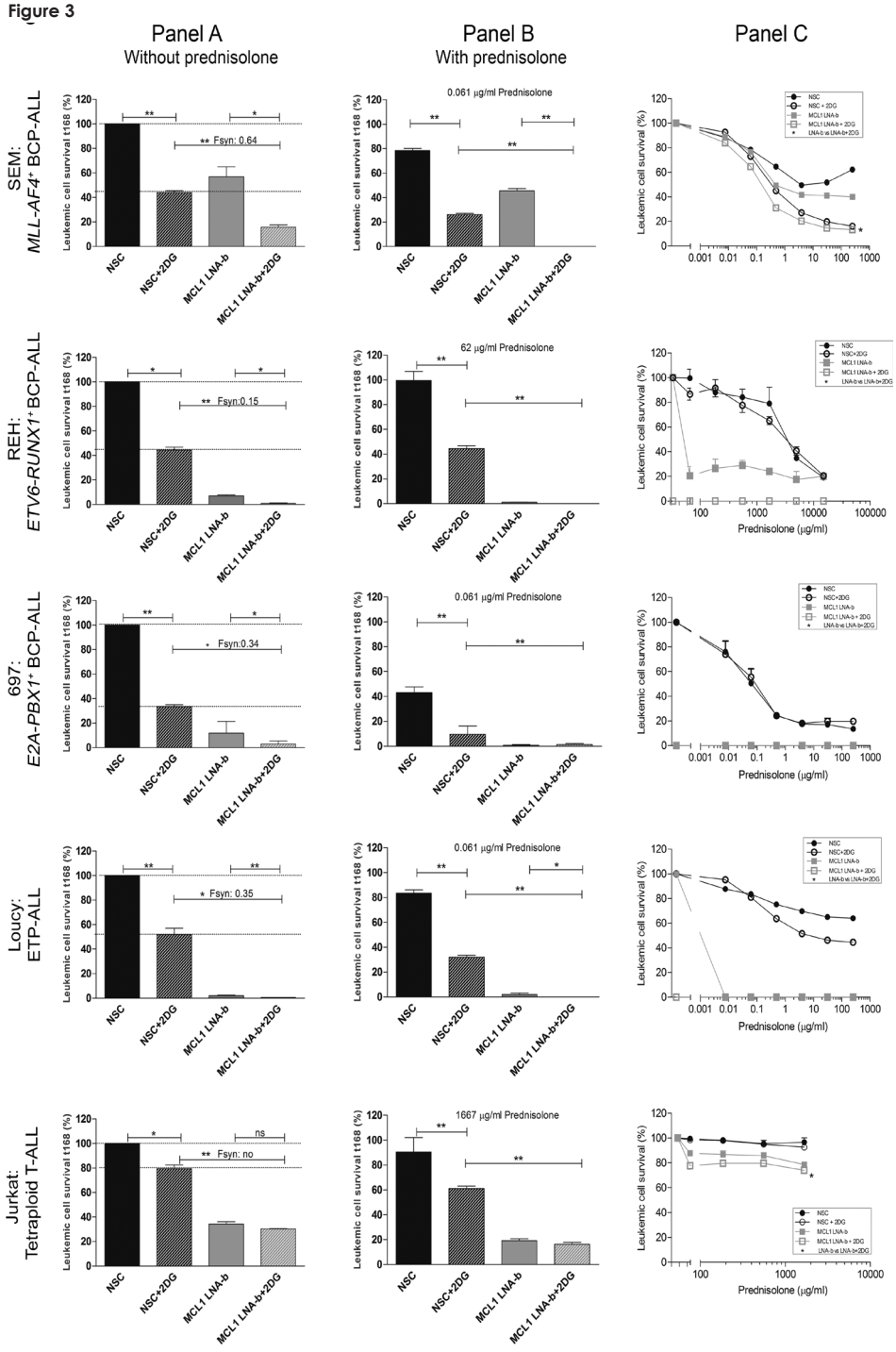
Figure 3. MCL1 silencing together with glycolysis inhibition synergistically inhibits leukemic cell survival and concomitantly sensitizes to prednisolone.

Panel A: Leukemic cell survival of a *MLL-AF4*$^+$ BCP-ALL, *ETV6-RUNX1*$^+$ BCP-ALL, *E2A-PBX1*$^+$ BCP-ALL, ETP-ALL and tetraploid T-ALL cell line after treatment with MCL1 LNA-b and/or 0.5mM 2-DG for 168 hours, compared to non-silencing control LNA (NSC, set at 100%). Data are presented as means plus SEM of three (*ETV6-RUNX1*$^+$ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$). F*syn* represents the synergy factor, where <1 indicates synergy.

Panel B: Leukemic cell survival after prednisolone exposure for three days, i.e. 96 till 168 hours after start of MCL1 LNA-b and/or 0.5mM 2-DG in equivalent cell lines as in panel A. Data were compared to a non-silencing control LNA (NSC) without prednisolone (see also panel A, set to 100%), to visualize the total effect on cell survival after prednisolone. Data are presented as means plus SEM of three (*ETV6-RUNX1*$^+$ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Panel C: Sensitivity of leukemic cells to prednisolone was measured in a 3-day MTT assay from t96 to t168 after treatment with MCL1 LNA-b and/or a non-silencing control LNA (NSC) with or without 0.5mM 2-DG. Cell survival depicted on the Y-axis was corrected for cell death induced by MCL1 knockdown and 0.5mM 2-DG itself in the absence of prednisolone, to visualize the absolute prednisolone effects. Data are presented as means plus SEM of three (*ETV6-RUNX1*$^+$ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

Figure 3


Panel A
Without prednisolone
Panel B
With prednisolone
Panel C
SEM:
MLL-AF4+ BCP-ALL
REH:
ETV6-RUNX1+ BCP-ALL
697:
E2A-PBX1+ BCP-ALL
Loucy:
ETP-ALL
Jurkat:
Tetraploid T-ALL
Leukemic cell survival t168 (%)
Leukemic cell survival (%)
NSC
NSC+2DG
MCL1 LNA-b
MCL1 LNA-b+2DG
** Fsyn: 0.64
0.061 µg/ml Prednisolone
** Fsyn:0.15
62 µg/ml Prednisolone
* Fsyn:0.34
0.061 µg/ml Prednisolone
* Fsyn: 0.35
0.061 µg/ml Prednisolone
** Fsyn: no
1667 µg/ml Prednisolone
Prednisolone (µg/ml)

amounts of the drugs to demonstrate an effect. Overall, these data indicate that MCL1-silenced cells upregulate glycolysis and that targeting glycolysis in MCL1 silenced cells synergistically inhibits leukemic survival and concomitantly reverses prednisolone resistance.

DISCUSSION

Prednisolone is the spearhead drug used in multi-drug treatment of ALL. Not only is the *in vivo* and *in vitro* response to prednisolone a strong prognostic factor for long-term clinical outcome [2], relapsed ALL patients also acquire prednisolone resistance disproportionately to other anti-leukemic agents [18]. To date, various mechanisms that sensitize leukemic cells *in vitro* to prednisolone have been described, including inhibition of the prednisolone interconverter 11β-HSD, knockdown of the glucocorticoid-dependent transcription regulator SMARCA4, inhibition of the voltage-dependent channel hERG1, which signals to ERK/PI3K/Akt survival pathways, knockdown of the calcium scavengers S100A8/S100A9, downregulation of anti-apoptotic MCL1, upregulation of pro-apoptotic BIM, and inhibition of glycolysis [6,8,9,19–22]. Sensitizing cells to prednisolone may therefore require a multifactorial approach. In this study, we provide evidence that reduction of MCL1 levels and inhibition of glycolysis synergistically inhibits leukemic survival and concomitantly sensitizes to prednisolone in ALL cells.

Downregulation of anti-apoptotic BCL-2 family members is hampered in prednisolone resistant cells [16]. In the present study, we observed that prednisolone exposure decreased the expression of the anti-apoptotic MCL1 in leukemic cells of sensitive patients, whereas that of resistant cells remained unchanged. Prednisolone did not affect the expression of other BCL-2 family members (BCL-XL and BCL-2) nor p53. In line with this are microarray data on primary BCP-ALL, which identified higher anti-apoptotic *MCL1* expression in prednisolone-resistant cells, but not higher Bcl-XL, BCL-2 or p53 expression [3]. We observed that silencing of MCL1 expression by two means, shMCL1 and MCL1 LNA, induced apoptosis and sensitized to prednisolone in BCP-ALL and T-ALL cell lines. This is consistent with previous findings in other leukemic cell lines that demonstrated prednisolone sensitization after direct knockdown of *MCL1* by shMCL1 or indirect downregulation of MCL1 via inhibition of mTOR [6,7]. Another study reported that disruption of the complex between beclin-1 and MCL1 by obatoclax and exposure to dexamethasone activated autophagy-dependent cell-death in otherwise dexamethasone-resistant cells [23]. However, inhibition of autophagy only slightly induced resistance to dexamethasone, implying direct anti-apoptotic effects

as well [23]. Overall, these data indicate MCL1 as a potent therapeutic target to convert glucocorticoid resistance. However, high expression of MCL1 is not predictive for an adverse clinical outcome, suggesting that additional mechanisms co-occur that induce prednisolone resistance in pediatric ALL [6]. Here, we observed that targeting MCL1 forces the glycolysis route thereby rescuing cells from prednisolone-induced apoptosis. It has been shown that BCL2 family members maintain the mitochondrial membrane potential by regulating the permeability transition pore and the ATP/ADP pump, both involved in oxidative phosphorylation [13,14]. Knockdown of MCL1 may therefore impair oxidative phosphorylation, forcing cells to produce ATP by glycolysis to ensure survival. This hypothesis is supported by our observation that treatment of leukemic cells with azide, a known inhibitor of oxidative phosphorylation, also increases glucose consumption (Supplemental Figure 15). Furthermore, an MCL1 amino-terminally truncated isoform was recently discovered that facilitates ATP production, respiration and maintenance of oligomeric ATP synthase in the mitochondria [24]. Our three MCL1 LNAs all target the 3' UTR of MCL1 and therefore also diminish this truncated MCL1 isoform. Furthermore, the glycolysis and apoptotic pathways may be connected via activity of the BCL2 family member BAD. The phosphorylation of aminoacid S112 in the BH3 domain of BAD acts like a switch between the metabolic and pro-apoptotic functions ascribed to BAD. BAD resides in a mitochondrial complex together with glucokinase and contributes to the activity of this glucose-metabolizing enzyme [25]. Glucokinase mediates the first step in glycolysis by converting glucose into glucose-6-phosphate. Although it has been shown that MCL1 does not bind BAD directly [26], silencing of MCL1 may indirectly induce BAD activity and/or may trigger glycolysis in a similar way as the BAD/glucokinase complex. As a net result the glycolytic rate (and hence glucose consumption) will increase and this may provide a rescue mechanism against prednisolone-induced cell death. This speculative functional explanation yet awaits further studies. Our study showed that inhibition of both glycolysis and MCL1 synergistically inhibit leukemic cell survival and concomitantly sensitizes leukemic cells to prednisolone. These results indicate the need to target both pathways and suggest that targeting MCL1 as a single target may not yield the desired clinical effect. We previously demonstrated that ALL cells increase glucose consumption to prevent prednisolone-induced apoptosis [8]. Glucocorticoids inhibit intracellular glucose uptake by regulating the expression of glucose transmembrane transporter (GLUT) [27–29]. Dexamethasone decreases GLUT-1 expression in ALL cells thereby decreasing glycolysis and inducing apoptosis [30]. We observed higher expression of GLUT-1 in prednisolone resistant ALL patients [3]. TXNIP, a negative regulator of glucose uptake [30] is correlated to GLUT-1 expres-

sion [31] and was also found upregulated after prednisolone treatment in sensitive patients.[32] Further studies are needed to demonstrate whether TXNIP and GLUT-1 facilitate the higher glucose metabolism observed after MCL1 knockdown.

Our results suggest that treatment with MCL1 LNA antisense and 2-DG represent a promising approach to decrease leukemic cell survival and to sensitize ALL patients to prednisolone. LNA antisense may offer a more direct and specific way of silencing MCL1 than the current BCL-2 family inhibitors R-(-)-gossypol (AT101) and obatoclax (GX-15-070), [33,34] which recently entered clinical phase I/II trials. Both inhibitors can block several members of the BCL-2 family, increasing the chance of side-effects in clinical practice. In contrast, LNA antisense specifically target the mRNA expression of one gene. We have shown in this study that MCL1 LNA effectively silences *MCL1* mRNA and protein expression in ALL cells, comparable to knockdown with the more stable shMCL1. Notably, no delivery vehicles were necessary to ensure uptake of MCL1 LNA antisense molecules by the ALL cells. Moreover, LNA's are conformationally structured to prevent most of the current hurdles in siRNA treatment, such as delivery, stability of the RNA molecules in circulation, strand bias and off-target effects [35]. LNA antisense molecules are currently investigated in phase I early clinical trials for three different target genes: EZN-3042, an inhibitor of Survivin is being investigated in children with relapsed ALL (www. Clinicaltrials.gov, NCT01186328); EZN-4176, which targets the androgen receptor, is being studied in adults with castration-resistant prostate cancer (NCT01337518); and EZN-2968, an HIF-1A-inhibitor, is being examined in advanced solid tumours and lymphoma (NCT00466583). We here provide functional *in vitro* proof that MCL1 LNA antisense molecules are effective in inhibiting leukemic cell survival and reversing prednisolone resistance and may offer merits to further investigate these MCL1-LNAs in clinical trials. However, as our data show that silencing of MCL1 increases glycolysis of cells, neither MCL1 LNA nor BH3 mimetics should be used as single agents. We have shown that this shift in energy metabolism can be exploited to concomitantly sensitize leukemic cells to prednisolone. 2DG may be a candidate agent since it has therapeutic potential and is proven to cause chemosensitisation in acute leukemia [8], breast cancer [36], and prostate cancer cells [37], and is now used in phase I clinical trials (www.ClinicalTrials.gov, NCT00096707).

In conclusion, MCL1 is a potent target to therapeutically inhibit leukemic survival and to reverse drug resistance in pediatric ALL. However, MCL1-silenced cells upregulate glycolysis, which may rescue cells from prednisolone-induced apoptosis. These data therefore provide evidence for concomitant causes of survival and resistance, and indicate that MCL1 and glycolysis should be

targeted simultaneously to reduce leukemic cell survival and prednisolone resistance in ALL.

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AUTHORSHIP AND DISCLOSURES

I.M.A. designed and performed research, analyzed and interpreted data, and wrote the paper; B.R.H. and T.K. developed the LNA oligonucleotides; R.V.D. assisted in the LNA synergy experiments. W.E.E. discussed data and revised the paper; R.P. and M.L.DB. designed research, analyzed and interpreted data, and revised the paper.

B.R.H. and T.K. are employed at Santaris Pharma A/S, Hørsholm, Denmark. The other authors declare to have no conflicts of interests.

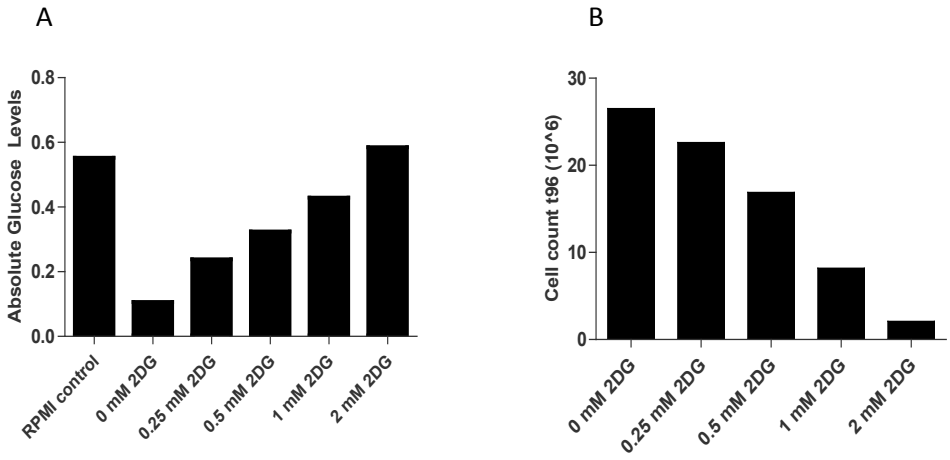
REFERENCES

1. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Körholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol. 2003;21(17):3262–8.
2. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood. 1998;92(1):259–66.
3. Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJP, Kazemier KM, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. New Engl J Med. 2004;351(6):533–42.
4. Placzek WJ, Wei J, Kitada S, Zhai D, Reed JC, Pellecchia M. A survey of the anti-apoptotic Bcl-2 subfamily expression in cancer types provides a platform to predict the efficacy of Bcl-2 antagonists in cancer therapy. Cell Death Dis. 2010;1(5):e40.
5. Lestini BJ, Goldsmith KC, Fluchel MN, Liu X, Chen NL, Goyal B, et al. Mcl1 downregulation sensitizes neuroblastoma to cytotoxic chemotherapy and small molecule Bcl2-family antagonists. Cancer Biol Ther. 2009;8(16):1587–95.
6. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, et al. Association of high-level MCL-1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia. Blood. 2010;115(5):1018–25.
7. Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, et al. Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. Cancer cell. 2006;10(4):331–42.
8. Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJC, et al. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood. 2009;113(9):2014–21.
9. Eberhart K, Renner K, Ritter I, Kastenberger M, Singer K, Hellerbrand C, et al. Low doses of 2-deoxy-glucose sensitize acute lymphoblastic leukemia cells to glucocorticoid-induced apoptosis. Leukemia. 2009;23(11):2167–70.
10. Mason EF, Zhao Y, Goraksha-Hicks P, Coloff JL, Gannon H, Jones SN, et al. Aerobic glycolysis suppresses p53 activity to provide selective protection from apoptosis upon loss of growth signals or inhibition of BCR-Abl. Cancer Res. 2010;70(20):8066–76.
11. Zhao Y, Altman BJ, Coloff JL, Herman CE, Jacobs SR, Wieman HL, et al. Glycogen synthase kinase 3alpha and 3beta mediate a glucose-sensitive antiapoptotic signaling pathway to stabilize Mcl-1. Mol Cell Biol. 2007;27(12):4328–39.
12. Pradelli L a, Bénéteau M, Chauvin C, Jacquin M a, Marchetti S, Muñoz-Pinedo C, et al. Glycolysis inhibition sensitizes tumor cells to death receptors-induced apoptosis by AMP kinase activation leading to Mcl-1

block in translation. Oncogene. 2010;29(11):1641-52.
13. Chen ZX, Pervaiz S. Bcl-2 induces pro-oxidant state by engaging mitochondrial respiration in tumor cells. Cell Death Differ. 2007;14(9):1617–27.
14. Vander Heiden MG, Chandel NS, Schumacker PT, Thompson CB. Bcl-xL prevents cell death following growth factor withdrawal by facilitating mitochondrial ATP/ADP exchange. Molecular cell. 1999;3(2):159–67.
15. Berenbaum MC. Synergy, additivism and antagonism in immunosuppression. A critical review. Clin Exp Immunol. 1977;28(1):1–18.
16. Laane E, Panaretakis T, Pokrovskaja K, Buentke E, Corcoran M, Söderhäll S, et al. Dexamethasone-induced apoptosis in acute lymphoblastic leukemia involves differential regulation of Bcl-2 family members. Haematologica. 2007;92(11):1460-9.
17. Brady HJ, Salomons GS, Bobeldijk RC, Berns AJ. T cells from baxalpha transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53. EMBO J. 1996;15(6):1221–30.
18. Klumper E, Pieters R, Veerman AJ, Huismans DR, Loonen AH, Hählen K, et al. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. Blood. 1995;86(10):3861–8.
19. Sai S, Nakagawa Y, Yamaguchi R, Suzuki M, Sakaguchi K, Okada S, et al. Expression of 11beta-hydroxysteroid dehydrogenase 2 contributes to glucocorticoid resistance in lymphoblastic leukemia cells. Leuk Res. 2011;35(12):1644–8.
20. Pillozzi S, Masselli M, De Lorenzo E, Accordi B, Cilia E, Crociani O, et al. Chemotherapy resistance in acute lymphoblastic leukemia requires hERG1 channels and is overcome by hERG1 blockers. Blood. 2011;117(3):902–14.
21. Spijkers-Hagelstein JAP, Schneider P, Hulleman E, de Boer J, Williams O, Pieters R, et al. Elevated S100A8/S100A9 expression causes glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. Leukemia. 2012;26(6):1255-65
22. Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. J Biol Chem . 2004;279(53):55809–17.
23. Bonapace L, Bornhauser BC, Schmitz M, Cario G, Ziegler U, Niggli FK, et al. Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. J Clin Invest. 2010;120(4):1310–23.
24. Perciavalle RM, Stewart DP, Koss B, Lynch J, Milasta S, Bathina M, et al. Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. Nat Cell Biol. 2012;14(6):575–83.
25. Danial NN, Gramm CF, Scorrano L, Zhang C-Y, Krauss S, Ranger AM, et al. BAD and glucokinase reside in a mitochondrial complex that integrates glycolysis and apoptosis. Nature. 2003;424(6951):952–6.
26. Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG, et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell. 2005;17(3):393–403.
27. Hajduch E, Hainault I, Meunier C, Jardel C, Hainque B, Guerre-Millo M,

et al. Regulation of glucose transporters in cultured rat adipocytes: synergistic effect of insulin and dexamethasone on GLUT4 gene expression through promoter activation. Endocrinology. 1995;136(11):4782–9.
28. Buentke E, Nordström A, Lin H, Björklund A-C, Laane E, Harada M, et al. Glucocorticoid-induced cell death is mediated through reduced glucose metabolism in lymphoid leukemia cells. Blood Cancer J. 2011;1(7):e31.
29. Boag JM, Beesley AH, Firth MJ, Freitas JR, Ford J, Hoffmann K, et al. Altered glucose metabolism in childhood pre-B acute lymphoblastic leukaemia. Leukemia. 2006;20(10):1731–7.
30. Wang Z, Rong YP, Malone MH, Davis MC, Zhong F, Distelhorst CW. Thioredoxin-interacting protein (txnip) is a glucocorticoid-regulated primary response gene involved in mediating glucocorticoid-induced apoptosis. Oncogene. 2006;25(13):1903–13.
31. Yu F-X, Goh S-R, Dai R-P, Luo Y. Adenosine-containing molecules amplify glucose signaling and enhance txnip expression. Mol Endocrinol. 2009;23(6):932–42.
32. Tissing WJE, den Boer ML, Meijerink JPP, Menezes RX, Swagemakers S, van der Spek PJ, et al. Genomewide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. Blood. 2007;109(9):3929–35.
33. Etxebarria A, Landeta O, Antonsson B, Basañez G. Regulation of anti-apoptotic MCL-1 function by gossypol: mechanistic insights from in vitro reconstituted systems. Biochem Pharmacol . 2008;76(11):1563-76.
34. Nguyen M, Marcellus RC, Roulston A, Watson M, Serfass L, Murthy Madiraju SR, et al. Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. Proc Natl Acad Sci. 2007;104(49):19512–7.
35. Koch T, Rosenbohm C, Hansen HF, Hansen B, Straarup EM, Kauppinen S. Locked Nucleic Acid: Properties and Therapeutic Aspects. In: Therapeutic Oligonucleotides. Cambridge: Royal Society of Chemistry; 2008
36. Zhang F, Aft RL. Chemosensitizing and cytotoxic effects of 2-deoxy-D-glucose on breast cancer cells. J Cancer Res Ther. 2009;5(Suppl 1):S41–3.
37. Sahra IB, Tanti J-F, Bost F. The combination of metformin and 2-deoxyglucose inhibits autophagy and induces AMPK dependent apoptosis in prostate cancer cells. Autophagy. 2010;6(5).

Supplemental Figure 1.

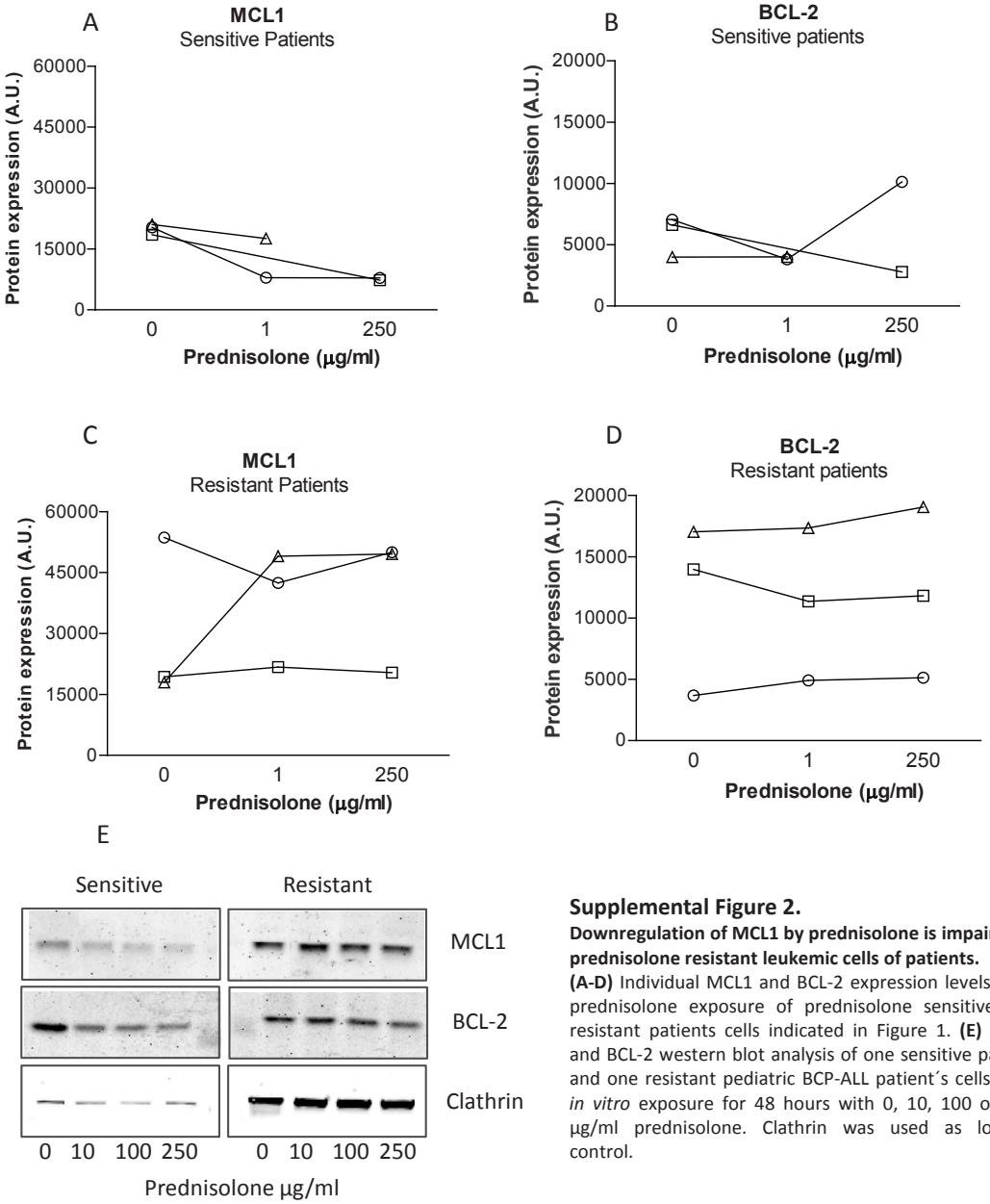


Supplemental Figure 1.

The effect of 2DG on glucose consumption and proliferation.

(A) Absolute glucose levels in the supernatant of *ETV6-RUNX1*$^+$ BCP-ALL (REH) cells were measured with a glucose assay. RPMI control represents the amount of glucose present in culture medium incubated for 96 hours in the absence of 2DG. Cells cultured without 2DG have consumed >90% of the glucose present in RPMI culture medium after 96 hrs. Exposure to increasing concentrations of 2DG reduces the amount of glucose that is being consumed. In cells treated with 2mM 2DG the glucose levels are virtually unaffected compared to RPMI control medium, indicating that no glucose has been consumed. (B) *ETV6-RUNX1*$^+$ BCP-ALL (REH) cells were counted by a trypan blue exclusion assay after exposure to 2DG for 96 hours. Glucose consumption (A) and cell viability (B) are correlated.

Supplemental Figure 2.

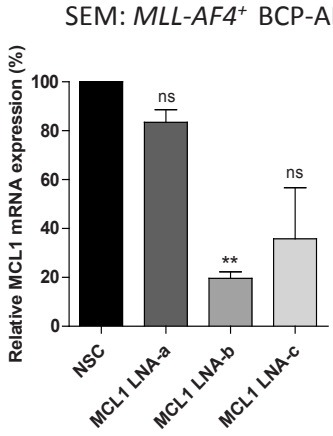


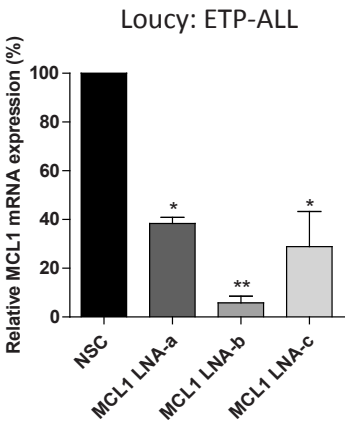
Supplemental Figure 2.

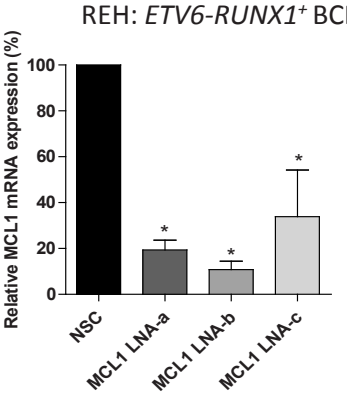
Downregulation of MCL1 by prednisolone is impaired in prednisolone resistant leukemic cells of patients.

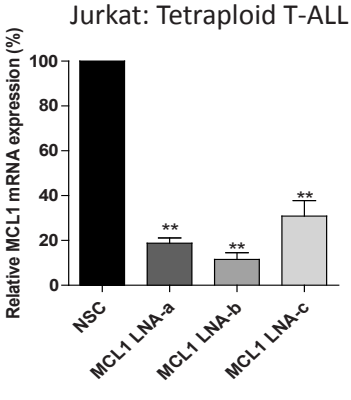
(A-D) Individual MCL1 and BCL-2 expression levels after prednisolone exposure of prednisolone sensitive and resistant patients cells indicated in Figure 1. **(E)** MCL1 and BCL-2 western blot analysis of one sensitive patient and one resistant pediatric BCP-ALL patient´s cells after *in vitro* exposure for 48 hours with 0, 10, 100 or 250 µg/ml prednisolone. Clathrin was used as loading control.

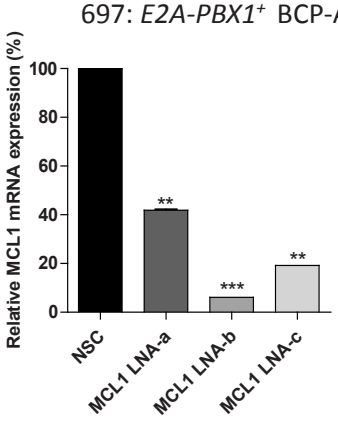
Supplemental Figure 3.





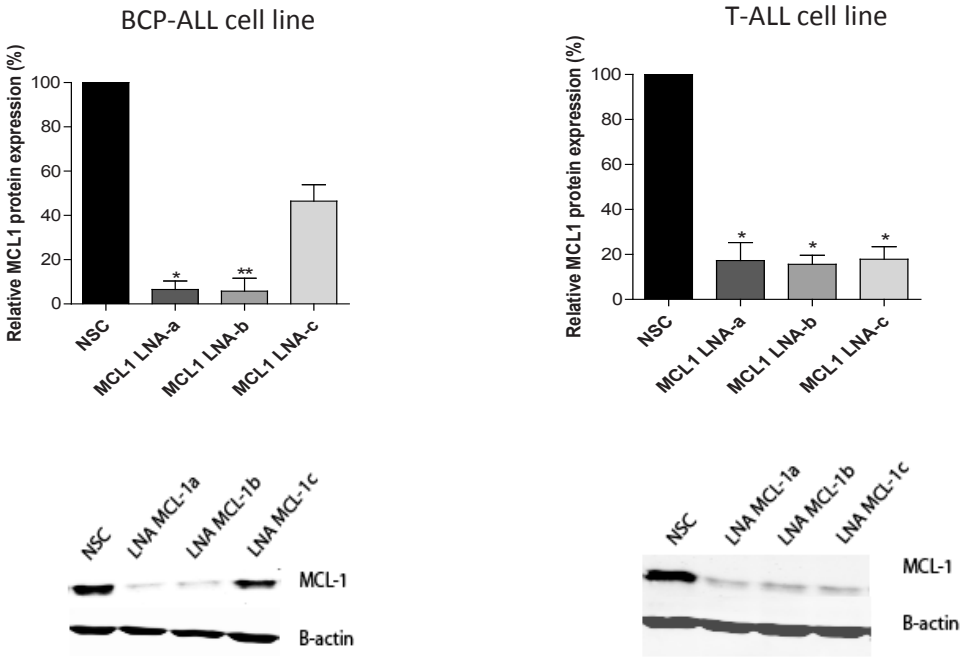






Supplemental Figure 3.
LNA antisense directed against *MCL1* efficiently silence the expression of *MCL1* mRNA. *MCL1* mRNA expression was measured after treatment with three different LNA antisense molecules against *MCL1*, i.e. MCL1 LNA-a , MCL1 LNA-b and MCL1 LNA-c in five distinct leukemic cell lines . Values were adjusted for expression of the housekeeping gene RPS20 and are relative to the non-silencing control (NSC). Data are presented as means plus SEM of three (*ETV6-RUNX1*+ BCP-ALL and tetraploid T-ALL*)* or two independent experiments (*p<0.05, **p<0.01, ***p<0.001).

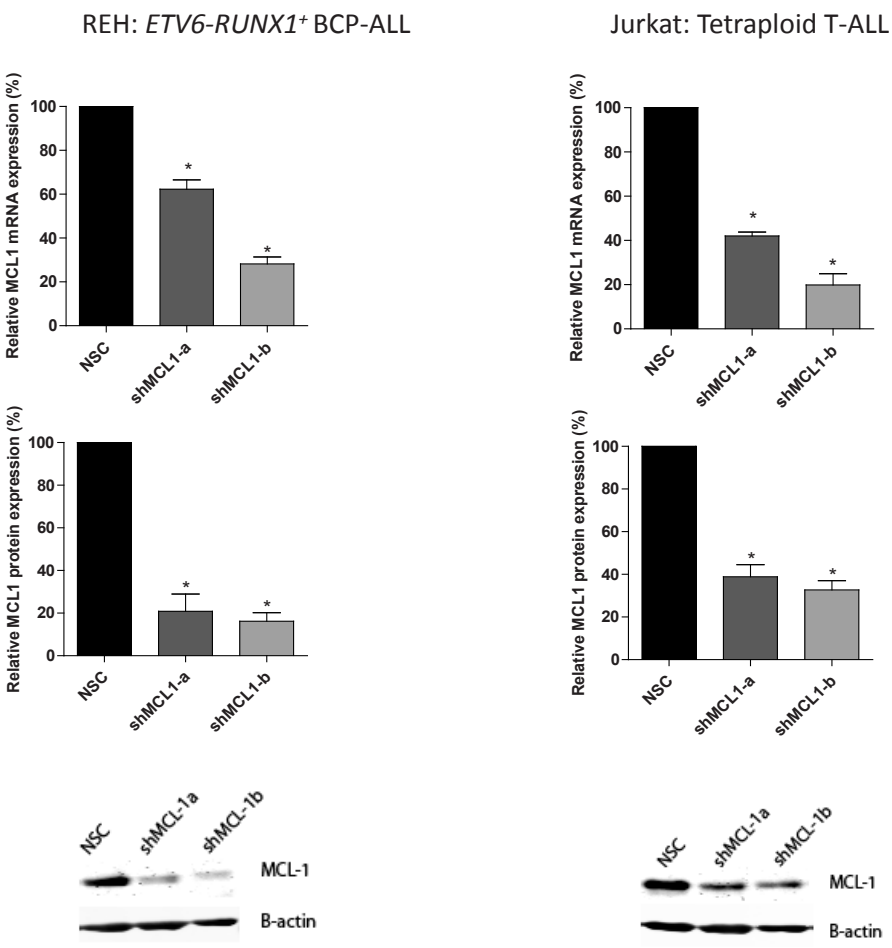
Supplemental Figure 4.



Supplemental Figure 4.

LNA antisense directed against MCL1 efficiently silence the expression of MCL1 protein. Protein expression of MCL1 was assessed at t168 with Western blot after LNA treatment. MCL1 protein expression was calculated with the Odyssey software, corrected for β-actin and is relative to the NSC. A representative Western blot for a BCP-ALL cell line, i.e. REH a *ETV6-RUNX1*$^{+}$ BCP-ALL cell line and a T-ALL, i.e. Jurkat a Tetraploid T-ALL cell line is illustrated. Data are presented as mean plus SEM of three independent experiments (*p<0.05, **p<0.01).

Supplemental Figure 5.

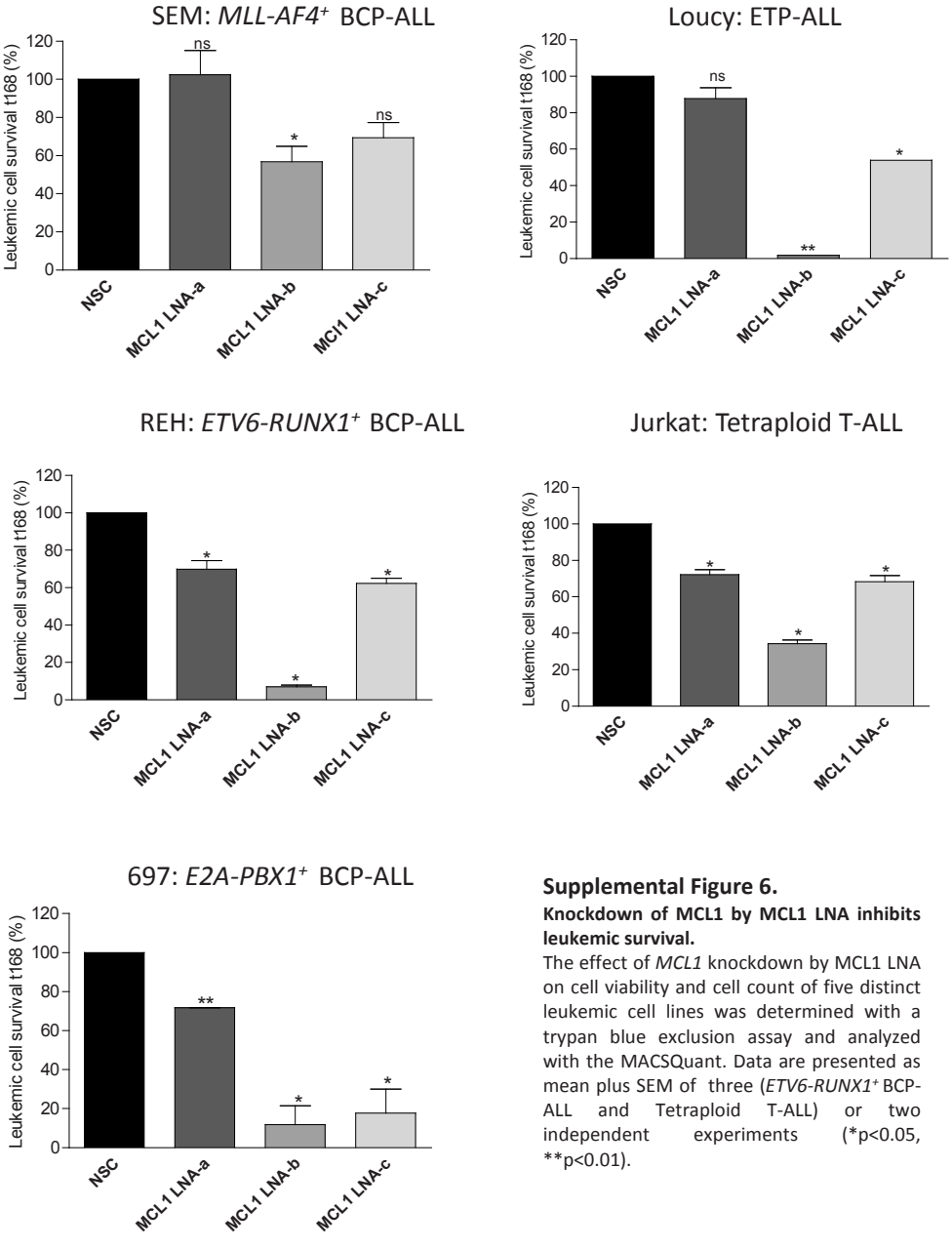


Supplemental Figure 5.

Short hairpins directed against *MCL1* efficiently silence the expression of MCL1.

MCL1 mRNA expression in *ETV6-RUNX1*$^{+}$ BCP-ALL cell line and Tetraploid T-ALL cell line was measured after lentiviral knockdown of *MCL1* with two different constructs, i.e. shMCL1-a and shMCL1-b. Values were adjusted for expression of the housekeeping gene RPS20 and are relative to the non-silencing control (NSC). Protein expression of MCL1 was assessed with Western blot after lentiviral knockdown. MCL1 protein expression was calculated with the Odyssey software, corrected for β-actin and is relative to the NSC. A representative Western blot is illustrated. Data are presented as mean plus SEM of three independent experiments (*p<0.05, **p<0.01).

Supplemental Figure 6.

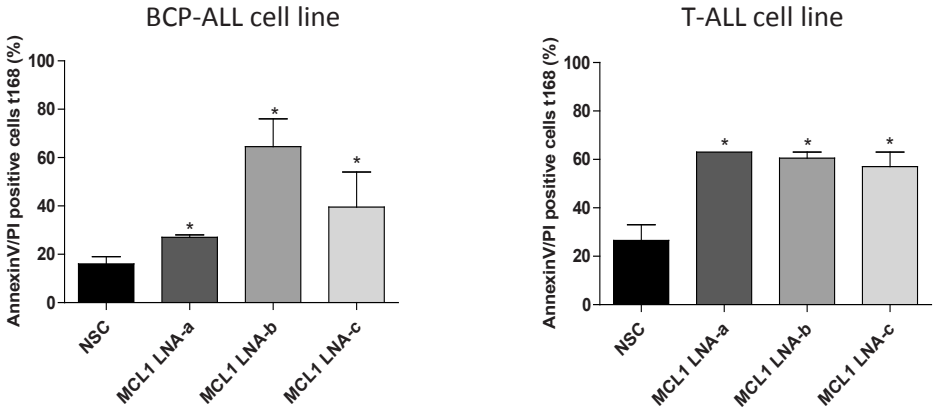


Supplemental Figure 6.

Knockdown of MCL1 by MCL1 LNA inhibits leukemic survival.

The effect of *MCL1* knockdown by MCL1 LNA on cell viability and cell count of five distinct leukemic cell lines was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. Data are presented as mean plus SEM of three (*ETV6-RUNX1*+ BCP-ALL and Tetraploid T-ALL) or two independent experiments (*p<0.05, **p<0.01).

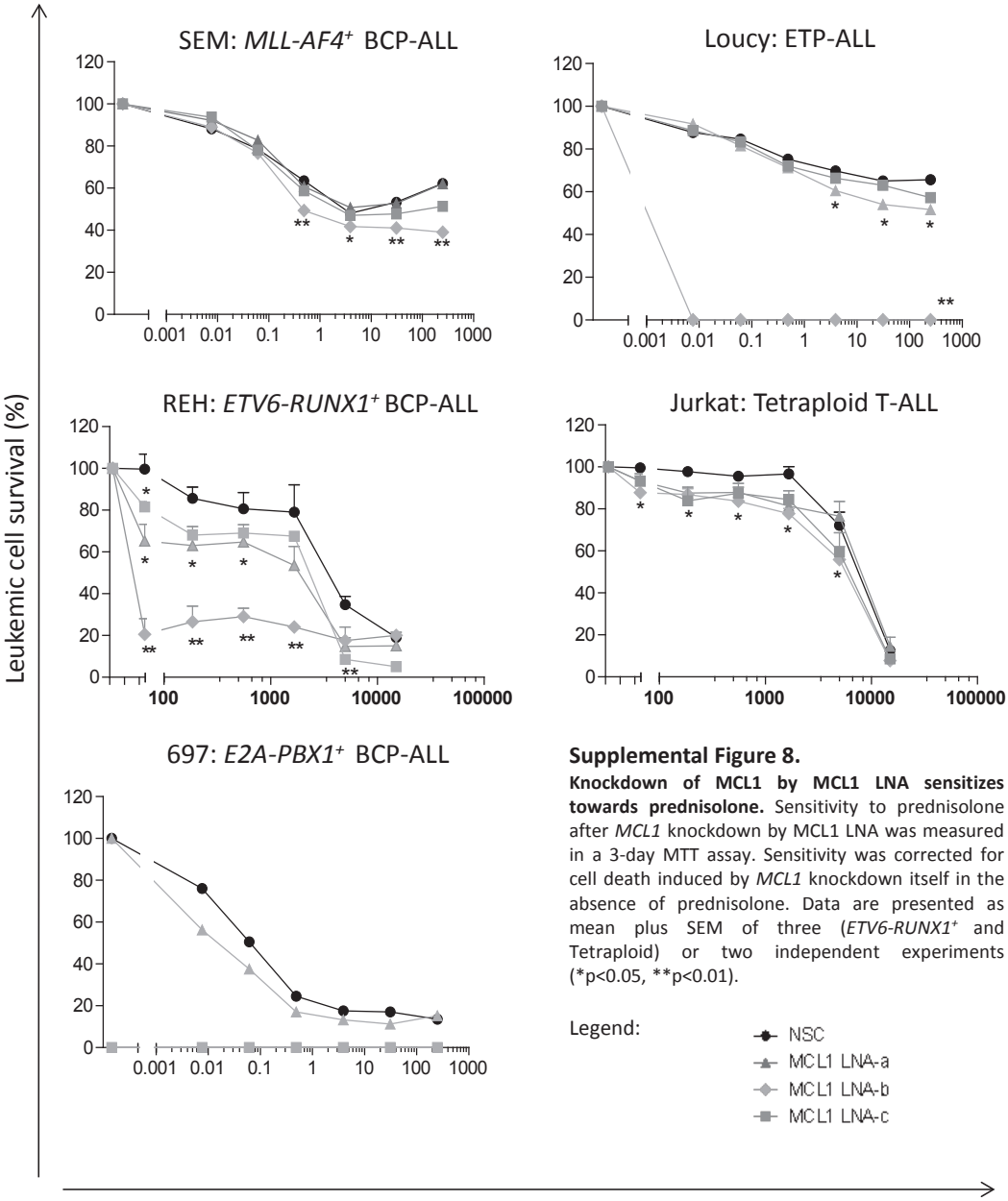
Supplemental Figure 7.



Supplemental Figure 7.

Knockdown of MCL1 by MCL1 LNA increases apoptosis. MCL1 knockdown-induced apoptosis in a representative BCP-ALL cell line, i.e. Reh an *ETV6-RUNX1*$^{+}$ BCP-ALL cell line and a T-ALL cell line, i.e. Jurkat a Tetraploid T-ALL, was assessed on a flowcytometer using an AnnexinV/PI staining. The percentage of apoptotic cells was calculated using the quadrant method. Data are presented as means plus SEM of three independent experiments (*p<0.05).

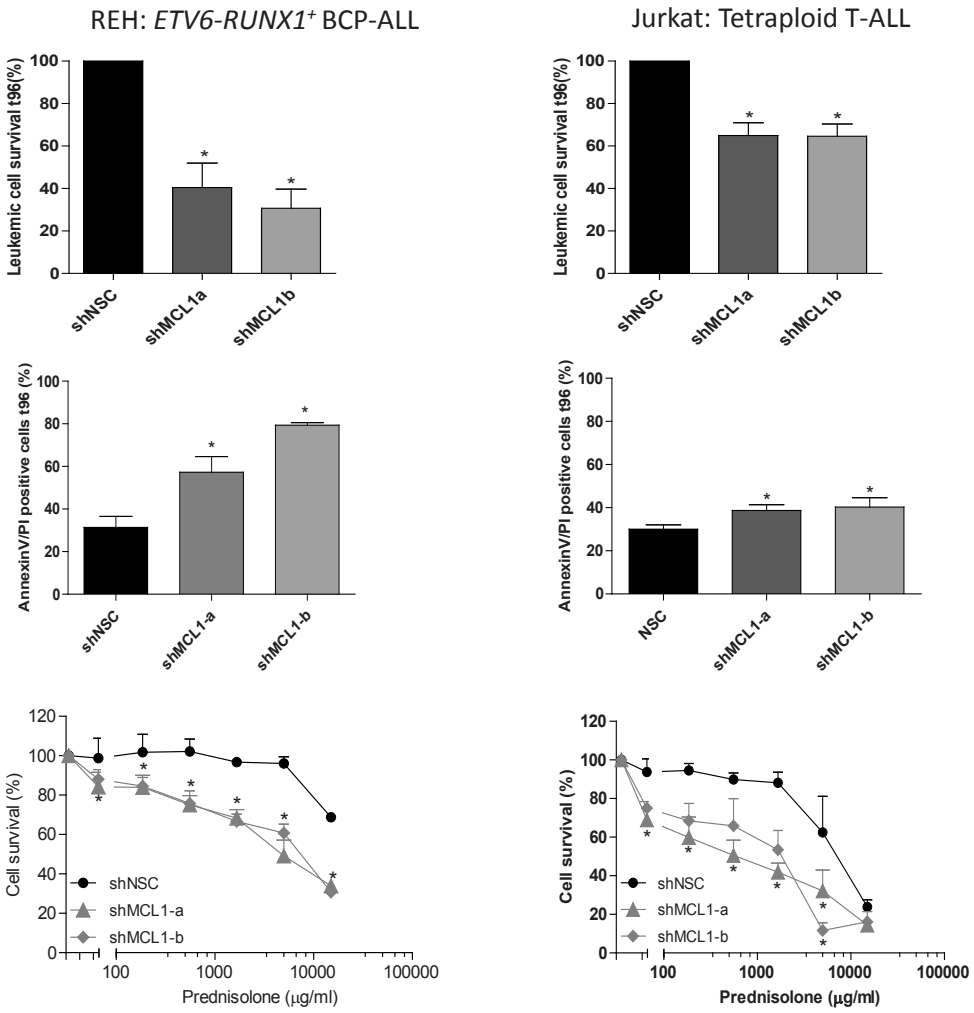
Supplemental Figure 8.



Supplemental Figure 8.

Knockdown of MCL1 by MCL1 LNA sensitizes towards prednisolone. Sensitivity to prednisolone after *MCL1* knockdown by MCL1 LNA was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by *MCL1* knockdown itself in the absence of prednisolone. Data are presented as mean plus SEM of three (*ETV6-RUNX1*+ and Tetraploid) or two independent experiments (*p<0.05, **p<0.01).

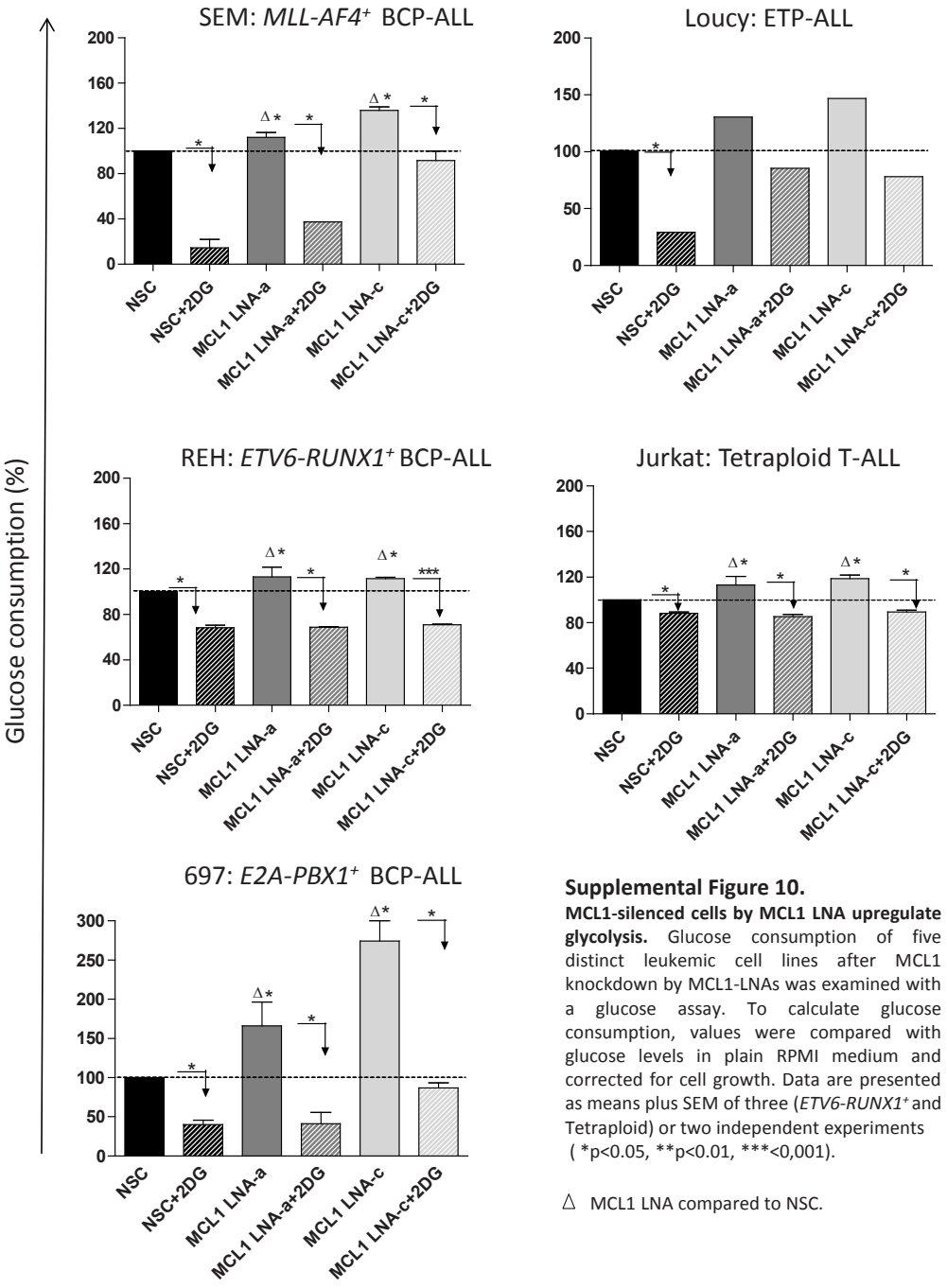
Supplemental Figure 9.



Supplemental Figure 9.

Knockdown of MCL1 by shMCL1 inhibits leukemic survival, increases apoptosis and sensitizes towards prednisolone. The effect of MCL1 knockdown by shMCL1 on cell viability and cell count of *ETV6-RUNX1+* BCP-ALL cells and T-ALL cells was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. *MCL1* knockdown-induced apoptosis in a BCP-ALL and T-ALL cell line was assessed on a flowcytometer using an AnnexinV/PI staining. The percentage of apoptotic cells was calculated using the quadrant method. Sensitivity to prednisolone after *MCL1* knockdown was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by *MCL1* knockdown itself in the absence of prednisolone. Data are presented as mean plus SEM of three independent experiments (*p<0.05, **p<0.01).

Supplemental Figure 10.

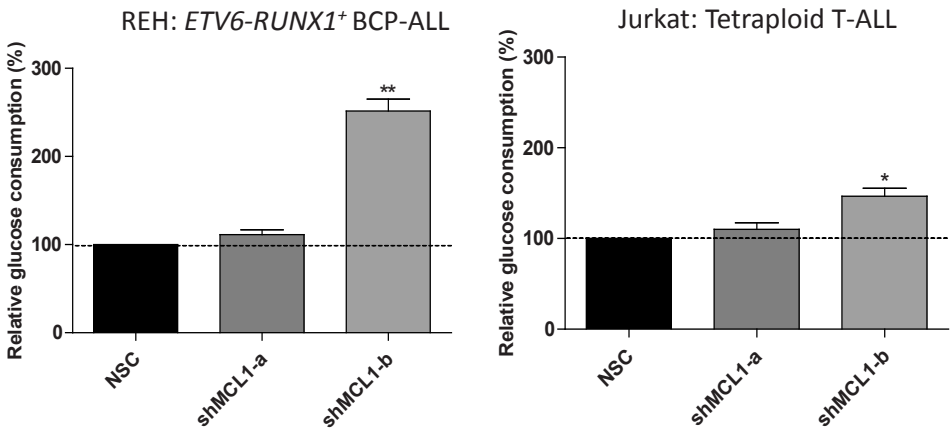


Supplemental Figure 10.

MCL1-silenced cells by MCL1 LNA upregulate glycolysis. Glucose consumption of five distinct leukemic cell lines after MCL1 knockdown by MCL1-LNAs was examined with a glucose assay. To calculate glucose consumption, values were compared with glucose levels in plain RPMI medium and corrected for cell growth. Data are presented as means plus SEM of three (*ETV6-RUNX1*+ and Tetraploid) or two independent experiments (*p<0.05, **p<0.01, ***<0,001).

Δ MCL1 LNA compared to NSC.

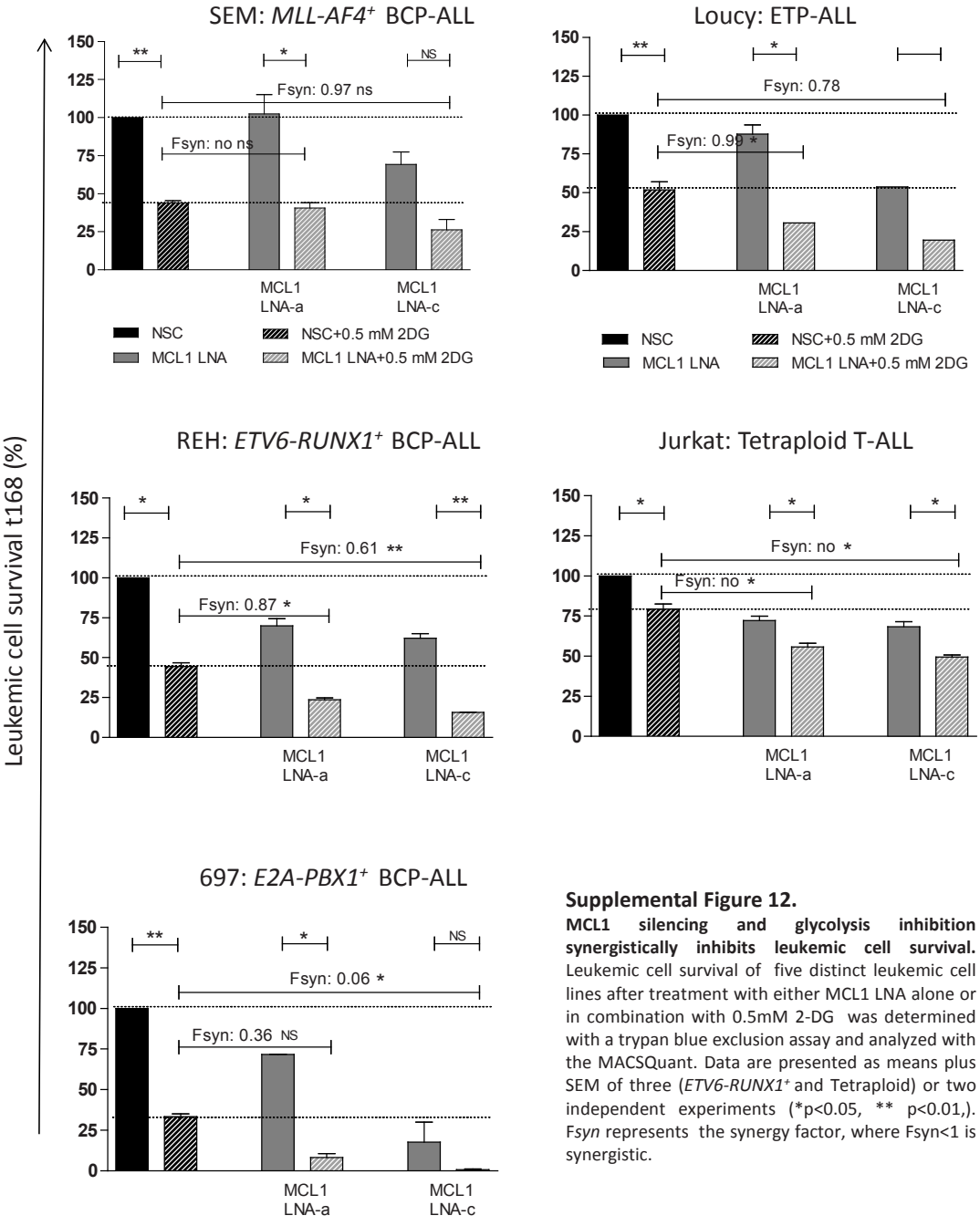
Supplemental Figure 11.



Supplemental Figure 11.

MCL1-silenced cells by shMCL1 upregulate glycolysis. Glucose consumption of a MCL1-silenced *ETV6-RUNX1+* BCP-ALL cell line and a tetraploid T-ALL cell line by shMCL1 was examined with a glucose assay. To calculate glucose consumption, values were compared with glucose levels in plain RPMI medium and corrected for cell growth. Data are presented as means plus SEM of three independent experiments (*p<0.05, **p<0.01). Off note, shMCL1-a is the only construct that targets exon 2 of MCL1, in contrast to shMCL1-b, LNA-a, LNA-b and LNA-c which are directed against exon 3. It may be that targeting exon 2 containing MCL1 transcripts does not affect glycolysis and/or that interference with exon 3 is more important for a functional effect on glycolysis.

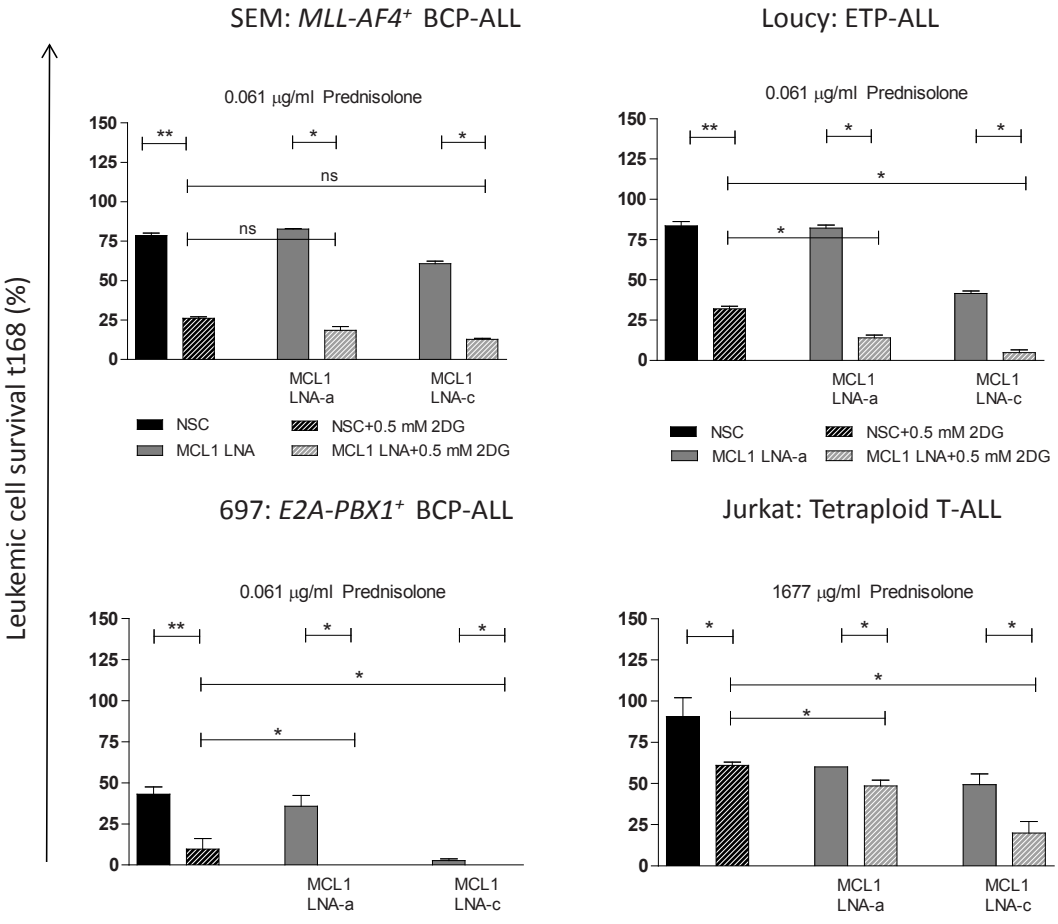
Supplemental Figure 12.



Supplemental Figure 12.

MCL1 silencing and glycolysis inhibition synergistically inhibits leukemic cell survival. Leukemic cell survival of five distinct leukemic cell lines after treatment with either MCL1 LNA alone or in combination with 0.5mM 2-DG was determined with a trypan blue exclusion assay and analyzed with the MACSQuant. Data are presented as means plus SEM of three (*ETV6-RUNX1*+ and Tetraploid) or two independent experiments (*p<0.05, ** p<0.01,). F*syn* represents the synergy factor, where Fsyn<1 is synergistic.

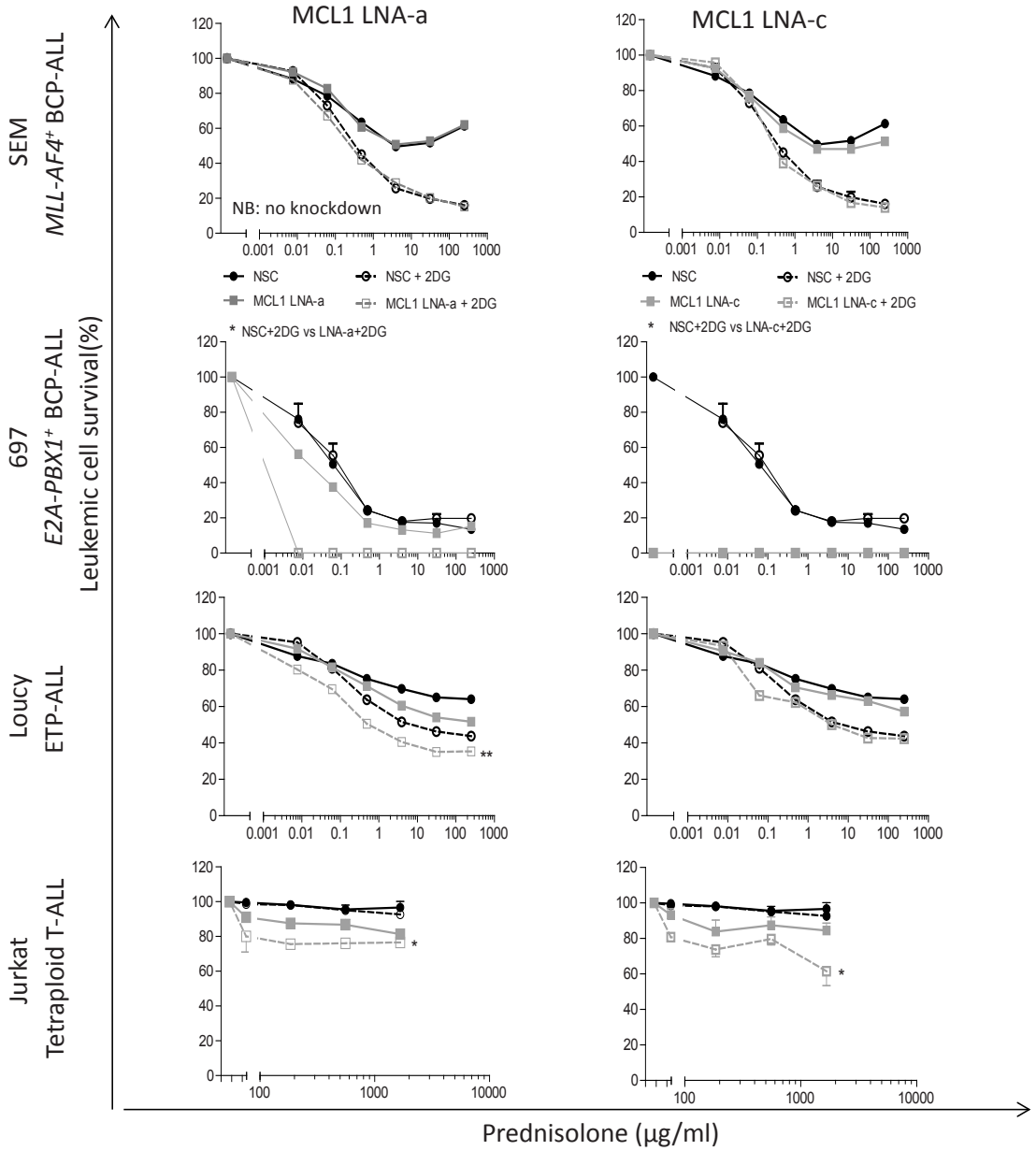
Supplemental Figure 13.



Supplemental Figure 13.

MCL1 knockdown, glycolysis inhibition and prednisolone treatment concomitantly inhibit leukemic cell survival. Leukemic cell survival after 3-day, i.e. from t96 untill t168, prednisolone exposure of *MLL-AF4+* BCP-ALL, *E2A-PBX1+* BCP-ALL, ETP-ALL and tetraploid T-ALL cells with either MCL1 LNA-b or 0.5mM 2-DG alone or in combination. Data were compared to NSC control without prednisolone, to visualize the total effect on cell survival of prednisolone, MCL LNA and 2-DG together. Data are presented as means plus SEM of three (*ETV6-RUNX1+* and Tetraploid) or two independent experiments (*p<0.05, **p<0.01).

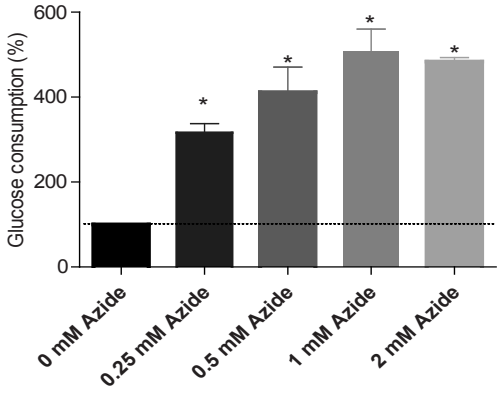
Supplemental Figure 14.



Supplemental Figure 14.

MCL1 silencing together with glycolysis inhibition concomitantly reverses drug resistance. Sensitivity of distinct leukemic cell lines to prednisolone after treatment with mock LNA control, MCL1 LNA –a and LNA-b and 0.5mM 2-DG alone or in combination was measured in a 3-day MTT assay. Sensitivity was corrected for cell death induced by *MCL1* knockdown and 0.5mM 2-DG itself in the absence of prednisolone. Data are presented as means plus SEM of three or two independent experiments (*p<0.05, **p<0.01, ***p<0.001)

Supplemental Figure 15.



Supplemental Figure 15.

Inhibition of oxidative phosphorylation by Azide augments glycolysis.

Glucose consumption was calculated after treatment with a concentration range of Azide, an inhibitor of oxidative phosphorylation, relative to untreated *ETV6-RUNX1*+ BCP-ALL cells (REH), and corrected for cell growth. Data are presented as means plus SEM of two independent experiments (*p<0.05).

EMP1, A NOVEL POOR PROGNOSTIC FACTOR IN PEDIATRIC LEUKEMIA REGULATES PREDNISOLONE RESISTANCE, CELL PROLIFERATION, MIGRATION AND ADHESION

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Submitted

ABSTRACT

Still 20% of pediatric precursor-B acute lymphoblastic leukemia (BCP-ALL) relapse on or after current treatment strategies. Treatment failure is associated with resistance to prednisolone. We aim to find new druggable targets that modulate prednisolone resistance. We generated microarray gene expression profiles of 256 pediatric ALL patient samples and identified a 3.4-fold increase in epithelial membrane protein 1 (*EMP1)* expression in *in vitro* prednisolone-resistant compared to -sensitive patients (p=0.003). EMP1-silencing in six BCP-ALL and T-ALL cell lines induced apoptosis and cell cycle arrest leading to 84.1±4.5% reduction in survival compared to non-silencing control transduced cells (shNSC) (p=0.014). Moreover, EMP1-silencing sensitized to prednisolone up to 8.8-fold (p<0.001). EMP1-silencing also abrogated migration and adhesion to mesenchymal stromal cells (MSCs) by 78.3±9.0% and 29.3±4.1% compared to shNSC (p<0.05). We discovered that EMP1 contributes to MSC-mediated prednisolone resistance. Pathway analysis indicated that EMP1 signals through the Src kinase family. *EMP1*-high BCP-ALL patients showed a poorer 5-year event-free survival compared to *EMP1*-low patients (77±2% vs. 89±2%, p=0.003). Multivariate analysis identified *EMP1* as an independent predictor for poor outcome in BCP-ALL (p=0.004, Hazard ratio:2.36(1.31-4.25). This study provides preclinical evidence that *EMP1* is an interesting target to develop drugs for to optimize treatment of BCP-ALL.

INTRODUCTION

Survival rates of pediatric acute lymphoblastic leukemia (ALL), the most common pediatric cancer, have improved enormously over the past decades. However, still 20% of B-cell precursor ALL (BCP-ALL) and 25% of T-ALL patients relapse during or shortly after completion of contemporary treatment protocols for newly diagnosed pediatric ALL [1]. Prednisolone is the spearhead drug used in multi-drug treatment of pediatric ALL and *in vivo* and *in vitro* response to prednisolone predicts long-term clinical outcome [2]. Moreover, relapsed ALL patients acquire prednisolone resistance disproportional to other anti-leukemic agents [3]. It is therefore necessary to find new druggable targets to reverse prednisolone resistance.

Prednisolone, a glucocorticoid, diffuses passively into the cell, where it binds the glucocorticoid receptor (GR). Consequently, GR chaperone proteins are released whereafter GR dimers can be formed. Dimerized GR acts as a transcription factor by either binding to positive or negative glucocorticoid response elements (GRE) in the DNA, or by binding to other transcription factors, such as NF-κB or AP-1 [4]. As a result a large number of genes are either turned on or off. Due to this complexity still little is known about the exact cause of resistance to glucocorticoids.

To examine the gene expression pattern that might cause prednisolone resistance in more detail, we performed microarray analysis of 256 children with newly diagnosed ALL that were tested *in vitro* to be either prednisolone sensitive, intermediate resistant or resistant. We identified epithelial membrane protein 1 (EMP1) as one of the most discriminative genes. EMP1 is a small hydrophobic four-transmembrane glycoprotein of 160 amino acids [5]. EMP1 has been implicated as a cell junction protein at the plasma membrane [6,7], however little is known about the exact function. It has been shown to be involved in cell proliferation, is identified as a c-Myc target [8], is correlated to brain [9] and breast carcinogenesis [10], and was demonstrated to be a biomarker of gefitinib resistance in non-small lung carcinoma [11].

We are the first to identify a significant role for EMP1 in pediatric ALL. We have discovered that silencing of EMP1 inhibited leukemic survival, sensitized leukemic cells to prednisolone and abrogated migration and adhesion to mesenchymal stromal cells (MSCs). Moreover, we identified EMP1 as an independent predictor for poor outcome in BCP-ALL. Taken together, these results point to EMP1 as a potential new druggable target to tackle leukemia and potentially increase current survival rates.

MATERIALS AND METHODS

Cell culture and Primary cells

Bone marrow and peripheral blood samples were collected from children alleged to suffer from newly diagnosed ALL and before start of initial therapy. Normal bone marrow samples were obtained from children who were suspected for a malignancy but turned out to be negative for a hematological disorder. Informed consent was given by patients or parents/guidance to use excess of diagnostic material for research purposes as approved by the local institutional review board. Mononuclear cells were isolated by lymphoprep density gradient centrifugation, as previously described [12,13]. Only samples with ≥90% leukemic blasts upon processing were used in the present study. The genetic subtype of each patient, i.e. hyperdiploid (>50 chromosomes), *ETV6-RUNX1*$^{+}$, *TCF3-PBX1*$^{+}$, MLL-rearrangement, *BCR-ABL1*$^{+}$, *BCR-ABL1*-like, or B-other (negative for aforementioned genomic lesions) was determined by means of FISH, PCR and by utilizing the 110-probeset classifier [14]. An MTT-assay (see below for details) was used to select cases being either *in vitro* highly sensitive, intermediate resistant or highly resistant to prednisolone; 74, 60 and 42 of BCP-ALL cases and 27, 34 and 19 T-ALL respectively, overall 256 samples. In total, data obtained from 373 BCP-ALL and 116 T-ALL cases were included to study the prognostic value of EMP1 expression levels.

All leukemic cell lines and HEK293T cells were obtained from DSMZ. Reh, 697, NALM6 and SEM are BCP-ALL cell lines with an *ETV6-RUNX1*, *TCF3-PBX1*, t(5;12) and an *MLL-AF4* rearrangement, respectively. Jurkat is a tetraploid T-ALL and SupT1 a T-lymphoblast cell line. All cell lines were resistant to prednisolone as determined by an MTS-assay. HEK293T, a human embryonal kidney cell line, was used for the production of viral particles. hMSC-TERT cells are human mesenchymal stromal cells modified with an increased telomerase activity and were a gift of Dr. D. Campana, St. Jude Childrens' Research Hospital, Memphis, USA [15]. Cell viability and cell count were determined by a trypan blue exclusion staining assay and analyzed by MACSQuant flow cytometer (Miltenyi Biotec).

Microarrays

RNA was extracted by means of Trizol isolation (Invitrogen) according to the manufacturer's protocol and RNA quality and integrity determined with the 2100 bioanalyzer (Agilent). The Affymetrix One-Cycle cDNA Synthesis kit and the GeneChip IVT Labeling kit were used to synthesize cRNA. RNA processing and hybridization to the Affymetrix U133 Plus 2.0 GeneChip oligonucleotide microarray were performed according to the manufacturer's protocol. Gene-expression

values were calculated with Affymetrix Microarray Suite version 5.0. Expression signals were scaled to the target intensity of 500 and log-transformed. Only arrays with scaling factor <10 and GAPDH cRNA integrity (3'/5') <3 were used for subsequent analysis. All arrays were Robust Multichip Average (RMA) and variance stabilization and normalization 2 (VSN2) normalized[16] and differentially expressed genes between *in vitro* prednisolone resistant and prednisolone sensitive patients were identified with Limma R Package taking along prednisolone intermediate patients and using subtype as a confounder in the statistical environment R, version 2.15.0. Correction for multiple tests was performed according to the false discovery rate (FDR) method [17]. All EMP1 probe sets were statistically differentially expressed between *in vitro* prednisolone sensitive and resistant patients. Probeset 201324_at was used for further analysis, as this probeset showed the most reliable differential expression between *in vitro* prednisolone sensitive and resistant BCP-ALL patients.

Lentiviral production and Infection

pLKO.1 Mission short hairpin RNA (shRNA) vectors were purchased from Sigma-Aldrich, i.e. a non-silencing control SHC002 (shNSC) and TRCN0000117944 against *EMP1* (sh*EMP1*). Lentiviral helper pMD2.G (Addgene plasmid 12259) and psPAX2 (Addgene plasmid 12260) were provided by Prof. D. Trono (Geneva, Switzerland). Infection occurred during 45' 1800 rpm spin-oculation of $0.5x10^6$ cells/ml with 2.5 transducing units/cell (Sigma). After 24h, infected cells were positively selected for puromycin resistance.

Quantitative RT-PCR

RNA was extracted using a Rneasy minikit (Qiagen) according to the manufacturer's protocol, whereafter cDNA was synthesized. *EMP1* mRNA levels were quantified by incorporation of SYBR Green (Thermo Scientific) by quantitative real-time PCR (Applied Biosystems 7900HT). Primers for *EMP1* were; 5'-TTGCTGGCTGGTATCTTT-3' (forward) and 5'-TTGAGGGCATCTTCACTG-3' (reverse). Primers used for the reference gene *RPS20*, were 5'-AAGGGCTGAGGATTTTTG-3' (forward) and 5'-CGTTGCGGCTTGTTAG-3' (reverse).

Protein isolation and Western blot analysis

Proteins were isolated with protein lysis buffer. Subsequently the supernatant was used for the luminex (see below) and the remaining pellet harboring membrane proteins was sonicated on ice (Branson Sonifier 250) in Laemmli Sample Buffer. Protein concentrations were quantified with the 2-D Quant Assay (Amersham Biosciences). Protein (25 µg) was loaded on Bio-Rad Mini-Protean gels (TGX

pre-cast anyKD) and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked with 5% BSA and probed with 1:200 anti-EMP1 (Santa Cruz, sc-50467) and anti-β-Actin 1:20,000 (Abcam, ab6276) in 5% BSA, followed by IRDye 800CW- and IRDye 680CW-labeled secondary antibody (Li-COR) respectively. Protein levels were quantified using the Odyssey 3.0 application software (Li-COR).

Luminex

Protein phosphorylation was determined with the 9-plex Multi-Pathway Magnetic Bead Panel (Millipore #46-680MAG) and Milliplex 8-plex Human Src Family kinase kit (Millipore #48-650MAG) following the manufacturer's protocol. To both kits β-Tubulin beads (Millipore #64-713MAG) and GAPDH beads (Millipore #46-667MAG) were added to correct for protein load.

Apoptosis measurement

Cell viability was assessed with an Annexin V/Propidium iodide (PI) staining. Fractions of Annexin V/PI double-positive and AnnexinV single-positive cells were quantified on a MACSQuant flow cytometer (Miltenyi Biotec).

Cell cycle distribution assay

Cell cycle distribution was analyzed using the FITC BrdU Flow Kit (B&D Pharmingen) following manufacturer's instructions.

In vitro drug-resistance assay

Cellular cytotoxicity of prednisolone (Bufa Pharmaceutical Products) was determined by the *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for patients´ samples and the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay for cell lines [2,13].

Cell adhesion assay

Subconfluent (80%) hMSC-TERT were irradiated with 30 Gy and either co-cultured in a 6-well plate with $2x10^6$ Maroon labeled (Cellvue, eBiosciences) Reh cells in plain AIMV (Gibco) for 24h in the case of flowsort experiments, or overnight in a 24-wells plate with $0.4x10^6$ leukemic cells in plain AIMV (Gibco) in the case of adhesion experiments of EMP1-silenced cells. Hereafter, non-adherent leukemic cells were harvested from the supernatant (suspension cells), and adherent leukemic cells were harvested by trypsinizing the supernatant-depleted hMSC-TERT layer with Trypsin-EDTA (Gibco) for 5 min at 37°C, 5% CO_2. Suspension and

adherent cells were quantified both by trypan blue exclusion assays and by the MACSQuant flow cytometer. In the case of the flowsort experiment, the adherent Maroon-labeled leukemic cells were separated from unlabeled hMSC-TERT cells by fluorescence activated cell sorting (BD Biosciences FACSAria cell sorter) and total RNA of the suspension and adherent cells was isolated.

Transwell migration assay

Subconfluent hMSC-TERT layers were cultured in a 24-wells plate at 37°C, 5% CO_2, whereafter they were irradiated with 30 Gy and placed on plain AIMV (Gibco) 48h prior to migration assay. In indicated experiments, medium was collected from hMSC-TERT layers after 48h of culture and was subsequently used as conditioned medium. Maroon-labeled leukemic cells ($4x10^5$) were transferred on top of a 3 µm pore polycarbonate membrane insert. In this transwell assay, leukemic cells were allowed to migrate overnight at 37°C in 5% CO_2 to the bottom compartment which contained either a hMSC-TERT layer, or conditioned medium collected from hMSC-TERT after 48h or plain AIMV medium. Subsequently, the amount of leukemic cells in the bottom compartment was quantified by both trypan blue exclusion assays and by the MACSQuant flow cytometer (Miltenyi Biotec).

MSCs and leukemic cells co-culture experiments

Leukemic cells ($4x10^5$) alone (condition 1) or co-cultured on top of a 80% subconfluent 30 Gy irradiated hMSC-TERT layer (condition 2) were cultured in 24-wells plates in RPMI 10% FCS with or without 0.488 ug/ml prednisolone for 3 days. Hereafter, leukemic cells in condition 1 were directly harvested from the culture medium. In condition 2, the non-adherent leukemic cells were collected from the supernatant, whereafter the adherent leukemic cells were harvested by trypsinizing the supernatant-depleted hMSC-TERT layer. Suspension and adherent cells were counted by trypan blue exclusion assays and the MACSQuant flow cytometer. To determine whether hMSC-TERT cells consume prednisolone, and thereby lower the available prednisolone-concentration in leukemia co-culture experiments, the hMSC-TERT layer was cultured with 0.488 µg/ml prednisolone at 37°C, 5% CO_2. After 3 days this MSC-derived prednisolone medium was collected and transferred to leukemic cells. In parallel, leukemic cells were exposed to prednisolone exposed to 37°C, 5% CO_2 for 3 days (similar to the MSC-medium), and to a freshly made dilution of prednisolone. After 3 days, the cytotoxic effect of prednisolone in these three media-conditions was compared by an MTS assay.

Statistical Analysis

Spearman's rank correlation coefficient was calculated to compare microarray gene expression results to qRT-PCR results. Data from other experiments were compared in unpaired *T* tests and Mann-Whitney *U* tests, $p<0.05$ was considered statistically significant. The dose-response curves of prednisolone in combination with shNSC compared to shEMP1 was analyzed by repeated measurement one-way ANOVA, testing the interaction between shRNA*prednisolone. Cumulative incidence of relapse (CIR) was calculated as time to relapse and non-response with death as a competing event in the statistical environment R version 2.15.0 using the method of Fine and Gray [18] with the software packages mstate 0.2.6 [19] and cmprsk 2.2-2 [20]. Event-free survival (EFS) was calculated as time to relapse, non-response, secondary malignancy and death in IBM SPPS Statistics 20 using Kaplan-Meier analysis and Cox proportional hazard analyses. Univariate and multivariate analysis on event-free survival were performed in IBM SPPS Statistics 20 using Cox proportional hazard analyses which were stratified for treatment protocol.

RESULTS

EMP1 expression is increased in prednisolone resistant leukemic cells

Gene expression profiling revealed that the mRNA levels of *EMP1* was median 3.4-fold higher in leukemic cells of 74 prednisolone resistant compared to 42 sensitive BCP-ALL patients calculated with limma taking along 60 prednisolone intermediate resistant patients and subtype as confounder ($p=0.003$, Supplemental Table S1). We verified this with qRT-PCR in 23 BCP-ALL patients (Figure 1A). qRT-PCR and microarray mRNA expression levels correlated significantly (Spearman $R=0.88$ $p<0.0001$ Figure 1A) and qRT-PCR confirmed that *EMP1* was higher expressed in prednisolone resistant patients compared to sensitive patients (Figure 1B $p<0.0001$). Moreover, *EMP1* expression was higher in BCP-ALL compared to normal bone marrow-derived mononuclear cells ($p<0.03$, Figure 1B). Furthermore, *EMP1* was most differentially expressed in prednisolone-resistant B-other cases (23.5-fold $p=0.003$), followed by *BCRABL1*-like cases (2.2-fold $p=0.04$), *ETV6-RUNX1*$^{+}$-rearranged cases (2.1-fold, $p=0.05$), and hyperdiploid cases (1.9-fold, $p=0.1$, Supplementary Figure S1). In T-ALL patients *EMP1* expression levels differed median 2.0-fold between 27 prednisolone-resistant and 19 sensitive cases ($p=0.04$ Supplementary Figure S1).

Figure 1.

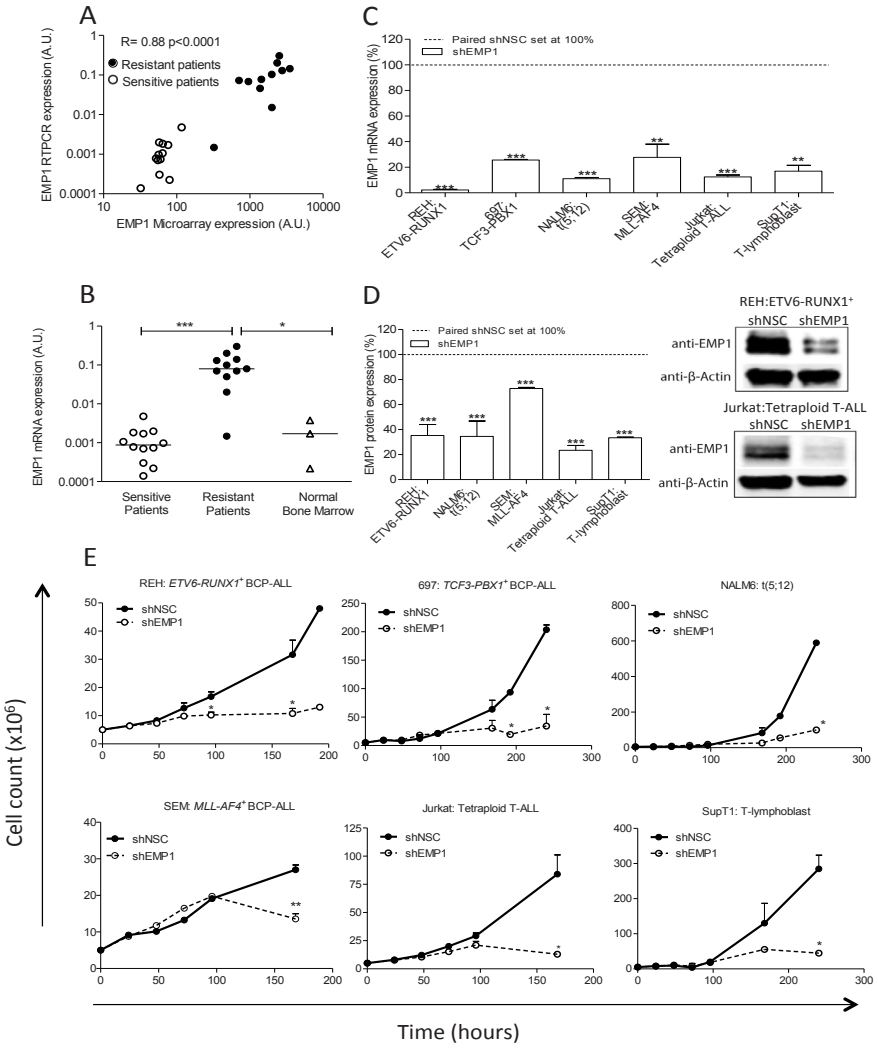

Figure 1. EMP1 expression is increased in prednisolone resistant leukemic cells. shEMP1 efficiently silences the expression of EMP1 and reduces cell proliferation.

(A) Microarray expression (x-axis) was confirmed with qRT-PCR (y-axis). qRT-PCR and microarray RNA expression correlated significantly (Spearman R=0.88 p<0.01). (B) EMP1 mRNA levels in 12 prednisolone sensitive and 11 prednisolone resistant BCP-ALL patients and 3 normal bone marrow samples were analyzed by means of qRT-PCR. (C) EMP1 mRNA levels were measured 168 hours after infection with shEMP1 or a non-silencing control short hairpin (shNSC) in six leukemic cell lines. Values were adjusted for expression of the housekeeping gene RPS20 and are relative to the levels seen in shNSC-transduced cells of each cell line (set to 100%). (D) Protein levels of EMP1 were assessed by Western blot at 168 hours after shEMP1 and shNSC transduction. EMP1 protein levels were corrected for β-actin and are relative to shNSC-transduced cells of each cell line (set to 100%). A representative Western blot for a BCP-ALL cell line, i.e. ETV6-RUNX1+ and a T-ALL cell line, i.e. tetraploid T-ALL, are shown. (E) Cell proliferation of EMP1 silenced cells and shNSC control cells was monitored over time by MACSQuant flow. Data are presented as mean plus SEM of three independent experiments (*p<0.05, **p<0.01, ***p<0.001).

EMP1 silencing increases apoptosis and induces partial cell cycle arrest

To assess the role of EMP1 in prednisolone-resistant ALL, we silenced EMP1 in six different prednisolone-resistant leukemic cell lines, i.e. an *ETV6-RUNX1*$^{+}$ BCP-ALL, a *TCF3-PBX1*$^{+}$ BCP-ALL, an t(5;12) BCP-ALL, an *MLL-AF4*$^{+}$ BCP-ALL, a tetraploid T-ALL and a T-lymphoblast cell line. Robust *EMP-1* knockdown was achieved following lentiviral transduction to levels varying between 2.3%±0.5% and 27.7%±20% of those observed in cells transduced with a non-silencing control short hairpin (shNSC; $p<0.001$ Figure 1C). Available shEMP1 constructs present in the Sigma-Aldrich shRNA library were tested and only one construct provided sufficient knockdown of *EMP1* (Supplemental Figure S2). Protein levels in EMP1 silenced cells were reduced up to 20.2%±3.7% ($p<0.05$) of those observed in shNSC-transduced cells after 168 hours (Figure 1D, Supplemental Figure S3). Silencing of EMP1 decreased cell proliferation in all six cell lines, resulting in a proliferation rate down to 16% of that seen for shNSC-transduced cells ($p<0.05$; Figure 1E). We were unable to study EMP1 knockdown in primary patients' ALL cells, since these primary cells only survive for 96 hours in ex vivo cultures and EMP1 protein knockdown only becomes evident after 168 hours.

Annexin-V/PI staining revealed a significant increase of apoptosis in five out of six cell lines, with a maximum increase of 2.4-fold ($p<0.05$, Figure 2A). BrdU incorporation assays showed a significant reduction of EMP1-silenced cells in S phase in all six cell lines up to 1.7-fold ($p<0.05$ Figure 2B). Four out of six cell lines demonstrated an increase of EMP1-silenced cells in G0/G1-phase and/or G2/M-phase, with a maximum increase of 1.5-fold and 2.1-fold, respectively ($p<0.01$ Figure 2B).

Knockdown of EMP1 sensitizes to prednisolone

We next evaluated whether inhibiting EMP1 expression sensitized leukemic cells towards prednisolone-induced apoptosis. Silencing of EMP1 expression rendered three out of six cell lines more sensitive towards prednisolone-induced apoptosis (Figure 3, Of note: The cytotoxic effect of prednisolone was corrected for death caused by silencing of EMP1 or shNSC.) The cytotoxicity of prednisolone in the other 3 cell lines did not change nor did the cells become more resistant to prednisolone (Figure 3). Silencing of EMP1 resulted in a fold-change reduction in LC50 values for prednisolone by 4.3-fold in the *TCF3-PBX1*$^{+}$ BCP-ALL, by 1.8-fold in Tetraploid T-ALL and by 8.8-fold in the T-lymphoblast cell lines (all $p<0.001$).

EMP1 is important for leukemic cell migration and cell adhesion

Recently, the bone marrow microenvironment was demonstrated to contribute to resistance of leukemic cells to drugs [21,22]. When we co-cultured leukemic cells

Figure 2.

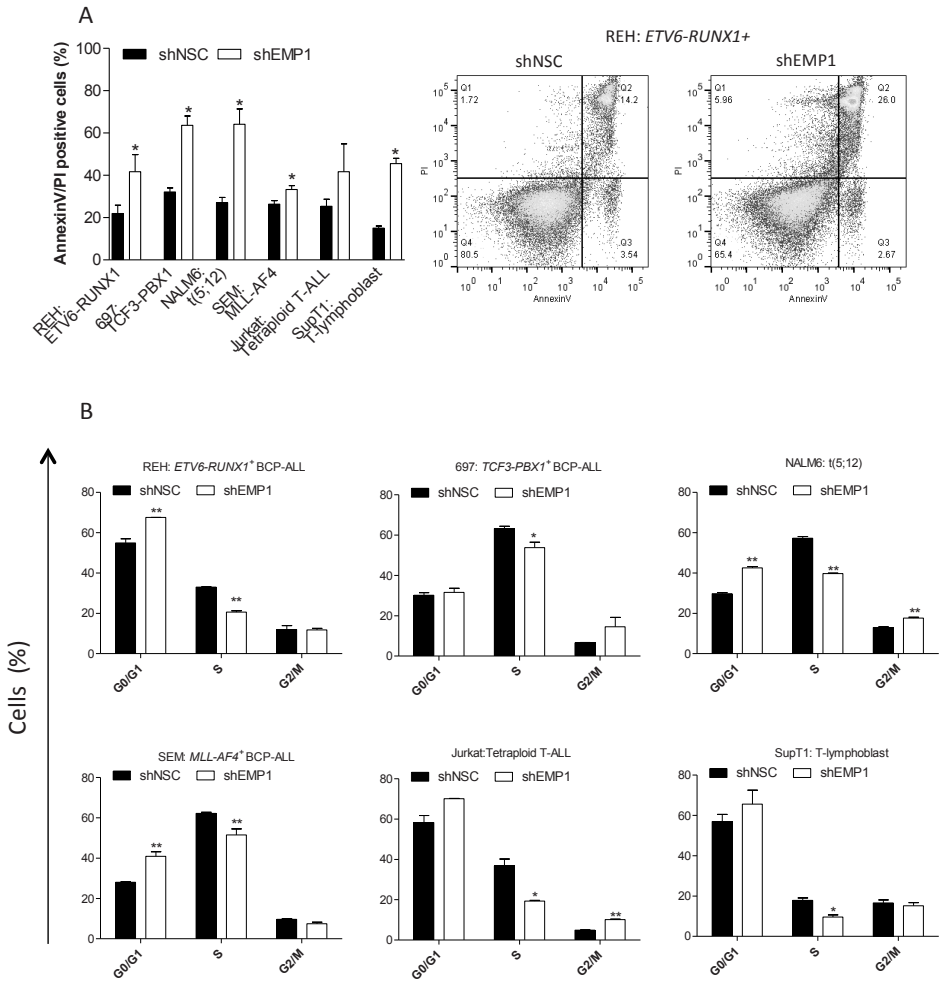

Figure 2. EMP1 silencing increases apoptosis and induces cell cycle arrest.
(A) EMP1 knockdown-induced apoptosis was assessed in six leukemic cell lines 168 hours after transduction with the MACSQuant using an AnnexinV/PI staining. The percentage of apoptotic cells was calculated using the quadrant method. (B) Cell cycle distribution of EMP1-silenced cells and non-silencing control cells was analyzed after 1h BrdU incorporation, followed by BrdU-FITC and 7-AAD staining. The percent of cells in G0/G1, S and G2/M is presented as means plus SEM of three independent experiments (*p<0.05, **p<0.01).

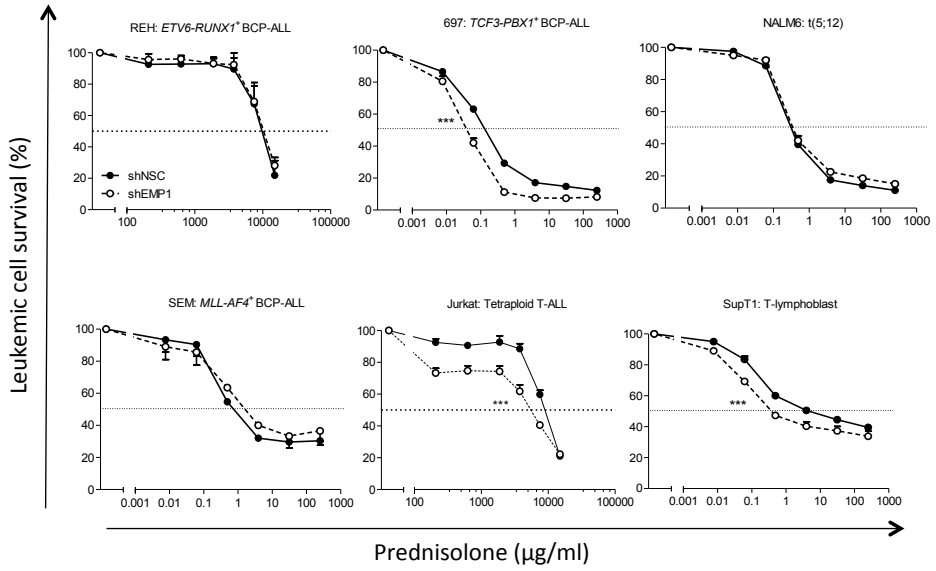

Figure 3. Knockdown of EMP1 sensitizes to prednisolone
Sensitivity to prednisolone after EMP1 knockdown was measured in a 3-day MTS assay. Sensitivity was corrected for cell death induced by EMP1 knockdown itself in the absence of prednisolone. Data are presented as mean plus SEM of three or more independent experiments (repeated measurement one-way ANOVA, ***p<0.001).

with a human mesenchymal stromal cell layer (hMSC-TERT) (Figure 4A), we observed a 4.1-fold increase in *EMP1* mRNA levels in the fraction of leukemic cells that adhered to hMSC-TERT compared to the fraction that remained in suspension (Figure 4B). EMP1 expressions levels returned to normal after the adhered ALL fraction was cultured without hMSC-TERT for 72 hours (Figure 4B). In five out of six cell lines viable EMP1-silenced cells adhered significantly less to hMSC-TERT, up to 29.3%±4.1% reduction, compared to shNSC-transduced control cells ($p<0.01$, Figure 4C).

Next, we evaluated migration of EMP1-silenced cells placed in an upper compartment of a transwell assay (Figure 4E and D). The bottom compartment contained either a hMSC-TERT layer that was pre-incubated with AIMV for 48h or did not contain a hMSC-TERT layer but only the preconditioned AIMV medium which had been collected from hMSC-TERT after 48h of incubation. A bottom compartment with only plain AIMV was used as negative control. Viable EMP1-silenced cells migrated less to the bottom compartment containing the hMSC-TERT layer or the preconditioned medium in all six cell line models, with a

maximal reduction in migration by 78.3%±9.0% in EMP1-silenced cells compared to shNSC-transduced control cells ($p<0.05$, Figure 4D).

Role of EMP1 in bone marrow microenvironment mediated prednisolone resistance

The observation that EMP1 is not only important in prednisolone resistance, but also in leukemic cell migration and adherence to hMSC-TERT prompted us to investigate the role of EMP1 in bone marrow microenvironment mediated prednisolone resistance. We cultured EMP1-silenced cells with or without hMSC-TERT layer and with or without 0.488 µg/ml prednisolone for 3 days. We discovered that an hMSC-TERT layer can rescue shNSC-transduced control cells from prednisolone-induced apoptosis (Figure 4F). This was not caused by prednisolone-induced apoptosis of the MSCs, since hMSC-TERT are prednisolone resistant, nor by reduced availability of prednisolone in the medium due to consumption by hMSC-TERT (Supplemental Figures S4A and B). Most importantly, EMP1 knockdown partly prevented hMSC-TERT mediated prednisolone resistance (Figure 4F).

EMP1 signals through the Src kinase family and further downstream to JNK, STAT3, STAT5, CREB and NF-κB

We next determined which signaling pathways are affected by EMP1. We demonstrate that EMP1 silencing in *ETV6-RUNX1*$^+$ and *MLL-AF4*$^+$ BCP-ALL and Tetraploid T-ALL cell lines decreased phosphorylation of several Src kinases, i.e. Src, Yes, Fgr, Blk and Hck, up to 55% ($p<0.05$ Figure 5A and B). Further downstream, EMP1 silencing resulted in decreased phosphorylation of JNK, STAT3, STAT5, CREB and NF-kB, up to 80% reduction ($p<0.05$ Figure 5A and B). p38 and ERK1/2 phosphorylation levels were below detection level in BCP-ALL cell lines (ND = not detectable).

EMP1 status is an independent prognostic factor for clinical outcome in BCP-ALL

EMP1 mRNA levels were determined by Affymetrix microarrays for 134 BCP-ALL and 60 T-ALL patients treated on the German COALL97-03-protocol, and 239 BCP-ALL and 56 T-ALL patients treated on the Dutch ALL10 (DCOG) protocol (Supplemental Table S1). *EMP1* expression status (i.e. low or high expression levels) was based on the median cut-off value of mRNA levels detected by microarrays among a batch of BCP-ALL and a batch of T-ALL cases.

In the combined analysis of the COALL and DCOG patients stratified for treatment protocol, *EMP1*-high BCP-ALL (n=187) patients had a significantly poorer event free survival (EFS) compared to *EMP1*-low BCP-ALL (n=186) patients

Figure 4

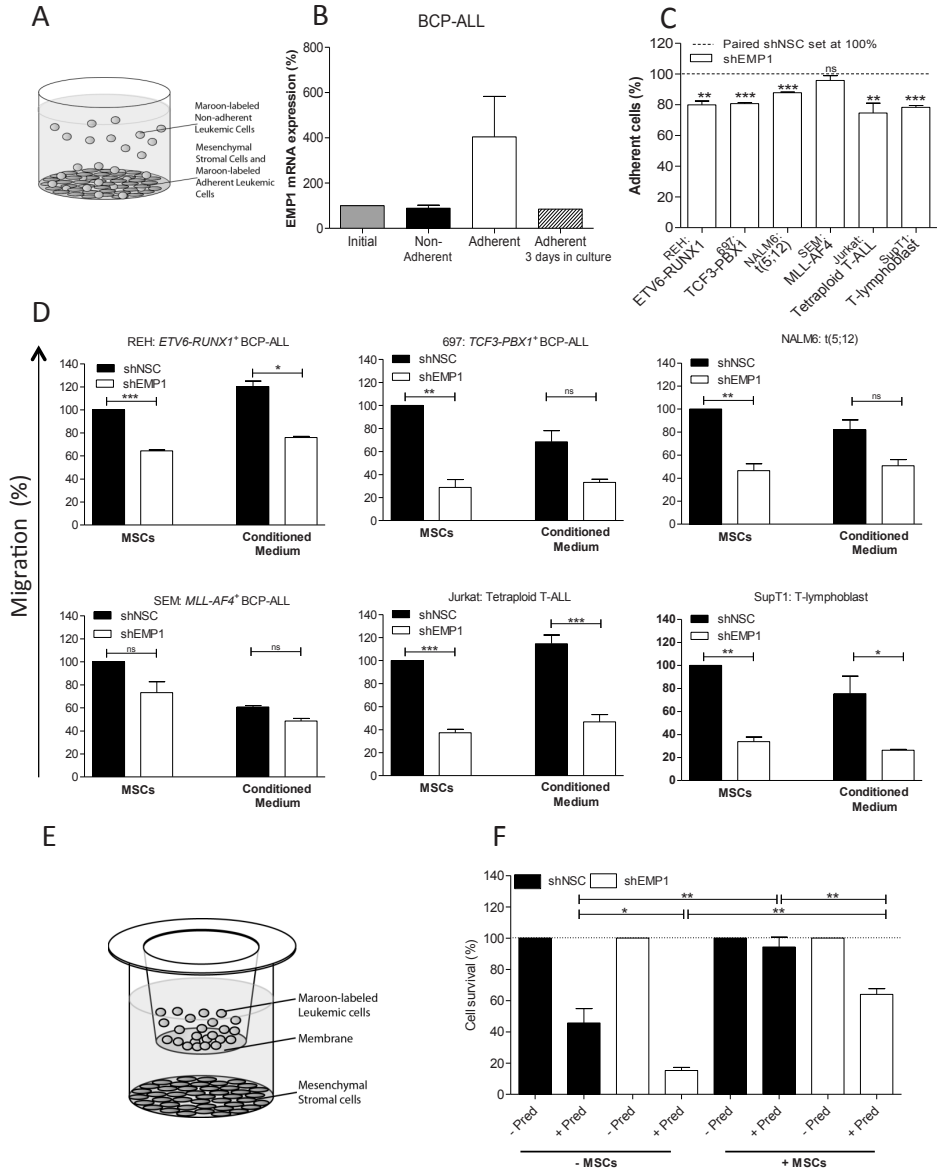
A
Maroon-labeled Non-adherent Leukemic Cells
Mesenchymal Stromal Cells and Maroon-labeled Adherent Leukemic Cells
B
BCP-ALL
EMP1 mRNA expression (%)
Initial
Non-Adherent
Adherent
Adherent 3 days in culture
C
Paired shNSC set at 100%
shEMP1
Adherent cells (%)
REH: ETV6-RUNX1
697: TCF3-PBX1
NALM6: t(5;12)
SEM: MLL-AF4
Jurkat: Tetraploid T-ALL
SupT1: T-lymphoblast
D
Migration (%)
REH: ETV6-RUNX1+ BCP-ALL
697: TCF3-PBX1+ BCP-ALL
NALM6: t(5;12)
SEM: MLL-AF4+ BCP-ALL
Jurkat: Tetraploid T-ALL
SupT1: T-lymphoblast
shNSC
shEMP1
MSCs
Conditioned Medium
E
Maroon-labeled Leukemic cells
Membrane
Mesenchymal Stromal cells
F
Cell survival (%)
shNSC
shEMP1
- Pred
+ Pred
- MSCs
+ MSCs

Figure 4. EMP1 is important in leukemic cell migration, adhesion to mesenchymal stromal cells and might be important in bone marrow microenvironment mediated prednisolone resistance.

(A-B) Maroon labeled *ETV6-RUNX1+* leukemic cells were cocultured with hMSC-TERT for 24h. EMP1 mRNA levels were measured in non-adherent leukemic cells and adherent leukemic cells that were separated from hMSC-TERT by fluorescence activated cell sorting. Separated adherent leukemic cells were subsequently cultured without hMSC-TERT for 72 hours. (C) Leukemic cells were cocultured on an MSC layer overnight, whereafter only viable non-adherent and adherent cells were quantified by means of the trypan blue exclusion assay and MACSQuant flow cytometer. (D-E) Non-silencing control leukemic cells or EMP1-silenced leukemic cells were placed in AIMV medium into an upper compartment of a transwell assay and allowed to migrate to a lower compartment containing hMSC-TERT preconditioned for 48h in AIMV, to a lower compartment with only preconditioned AIMV medium taken from MSCs or to plain AIMV. Migration of viable cells was assessed by trypan blue exclussion assay and MACSQuant flow cytometer. (F) Non-silencing control leukemic cells and EMP1-silenced leukemic cells were cultured for three days with or without MSCs and with or without 0.488 µg/ml prednisolone. Hereafter, leukemic cells were harvested and cell survival was assessed by trypan blue exclusion assay and MACSQuant flow. Data are presented as mean plus SEM of three or more independent experiments (*p<0.05, **p<0.01, ***p<0.001).

with 5-year EFS rates of 77%±3% and 89%±2%, respectively (p<0.004, Figure 6A and Table 1). Cumulative incidence of relapse (CIR) analysis demonstrated a significant higher relapse and non-response risk for *EMP1*-high than *EMP1*-low cases in BCP-ALL (5-year CIR: 17%±3% vs. 9%±2% p=0.02, Figure 6B). EFS and CIR curves per treatment protocol are depicted in Supplemental Figure S5. To evaluate the independent prognostic value of *EMP1*, we performed Cox's multivariate regression analysis on all 373 BCP-ALL patients stratified according to treatment protocol using white blood cell count, age and *EMP1* expression status as covariates. We identified *EMP1*-high expression levels at diagnosis as

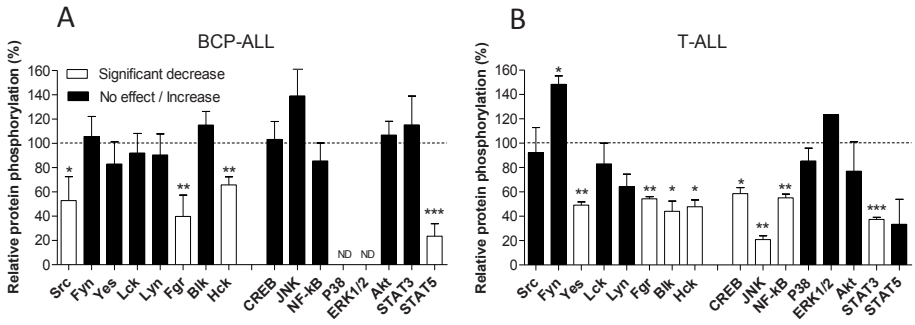

Figure 5. EMP1 signals through the Src kinase family to activate JNK, STAT3, STAT5, CREB and NF-κB.

(A-B) Phosphorylation levels of Src kinase family members and several key-members of important cellular pathways were determined by means of a luminex assay in protein harvested from non-silencing control cells and EMP1-silenced cells at 168 hours after infection. Phosphorylation levels in shNSC transduced control cells were put to 100% (dashed line). Phosphorylation levels were corrected for protein load by GAPDH and β-tubulin levels in the same sample. Data are presented as mean plus SEM of REH and SEM BCP-ALL cell lines (n=4) (A) and Jurkat T-ALL cell line experiments (n=2) (B) (*p<0.05, **p<0.01, ***p<0.001). ND: not detectable, P38 and ERK1/2 measurements in BCP-ALL were below the background level

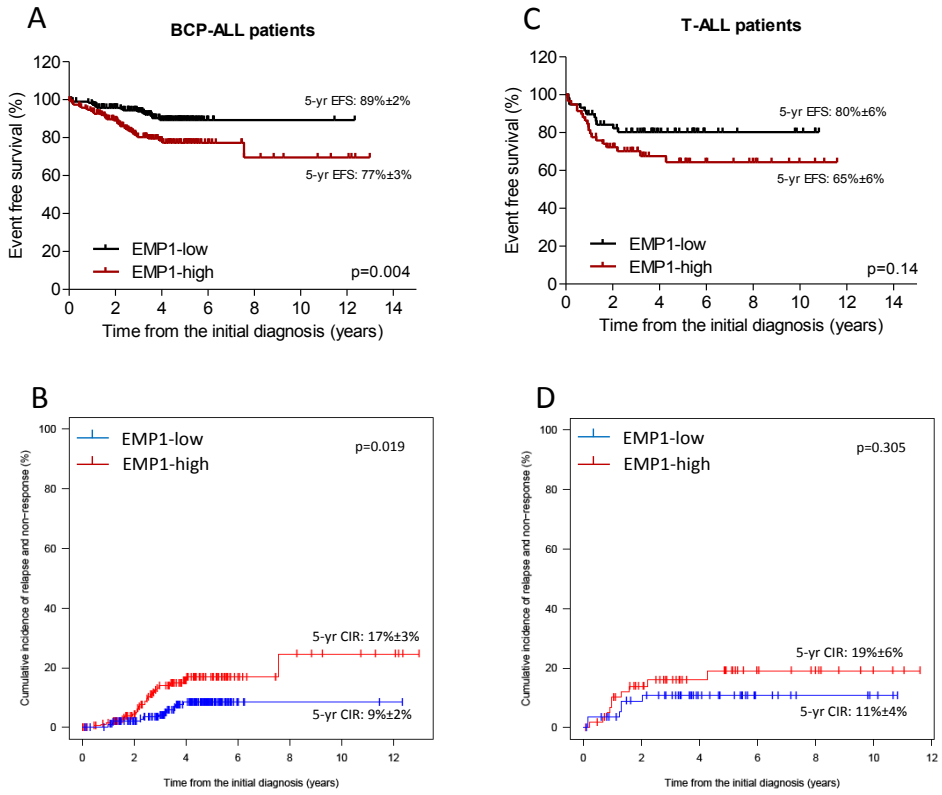

Figure 6. EMP1 status is an independent prognostic factor in BCP-ALL.
EMP1 status was based on the median cut-off value of microarray mRNA expression levels seen in 373 BCP-ALL (panel A and B) and 116 T-ALL (panel C and D). Event-free survival (EFS) (upper panels) and cumulative incidence of relapse and non-response (CIR) (lower panels) was calculated. Event-free survival (EFS) was calculated as time to relapse, non-response, secondary malignancy and death using Kaplan-Meier analysis and Cox proportional hazard analyses. Cumulative incidence of relapse (CIR) was calculated as time to relapse and non-response with death as a competing event using the method of Fine and Gray [18].

an independent predictor ($p<0.01$) for poor outcome in BCP-ALL with a hazard-ratio of 2.36 (95% confidence interval: 1.31–4.25, Table 1). The poor prognosis of *EMP1*-high expressing BCP-ALL patients at diagnosis is independent of *in vitro* and *in vivo* prednisolone response (Supplemental Table S1) and subtype of ALL (Supplemental Table S2).

Although we found a similar functional role for EMP1 in T-ALL cell line models and a trend for poorer outcome of *EMP1*-high cases in the T-ALL cohort, this was not statistically significant neither in a treatment-protocol stratified analysis (Figure 6C/D, Table 1B) nor in each of the protocols separately (Supplemental Figure S5, Supplemental Table S2 and S3).

Table 1. Univariate and multivariate analysis of EMP1 status and event-free survival.
Univariate and multivariate analysis of the indicated prognostic factors stratified according to treatment protocol (DCOG: Dutch, COALL: German) were performed by a Cox regression model in BCP-ALL (A) and T-ALL (B).

A

BCP-ALL						
			Univariate Analysis		Multivariate Analysis	
	Patients	Events	HR (95%-CI)	p-value	HR (95%-CI)	p-value
***EMP1* expression (mRNA)**						
<Median	186	16				
≥Median	187	37	2.42(1.34-4.35)	<0.01	2.36(1.31-4.25)	<0.01
Age at diagnosis (years)						
Low <10	255	35				
High ≥10	118	18	1.50(0.84-2.67)	0.17	1.44(0.80-2.56)	0.22
WBC count (x 10^9/ L)						
Low < 50	288	37				
High ≥ 50	85	16	1.58(0.88-2.83)	0.13	1.46(0.81-2.63	0.21

B

T-ALL						
			Univariate Analysis		Multivariate Analysis	
	Patients	Events	HR (95%-CI)	p-value	HR (95%-CI)	p-value
***EMP1* expression (mRNA)**						
<Median	58	11				
≥Median	58	19	1.75(0.83-3.68)	0.14	1.76(0.83-3.71)	0.14
Age at diagnosis (years)						
Low <10	63	16				
High >10	53	14	1.09(0.53-2.25)	0.81	1.10(0.53-2.25)	0.80
WBC count (x 10^9/ L)						
Low < 50	36	8				
High ≥ 50	79	22	1.17(0.52-2.64)	0.71	1.09(0.48-2.48)	0.83

DISCUSSION

Prednisolone, a glucocorticoid, regulates transcription of numerous genes eventually leading to cell cycle arrest and apoptosis in lymphocytes. These properties make glucocorticoids pivotal in the treatment of hematologic malignancies and have led to their inclusion in all chemotherapy protocols for lymphoid malignancies [23]. Elucidating causes of resistance to prednisolone remains however

challenging due to the multifactorial processes in which these glucocorticoids are involved.

In this study, we identified a significant overexpression of *EMP1* mRNA in leukemic cells taken from prednisolone resistant ALL patients. We show that knockdown of EMP1 moderately sensitized leukemic cells to prednisolone. Interestingly, a link between glucocorticoids and EMP1 family members has been shown before, as glucocorticoids can regulate the promoter activity of the homologues family members PMP22 and EMP2 [24,25]. We show that EMP1 is important for leukemic cell survival, as silencing of EMP1 results into cell cycle arrest and apoptosis. In line with this are several studies associating *EMP1* expression levels with cell cycle and cell survival [5,26,27]. The observed role of EMP1 in migration and adhesion of leukemic cells is supported by the notion that EMP1 regulates cell-cell contact [6,7], and contributes to integrin mediated leukocyte migration and adhesion [11]. These findings are important in relation to the tumor microenvironment, as this plays a significant role in leukemic cell survival and in chemotherapy resistance. Chemokines secreted by MSCs, such as stem cell factor 1 and CXCL12 stimulate leukemic cell homing and survival [28,29]. MSCs can also induce leukemic drug resistance through upregulation of the voltage-dependant channel hERG1 [21], or rescue cells from L-asparaginase by secreting asparagine [22]. Furthermore, sequestration of ALL cells by MSCs may provide a physical barrier for chemotherapeutics. We show here that MSCs can protect leukemic cells against prednisolone-induced apoptosis and, moreover, that silencing of EMP1 reduces the protection by MSCs. These data suggest that EMP1 contributes to microenvironment-induced prednisolone resistance.

We furthermore determined which signaling pathways underlie EMP1 mediated effects. Recent studies in non-small lung carcinoma suggest that EMP1 increases EGFR signaling [11] , and showed that EMP1 activates the PI3K/AKT pathway [30]. We did, however, not find an association between EMP1 and EGFR signaling (Ariës *et al*, unpublished data), nor could we demonstrate an effect on AKT phosphorylation after EMP1 silencing in ALL (Figure 5). In contrast, we demonstrated that EMP1 mediates its effect through the Src-kinase family, including Src, Fgr, Hck, Yes and Blk. This is in line with data showing that the family member EMP2 promotes Src and FAK phosphorylation [31]. Src kinase family members are oncogenes which are essential in B-cell and T-cell receptor signaling [32,33]. Silencing of EMP1 also decreased the phosphorylation of CREB, NF-kB, JNK, STAT3 and STAT5 in our study. Src kinase activation was previously shown to affect the proliferation and migration of cells via these proteins [34]. Furthermore, JNK and STAT pathways have proven to be important in cell survival of both BCP-ALL and T-ALL[35–37]. We propose the following model; EMP1 most likely serves together

with integrines [11] as an anchor molecule essential for proper function of receptor tyrosine kinases (RTKs). Knockdown of EMP1 probably results in destabilized RTKs, impairing Src kinase phosphorylation and context-dependent downstream signaling to STAT5 in BCP-ALL and JNK, STAT3, CREB and NF-κB in T-ALL, leading to decreased proliferation, migration, adhesion, and a modest decrease in prednisolone resistance. Most importantly, in addition to our functional studies, we identified that *EMP1* expression status is an independent prognostic factor for clinical outcome in BCP-ALL. Although we showed that EMP1 has a functional role in T-ALL cell line models, the *EMP1* expression status was not a strong prognostic indicator in T-ALL patients.

Taken together, this study substantiates an important pathobiological role for EMP1 in ALL. The membrane protein property of EMP1 potentiates it as an interesting candidate for drug-targeting since problems of drug penetration and partitioning in the cytoplasm or elimination of the drug by drug-efflux pumps can be avoided [38]. The development of an EMP1 inhibitory small compound or antibody is desirable and may serve as a potential new therapeutic option for ALL.

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AUTHORSHIP CONTRIBUTIONS

IMA designed and performed experiments, analyzed and interpreted data, and wrote the paper; ISJ and ERD performed experiments; LCJVDB performed flowsort experiment; JMB assisted with the statistical analysis of microarray data; MH and GE provided COALL survival data; RP and MLDB designed research, interpreted data, and revised the paper.

REFERENCES

1. Pui C-H, Carroll WL, Meshinchi S, Arceci RJ. Biology, risk stratification, and therapy of pediatric acute leukemias: an update. *Journal of clinical oncology*. 2011;29(5):551–65.
2. Kaspers GJ, Pieters R, Van Zantwijk CH, et al. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood*. 1998;92(1):259–66.
3. Klumper E, Pieters R, Veerman AJ, et al. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. *Blood*. 1995;86(10):3861–8.
4. Kfir-Erenfeld S, Sionov RV, Spokoini R, Cohen O, Yefenof E. Protein kinase networks regulating glucocorticoid-induced apoptosis of hematopoietic cancer cells: fundamental aspects and practical considerations. *Leukemia & lymphoma*. 2010;51(11):1968–2005.
5. Taylor V, Welcher AA, Program AE, Suter U. Epithelial membrane protein-1, peripheral myelin protein 22, and lens membrane protein 20 define a novel gene family. *The Journal of biological chemistry*. 1995;270(48):28824–33.
6. Bangsow T, Baumann E, Bangsow C, et al. The epithelial membrane protein 1 is a novel tight junction protein of the blood-brain barrier. *Journal of cerebral blood flow and metabolism*. 2008;28(6):1249–60.
7. Lee H-S, Sherley JL, Chen JJW, et al. EMP-1 is a junctional protein in a liver stem cell line and in the liver. *Biochemical and biophysical research communications*. 2005;334(4):996–1003.
8. Ben-Porath I, Yanuka O, Benvenisty N. The tmp gene, encoding a membrane protein, is a c-Myc target with a tumorigenic activity. *Molecular and cellular biology*. 1999;19(5):3529–39.
9. Bredel M, Bredel C, Juric D, et al. Functional network analysis reveals extended gliomagenesis pathway maps and three novel MYC-interacting genes in human gliomas. *Cancer research*. 2005;65(19):8679–89.
10. Turashvili G, Bouchal J, Ehrmann J, et al. Novel immunohistochemical markers for the differentiation of lobular and ductal invasive breast carcinomas. *Biomedical papers*. 2007;151(1):59–64.
11. Jain A, Tindell CA, Laux I, et al. Epithelial membrane protein-1 is a biomarker of gefitinib resistance. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(33):11858–63.
12. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *The New England journal of medicine*. 2004;351(6):533–542.
13. Den Boer ML, Harms DO, Pieters R, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *Journal of clinical oncology*. 2003;21(17):3262–8.
14. Den Boer ML, van Slegtenhorst M, De Menezes RX, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classifi-

cation study. *The lancet oncology*. 2009;10(2):125–34.
15. Mihara K, Imai C, Coustan-Smith E, et al. Development and functional characterization of human bone marrow mesenchymal cells immortalized by enforced expression of telomerase. *British journal of haematology*. 2003;120(5):846–9.
16. Huber W, von Heydebreck A, Sültmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics*. 2002;18 Suppl 1:S96–104.
17. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*. 1995;57(1):289–300.
18. Fine JP, Gray RJ. A Proportional Hazards Model for the Subdistribution of a Competing Risk. *Journal of the American Statistical Association*. 1999;94(June):496–509.
19. De Wreede LC, Fiocco M, Putter H. mstate : An R Package for the Analysis of Competing Risks and Multi-State Models. *Journal of Statistical Software*. 2011;38(7):
20. Gray R. cmprsk: Subdistribution Analysis of Competing Risks. R package version 2.2-2. 2011;
21. Pillozzi S, Masselli M, De Lorenzo E, et al. Chemotherapy resistance in acute lymphoblastic leukemia requires hERG1 channels and is overcome by hERG1 blockers. *Blood*. 2011;117(3):902–14.
22. Iwamoto S, Mihara K, Downing JR, Pui C-H, Campana D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. *The Journal of clinical investigation*. 2007;117(4):1049–57.
23. Sionov RV, Spokoini R, Kfir-Erenfeld S, Cohen O, Yefenof E. Mechanisms regulating the susceptibility of hematopoietic malignancies to glucocorticoid-induced apoptosis. *Advances in cancer research*. 2008;101:127–248.
24. Désarnaud F, Bidichandani S, Patel PI, Baulieu E-E, Schumacher M. Glucocorticosteroids stimulate the activity of the promoters of peripheral myelin protein-22 and protein zero genes in Schwann cells. *Brain Research*. 2000;865(1):12–16.
25. Wadehra M, Mainigi M, Morales SA, et al. Steroid hormone regulation of EMP2 expression and localization in the endometrium. *Reproductive biology and endocrinology*. 2008;6:15.
26. Li Z-Y, Xiong S-H, Hu M, Zhang C-S. Epithelial membrane protein 1 inhibits human spinal chondrocyte differentiation. *The Anatomical record*. 2011;294(6):1015–24.
27. Ben-Porath I, Benvenisty N. Characterization of a tumor-associated gene, a member of a novel family of genes encoding membrane glycoproteins. *Gene*. 1996;183(1-2):69–75.
28. Sison EAR, Brown P. The bone marrow microenvironment and leukemia: biology and therapeutic targeting. *Expert review of hematology*. 2011;4(3):271–83.
29. Masson K, Rönnstrand L. Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3. *Cellular signalling*. 2009;21(12):1717–26.
30. Lai S, Wang G, Cao X, et al. EMP-1 promotes tumorigenesis of NSCLC through PI3K/AKT pathway. *Journal of Huazhong University of Science and Technology. Medical Sciences*. 2012;32(6):834–8.

31. Morales SA, Mareninov S, Coulam P, et al. Functional consequences of interactions between FAK and epithelial membrane protein 2 (EMP2). *Investigative ophthalmology & visual science*. 2009;50(10):4949–56.
32. Gauld SB, Cambier JC. Src-family kinases in B-cell development and signaling. *Oncogene*. 2004;23(48):8001–6.
33. Palacios EH, Weiss A. Function of the Src-family kinases, Lck and Fyn, in T-cell development and activation. *Oncogene*. 2004;23(48):7990–8000.
34. Silva CM. Role of STATs as downstream signal transducers in Src family kinase-mediated tumorigenesis. *Oncogene*. 2004;23(48):8017–23.
35. Quintas-Cardama A, Verstovsek S. Molecular Pathways: JAK/STAT Pathway: Mutations, Inhibitors, and Resistance. *Clinical Cancer Research*. 2013;19(8):1933–1940.
36. Malin S, McManus S, Busslinger M. STAT5 in B cell development and leukemia. *Current opinion in immunology*. 2010;22(2):168–76.
37. Mangolini M, de Boer J, Walf-Vorderwülbecke V, et al. STAT3 mediates oncogenic addiction to TEL-AML1 in t(12;21) acute lymphoblastic leukemia. *Blood*. 2013;122(4):542–9.
38. Pignatello R, Musumeci T, Basile L, Carbone C, Puglisi G. Biomembrane models and drug-biomembrane interaction studies: Involvement in drug design and development. *Journal of pharmacy & bioallied sciences*. 2011;3(1):4–14.

Figure S1.

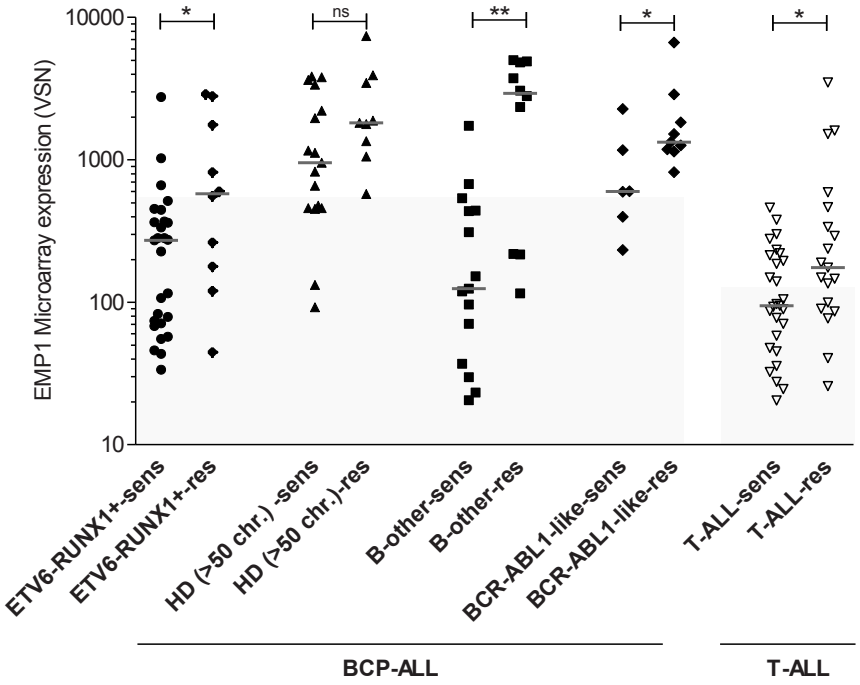

Figure S1. Differential expression of EMP1 between prednisolone resistant and sensitive patients per subtype of ALL. Microarray expression levels of EMP1 probeset 201324_at are compared between *in vitro* prednisolone sensitive (sens) and resistant (res) *ETV6-RUNX1*$^{+}$, hyperdiploid (HD) (>50 chromosomes), *BCR-ABL1*-like, B-other BCP-ALL patients and T-ALL patients. Green block indicates patients with an EMP1 expression below the median. The median expression level differed between BCP and T-ALL cases because these patients were tested in two different batches of microarrays.

Figure S2.

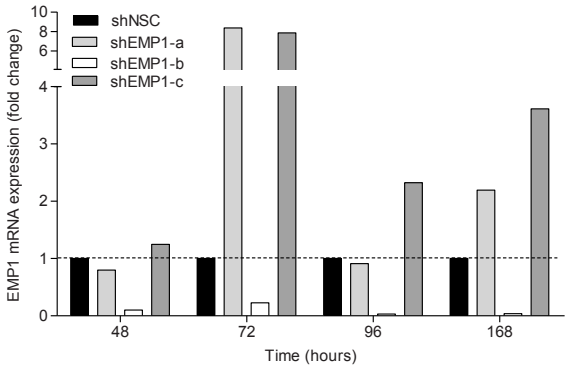

Figure S2. Only shEMP1-b yields sufficient *EMP1* mRNA knockdown.
EMP1 mRNA expression was assessed at different timepoints after transfection with three different shEMP1 constructs available TRCN0000117943 (shEMP1-a), TRCN0000117944 (shEMP1-b) and TRCN0000117945 (shEMP1-c). Only shEMP1-b yielded sufficient knockdown at all timepoints.

Figure S3.

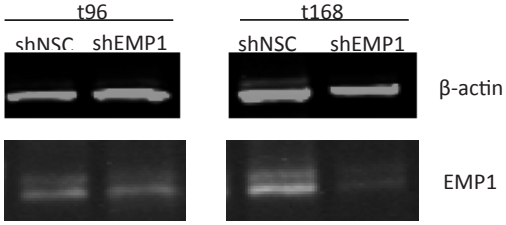

Figure S3. Knockdown of EMP1 protein expression became apparent after 168h.
EMP1 protein expression in NALM6 was assessed after 96h and 168h. Knockdown of EMP1 protein expression became apparent after 168h. We were therefore not able to achieve EMP1 knockdown in primary patients' ALL cells, since these cells only survive for 96 hours.

Figure S4.

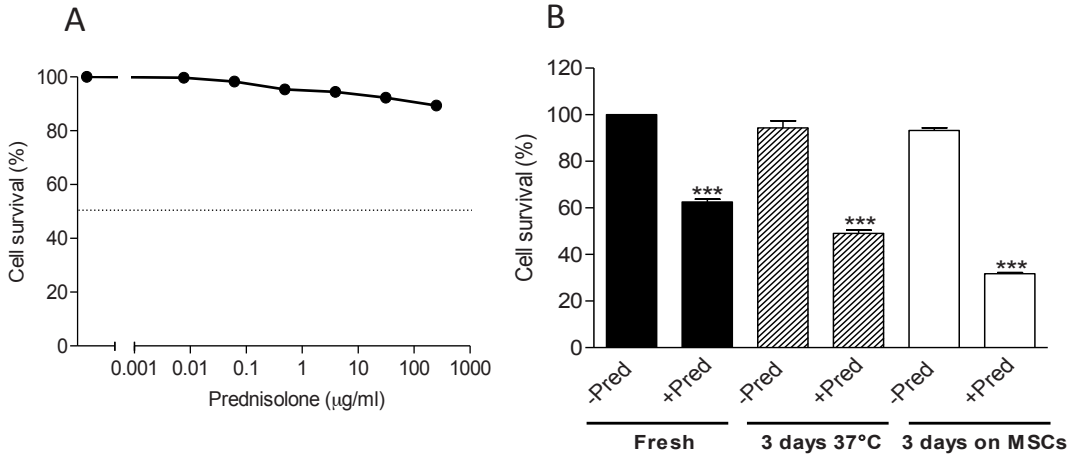

Figure S4. Leukemic cell proliferation, MSCs and prednisolone. (A) Cell survival of hMSC-TERT after 3-day prednisolone exposure determined by an MTS assay. (B) Leukemic cell survival determined by a 3-day MTS assay with either fresh prednisolone in RPMI medium, prednisolone that was incubated for three days at 37 °C in RPMI medium, and prednisolone in RPMI medium that was harvested from hMSC-TERT after three days of incubation at 37 °C . Data are presented as mean plus SEM of three independent experiments (***p<0.001).

Figure S5.

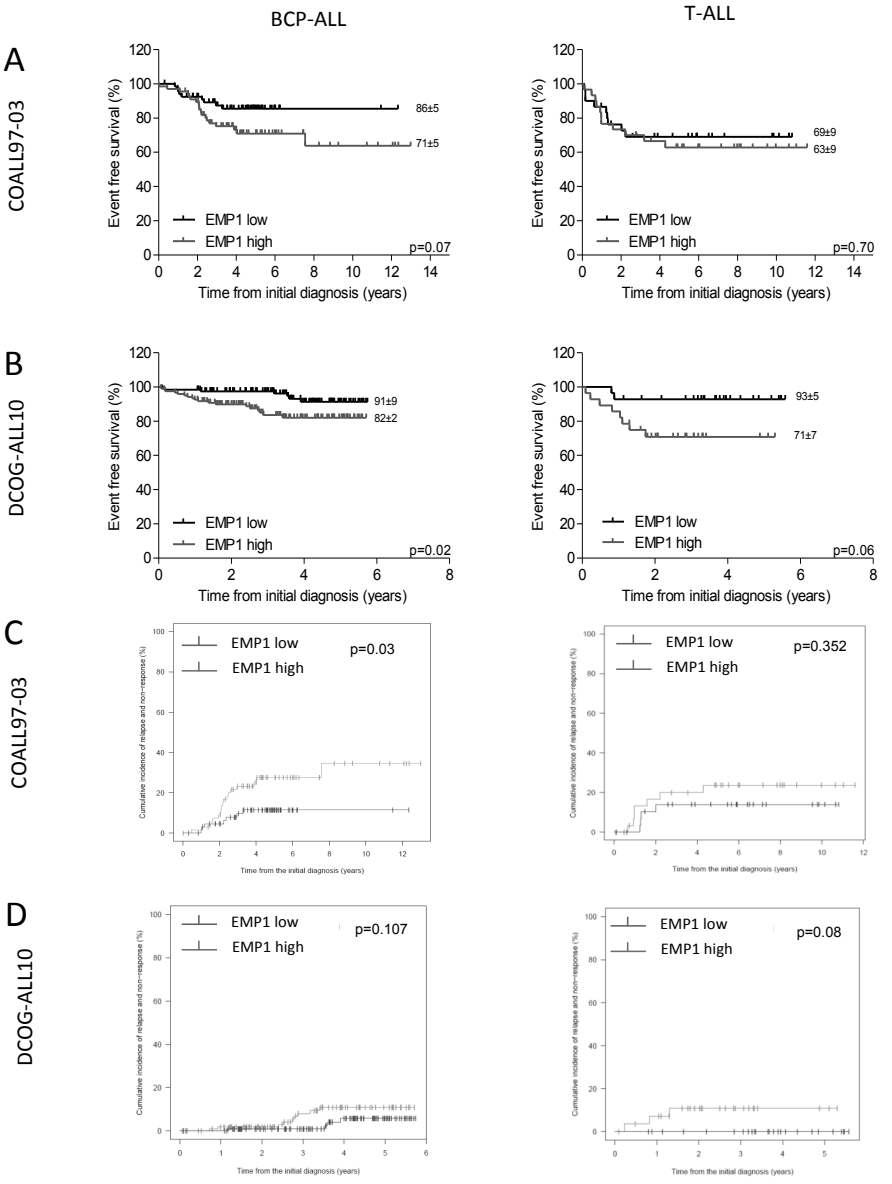

Figure S5. EFS and CIR of *EMP1* high versus *EMP1* low patients per treatment protocol. *EMP1* status was determined by the median value of microarray mRNA expression for 134 BCP-ALL (left) and 60 T-ALL patients (right) treated on the German COALL97-03-protocol (A and C panel), and 249 BCP-ALL (left) and 56 T-ALL (right) patients treated on the Dutch ALL10 protocol (B and D panel). 5-year event-free survival were analyzed using Kaplan-Meier statistics. Cumulative incidence of relapse was calculated with the method of Fine and Gray.

Supplemental Table S1
EMP1 microarray expression of 512 patients used for limma analysis and survival analysis.

Patient Nr.	Protocol	Genetic	*In vitro* Prednisolone	*In vitro* Prednisolone2	*EMP1*	BCP-ALL Limma	Survival
	COALL/ALL10/other	Subtype	LC50	Category	201324_at	analysis	Analysis
1	COALL	HD	0.106	Intermediate	11.09	x	x
2	COALL	HD	2.287	Intermediate	9.95	x	x
3	COALL	T	0.051	Sensitive	7.79		x
4	COALL	BAL	0.337	Intermediate	11.28	x	x
5	COALL	BO	0.334	Intermediate	8.15	x	x
6	COALL	BAL	>250	Resistant	12.70	x	x
7	COALL	ER	0.042	Sensitive	6.74	x	x
8	COALL	T	0.371	Intermediate	6.91		x
9	COALL	BAL	>250	Resistant	10.21	x	x
10	COALL	T	0.412	Intermediate	8.70		x
11	COALL	BO	0.225	Intermediate	11.40	x	x
12	COALL	T	0.310	Intermediate	9.21		x
13	COALL	HD	14.8	Intermediate	9.61	x	x
14	COALL	T	>250	Resistant	7.45		x
15	COALL	T	3.08	Intermediate	8.12		x
16	COALL	MLL	0.283	Intermediate	9.90	x	x
17	COALL	HD	0.450	Intermediate	12.40	x	x
18	COALL	T	0.367	Intermediate	7.25		x
19	COALL	ER	0.020	Sensitive	8.09	x	x
20	COALL	T	0.397	Intermediate	5.94		x
21	COALL	T	0.082	Sensitive	6.44		x
22	COALL	T	>250	Resistant	6.27		x
23	COALL	T	>250	Resistant	6.43		x
24	COALL	T	0.048	Sensitive	6.14		x
25	COALL	T	>250	Resistant	8.40		x
26	COALL	T	>250	Resistant	5.34		x
27	COALL	T	0.098	Sensitive	6.28		x
28	COALL	T	>250	Resistant	10.57		x
29	COALL	HD	>250	Resistant	10.83	x	x
30	COALL	T	0.351	Intermediate	6.66		x
31	COALL	BAL	>250	Resistant	9.68	x	x
32	COALL	T	1.95	Intermediate	7.69		x
33	COALL	T	>250	Resistant	9.21		x
34	COALL	BAL	>250	Resistant	11.49	x	x
35	COALL	BAL	195	Resistant	10.30	x	x
36	COALL	T	29.5	Intermediate	5.32		x
37	COALL	T	0.337	Intermediate	7.18		x
38	COALL	T	2.36	Intermediate	6.78		x
39	COALL	T	0.014	Sensitive	8.85		x
40	COALL	T	0.047	Sensitive	8.12		x
41	COALL	ER	229	Resistant	9.16	x	x
42	COALL	T	>250	Resistant	7.08		x
43	COALL	BO	154	Resistant	11.20	x	x
44	COALL	T	>250	Resistant	8.19		x
45	COALL	T	0.038	Sensitive	7.61		x
46	COALL	T	>250	Resistant	7.89		x
47	COALL	T	>250	Resistant	7.19		x
48	COALL	T	>250	Resistant	8.86		x
49	COALL	T	0.057	Sensitive	7.13		x
50	COALL	T	0.081	Sensitive	6.71		x
51	COALL	BAL	>250	Resistant	10.16	x	x
52	COALL	ER	>250	Resistant	11.45	x	x
53	COALL	BO	>250	Resistant	11.58	x	x
54	COALL	T	0.199	Intermediate	5.60		x
55	COALL	T	0.037	Sensitive	8.23		x
56	COALL	T	0.055	Sensitive	7.74		x

57	COALL	T	>250	Resistant	7.22		x
58	COALL	BAL	>250	Resistant	10.38	x	x
59	COALL	T	0.286	Intermediate	5.15		x
60	COALL	BO	>250	Resistant	11.87	x	x
61	COALL	T	0.488	Intermediate	6.18		x
62	COALL	T	126	Intermediate	6.33		x
63	COALL	ER	0.031	Sensitive	8.39	x	x
64	COALL	BO	0.040	Sensitive	6.90	x	x
65	COALL	ER	0.068	Sensitive	8.10	x	x
66	COALL	BAL	27.1	Intermediate	11.73	x	x
67	COALL	BO	0.014	Sensitive	8.78	x	x
68	COALL	HD	>250	Resistant	12.85	x	x
69	COALL	BO	0.031	Sensitive	9.07	x	x
70	COALL	ER	>250	Resistant	10.78	x	x
71	COALL	ER	>250	Resistant	8.04	x	x
72	COALL	T	223	Resistant	11.77		x
73	COALL	BO	17.6	Intermediate	10.97	x	x
74	COALL	ER	<0,008	Sensitive	6.21	x	x
75	COALL	T	<0,008	Sensitive	5.58		x
76	COALL	T	0.373	Intermediate	6.85		x
77	COALL	T	1.42	Intermediate	6.36		x
78	COALL	BAL	0.324	Intermediate	9.58	x	x
79	COALL	E2A	0.030	Sensitive	8.09	x	x
80	COALL	BAL	0.061	Sensitive	7.86	x	x
81	COALL	HD	0.026	Sensitive	8.90	x	x
82	COALL	BA	0.032	Sensitive	11.27	x	x
83	COALL	T	0.031	Sensitive	4.62		x
84	COALL	T	0.045	Sensitive	6.54		x
85	COALL	BO	0.024	Sensitive	9.40	x	x
86	COALL	T	0.043	Sensitive	5.50		x
87	COALL	ER	0.040	Sensitive	11.43	x	x
88	COALL	BO	0.243	Intermediate	9.02	x	x
89	COALL	ER	0.057	Sensitive	10.00	x	x
90	COALL	T	0.298	Intermediate	7.01		x
91	COALL	HD	0.280	Intermediate	9.73	x	x
92	COALL	BO	0.028	Sensitive	6.14	x	x
93	COALL	ER	0.384	Intermediate	9.20	x	x
94	COALL	BAL	0.275	Intermediate	10.80	x	x
95	COALL	ER	0.045	Sensitive	8.14	x	x
96	COALL	BAL	0.553	Intermediate	9.61	x	x
97	COALL	ER	0.302	Intermediate	6.56	x	x
98	COALL	T	0.259	Intermediate	7.07		x
99	COALL	BAL	0.583	Intermediate	11.80	x	x
100	COALL	ER	0.247	Intermediate	6.87	x	x
101	COALL	T	177	Resistant	7.57		x
102	COALL	ER	0.091	Sensitive	6.85	x	x
103	COALL	ER	0.397	Intermediate	6.68	x	x
104	COALL	BO	>250	Resistant	12.26	x	x
105	COALL	HD	0.306	Intermediate	9.77	x	x
106	COALL	HD	0.208	Intermediate	6.60	x	x
107	COALL	NA	0.271	Intermediate	9.31	x	x
108	COALL	HD	0.421	Intermediate	11.69	x	x
109	COALL	HD	0.417	Intermediate	8.66	x	x
110	COALL	BO	>250	Resistant	7.76	x	x
111	COALL	ER	0.050	Sensitive	6.30	x	x
112	COALL	T	>250	Resistant	10.65		x
113	COALL	HD	0.253	Intermediate	10.22	x	x
114	COALL	ER	0.427	Intermediate	9.09	x	x
115	COALL	BA	3.62	Intermediate	8.98	x	x
116	COALL	BO	3.87	Intermediate	4.96	x	x
117	COALL	T	0.027	Sensitive	7.87		x

118	COALL	T	0.061	Sensitive	6.56		x
119	COALL	BO	0.074	Sensitive	8.28	x	x
120	COALL	BO	0.179	Intermediate	9.71	x	x
121	COALL	ER	155	Resistant	6.91	x	x
122	COALL	ER	0.133	Intermediate	6.07	x	x
123	COALL	BAL	0.039	Sensitive	8.64	x	x
124	COALL	HD	>250	Resistant	11.94	x	x
125	COALL	BO	0.023	Sensitive	4.90	x	x
126	COALL	ER	<0,008	Sensitive	5.44	x	x
127	COALL	HD	0.034	Sensitive	8.84	x	x
128	COALL	ER	>250	Resistant	9.12	x	x
129	COALL	BO	>250	Resistant	12.29	x	x
130	COALL	BO	0.009	Sensitive	4.36	x	x
131	COALL	HD	0.025	Sensitive	10.18	x	x
132	COALL	HD	0.058	Sensitive	8.82	x	x
133	COALL	BO	<0,008	Sensitive	10.76	x	x
134	COALL	HD	<0,008	Sensitive	11.11	x	x
135	COALL	ER	0.346	Intermediate	8.54	x	x
136	COALL	HD	0.199	Intermediate	9.44	x	x
137	COALL	HD	0.049	Sensitive	9.36	x	x
138	COALL	HD	0.059	Sensitive	10.94	x	x
139	COALL	HD	>250	Resistant	10.04	x	x
140	COALL	HD	0.040	Sensitive	11.72	x	x
141	COALL	ER	0.053	Sensitive	9.01	x	x
142	COALL	BAL	0.338	Intermediate	9.15	x	x
143	COALL	HD	0.057	Sensitive	11.91	x	x
144	COALL	BAL	0.034	Sensitive	11.15	x	x
145	COALL	BO	>250	Resistant	12.24	x	x
146	COALL	T	0.021	Sensitive	4.79		x
147	COALL	ER	0.026	Sensitive	5.52	x	x
148	COALL	BO	0.046	Sensitive	5.21	x	x
149	COALL	HD	211	Resistant	11.76	x	x
150	COALL	ER	0.025	Sensitive	9.37	x	x
151	COALL	BO	2.77	Intermediate	6.69	x	x
152	COALL	BO	>250	Resistant	7.77	x	x
153	COALL	BO	0.429	Intermediate	5.95	x	x
154	COALL	T	0.488	Intermediate	5.15		x
155	COALL	BO	2.20	Intermediate	8.69	x	x
156	COALL	T	>250	Resistant	6.49		x
157	COALL	HD	0.129	Intermediate	9.49	x	x
158	COALL	T	0.032	Sensitive	7.54		x
159	COALL	BAL	172	Resistant	10.84	x	x
160	COALL	ER	<0,008	Sensitive	7.83	x	x
161	COALL	ER	0.025	Sensitive	8.82	x	x
162	COALL	HD	0.015	Sensitive	11.89	x	x
163	COALL	BAL	0.106	Intermediate	7.86	x	x
164	COALL	HD	0.011	Sensitive	10.13	x	x
165	COALL	E2A	>250	Resistant	9.49	x	x
166	COALL	BO	<0,008	Sensitive	6.96	x	x
167	COALL	HD	0.050	Sensitive	11.83	x	x
168	COALL	BAL	0.027	Sensitive	9.23	x	x
169	COALL	BAL	0.054	Sensitive	10.19	x	x
170	COALL	HD	206	Resistant	9.17	x	x
171	COALL	ER	0.465	Intermediate	8.88	x	x
172	COALL	ER	>250	Resistant	9.68	x	x
173	COALL	BO	0.045	Sensitive	8.77	x	x
174	COALL	BAL	2.61	Intermediate	8.07	x	x
175	COALL	HD	0.186	Intermediate	9.07	x	x
176	COALL	T	0.055	Sensitive	6.48		x
177	COALL	ER	0.049	Sensitive	8.51	x	x
178	COALL	T	0.046	Sensitive	8.57		x

179	COALL	HD	0.028	Sensitive	9.90	x	x
180	COALL	HD	0.049	Sensitive	9.69	x	x
181	COALL	BO	0.042	Sensitive	7.25	x	x
182	COALL	T	0.221	Intermediate	6.87		x
183	COALL	HD	0.038	Sensitive	7.05	x	x
184	COALL	BO	>250	Resistant	11.45	x	x
185	COALL	HD	>250	Resistant	10.88	x	x
186	COALL	BAL	0.409	Intermediate	7.24	x	x
187	COALL	HD	>250	Resistant	10.40	x	x
188	COALL	ER	0.131	Intermediate	6.96	x	x
189	COALL	ER	0.054	Sensitive	5.07	x	x
190	COALL	ER	0.173	Intermediate	6.26	x	x
191	COALL	T	0.312	Intermediate	4.74		x
192	COALL	ER	0.032	Sensitive	6.09	x	x
193	COALL	ER	0.396	Intermediate	11.32	x	x
194	COALL	ER	0.175	Intermediate	8.82	x	x
195	ALL10	ER	0.040	Sensitive	8.80	x	x
196	ALL10	ER	0.060	Sensitive	8.50	x	x
197	ALL10	ER	NA	NA	9.84		x
198	ALL10	MLL	>250	Resistant	8.87	x	x
199	ALL10	T	0.055	Sensitive	5.86		x
200	ALL10	BO	0.036	Sensitive	4.54	x	x
201	ALL10	T	NA	NA	5.20		x
202	ALL10	T	0.163	Intermediate	6.97		x
203	ALL10	HD	0.363	Intermediate	8.59	x	x
204	ALL10	ER	0.219	Intermediate	5.93	x	x
205	ALL10	HD	NA	NA	8.21		x
206	ALL10	HD	0.357	Intermediate	8.92	x	x
207	ALL10	ER	0.090	Sensitive	5.79	x	x
208	ALL10	ER	>250	Resistant	5.48	x	x
209	ALL10	HD	NA	NA	8.00		x
210	ALL10	ER	NA	NA	5.67		x
211	ALL10	ER	>250	Resistant	11.50	x	x
212	ALL10	BO	>250	Resistant	6.85	x	x
213	ALL10	ER	0.159	Intermediate	5.16	x	x
214	ALL10	ER	>250	Resistant	7.48	x	x
215	ALL10	ER	<0,008	Sensitive	5.84	x	x
216	ALL10	HD	0.208	Intermediate	10.67	x	x
217	ALL10	BO	NA	NA	7.79		x
218	ALL10	ER	0.061	Sensitive	6.37	x	x
219	ALL10	BAL	>250	Resistant	10.57	x	x
220	ALL10	ER	NA	NA	9.97		x
221	ALL10	BAL	NA	NA	5.22		x
222	ALL10	BO	NA	NA	8.81		x
223	ALL10	BAL	0.284	Intermediate	10.11	x	x
224	ALL10	HD	0.330	Intermediate	7.49	x	x
225	ALL10	HD	NA	NA	10.73		x
226	ALL10	E2A	<0,008	Sensitive	4.64	x	x
227	ALL10	HD	<0,008	Sensitive	8.84	x	x
228	ALL10	HD	>250	Resistant	10.80	x	x
229	ALL10	BAL	0.053	Sensitive	9.22	x	x
230	ALL10	HD	NA	NA	10.55		x
231	ALL10	HD	0.151	Intermediate	10.59	x	x
232	ALL10	E2A	0.087	Sensitive	10.75	x	x
233	ALL10	T	NA	NA	7.28		x
234	ALL10	ER	0.102	Intermediate	6.95	x	x
235	ALL10	ER	>250	Resistant	6.15	x	x
236	ALL10	T	0.334	Intermediate	6.02		x
237	ALL10	ER	0.047	Sensitive	8.14	x	x
238	ALL10	T	0.040	Sensitive	5.02		x
239	ALL10	HD	0.041	Sensitive	6.53	x	x

240	ALL10	BO	<0,008	Sensitive	6.59	x	x
241	ALL10	T	0.051	Sensitive	5.16		x
242	ALL10	T	0.348	Intermediate	6.78		x
243	ALL10	ER	0.041	Sensitive	8.53	x	x
244	ALL10	BAL	NA	NA	8.94		x
245	ALL10	HD	NA	NA	8.55		x
246	ALL10	BAL	NA	NA	8.68		x
247	ALL10	BO	NA	NA	9.75		x
248	ALL10	BO	NA	NA	8.13		x
249	ALL10	ER	0.343	Intermediate	9.85	x	x
250	ALL10	HD	0.216	Intermediate	10.48	x	x
251	ALL10	HD	NA	NA	9.31		x
252	ALL10	BAL	NA	NA	8.89		x
253	ALL10	ER	NA	NA	6.17		x
254	ALL10	ER	NA	NA	8.36		x
255	ALL10	ER	NA	NA	7.51		x
256	ALL10	BO	NA	NA	9.00		x
257	ALL10	T	NA	NA	5.91		x
258	ALL10	E2A	NA	NA	7.63		x
259	ALL10	HD	NA	NA	10.36		x
260	ALL10	HD	NA	NA	10.01		x
261	ALL10	ER	NA	NA	7.08		x
262	ALL10	T	NA	NA	6.35		x
263	ALL10	T	NA	NA	5.86		x
264	ALL10	ER	NA	NA	6.52		x
265	ALL10	HD	NA	NA	10.42		x
266	ALL10	ER	NA	NA	7.75		x
267	ALL10	T	NA	NA	8.41		x
268	ALL10	HD	NA	NA	10.29		x
269	ALL10	E2A	NA	NA	7.38		x
270	ALL10	HD	NA	NA	9.28		x
271	ALL10	HD	NA	NA	11.54		x
272	ALL10	HD	NA	NA	13.06		x
273	ALL10	HD	NA	NA	10.59		x
274	ALL10	MLL	NA	NA	11.33		x
275	ALL10	HD	NA	NA	12.26		x
276	ALL10	T	NA	NA	7.01		x
277	ALL10	HD	NA	NA	11.31		x
278	ALL10	HD	NA	NA	9.98		x
279	ALL10	HD	NA	NA	10.33		x
280	ALL10	HD	NA	NA	11.29		x
281	ALL10	ER	NA	NA	8.21		x
282	ALL10	E2A	NA	NA	9.00		x
283	ALL10	ER	NA	NA	9.13		x
284	ALL10	BO	NA	NA	10.81		x
285	ALL10	BO	NA	NA	9.99		x
286	ALL10	ER	NA	NA	7.31		x
287	ALL10	ER	NA	NA	7.96		x
288	ALL10	BAL	NA	NA	8.36		x
289	ALL10	HD	NA	NA	11.57		x
290	ALL10	T	NA	NA	8.04		x
291	ALL10	ER	NA	NA	8.10		x
292	ALL10	T	NA	NA	12.32		x
293	ALL10	HD	NA	NA	12.36		x
294	ALL10	T	NA	NA	5.15		x
295	ALL10	T	NA	NA	6.73		x
296	ALL10	ER	NA	NA	6.38		x
297	ALL10	E2A	NA	NA	6.05		x
298	ALL10	HD	NA	NA	10.44		x
299	ALL10	HD	NA	NA	9.67		x
300	ALL10	HD	NA	NA	10.05		x

301	ALL10	ER	NA	NA	8.23		x
302	ALL10	ER	NA	NA	7.66		x
303	ALL10	BO	NA	NA	6.74		x
304	ALL10	E2A	NA	NA	5.97		x
305	ALL10	T	NA	NA	6.94		x
306	ALL10	ER	NA	NA	7.31		x
307	ALL10	HD	NA	NA	11.53		x
308	ALL10	BAL	NA	NA	10.28		x
309	ALL10	HD	NA	NA	12.03		x
310	ALL10	HD	NA	NA	11.28		x
311	ALL10	HD	NA	NA	11.47		x
312	ALL10	ER	NA	NA	8.59		x
313	ALL10	ER	NA	NA	7.91		x
314	ALL10	HD	NA	NA	10.47		x
315	ALL10	ER	NA	NA	7.92		x
316	ALL10	ER	NA	NA	8.78		x
317	ALL10	ER	NA	NA	10.34		x
318	ALL10	HD	NA	NA	11.26		x
319	ALL10	T	NA	NA	5.76		x
320	ALL10	MLL	NA	NA	6.07		x
321	ALL10	ER	NA	NA	6.18		x
322	ALL10	E2A	NA	NA	6.24		x
323	ALL10	BO	NA	NA	7.96		x
324	ALL10	ER	NA	NA	9.93		x
325	ALL10	HD	NA	NA	10.69		x
326	ALL10	BO	NA	NA	10.76		x
327	ALL10	HD	NA	NA	11.69		x
328	ALL10	ER	NA	NA	5.28		x
329	ALL10	BAL	NA	NA	7.53		x
330	ALL10	HD	NA	NA	11.53		x
331	ALL10	ER	NA	NA	6.39		x
332	ALL10	BAL	NA	NA	5.94		x
333	ALL10	BAL	NA	NA	7.92		x
334	ALL10	ER	NA	NA	7.03		x
335	ALL10	T	NA	NA	5.83		x
336	ALL10	ER	NA	NA	7.35		x
337	ALL10	ER	NA	NA	7.21		x
338	ALL10	HD	NA	NA	8.89		x
339	ALL10	BAL	NA	NA	8.72		x
340	ALL10	ER	NA	NA	10.60		x
341	ALL10	T	NA	NA	6.52		x
342	ALL10	BAL	NA	NA	10.02		x
343	ALL10	ER	NA	NA	6.68		x
344	ALL10	ER	NA	NA	7.77		x
345	ALL10	T	NA	NA	5.97		x
346	ALL10	BAL	NA	NA	9.09		x
347	ALL10	HD	NA	NA	12.31		x
348	ALL10	T	NA	NA	8.12		x
349	ALL10	T	NA	NA	4.85		x
350	ALL10	ER	NA	NA	8.83		x
351	ALL10	T	NA	NA	6.88		x
352	ALL10	BO	NA	NA	6.94		x
353	ALL10	T	NA	NA	6.02		x
354	ALL10	ER	NA	NA	6.70		x
355	ALL10	BAL	NA	NA	10.58		x
356	ALL10	BO	NA	NA	11.20		x
357	ALL10	BO	NA	NA	7.08		x
358	ALL10	ER	NA	NA	6.34		x
359	ALL10	BAL	NA	NA	8.65		x
360	ALL10	BO	NA	NA	5.72		x
361	ALL10	BAL	NA	NA	8.43		x

362	ALL10	HD	NA	NA	11.17		x
363	ALL10	BAL	NA	NA	6.85		x
364	ALL10	HD	NA	NA	10.42		x
365	ALL10	BAL	NA	NA	9.63		x
366	ALL10	ER	NA	NA	7.58		x
367	ALL10	HD	NA	NA	9.64		x
368	ALL10	HD	NA	NA	10.22		x
369	ALL10	E2A	NA	NA	5.96		x
370	ALL10	HD	NA	NA	9.35		x
371	ALL10	T	NA	NA	9.11		x
372	ALL10	T	NA	NA	6.77		x
373	ALL10	ER	NA	NA	7.86		x
374	ALL10	T	NA	NA	6.26		x
375	ALL10	BO	NA	NA	10.60		x
376	ALL10	T	NA	NA	7.25		x
377	ALL10	T	NA	NA	5.64		x
378	ALL10	HD	NA	NA	10.88		x
379	ALL10	ER	NA	NA	7.39		x
380	ALL10	ER	NA	NA	9.69		x
381	ALL10	T	NA	NA	7.67		x
382	ALL10	ER	NA	NA	8.23		x
383	ALL10	MLL	NA	NA	8.65		x
384	ALL10	T	NA	NA	9.33		x
385	ALL10	T	NA	NA	7.99		x
386	ALL10	E2A	NA	NA	5.78		x
387	ALL10	ER	NA	NA	6.66		x
388	ALL10	ER	NA	NA	5.63		x
389	ALL10	HD	NA	NA	11.35		x
390	ALL10	T	NA	NA	8.12		x
391	ALL10	HD	NA	NA	11.49		x
392	ALL10	T	NA	NA	7.06		x
393	ALL10	BAL	NA	NA	10.01		x
394	ALL10	BO	NA	NA	10.49		x
395	ALL10	T	NA	NA	8.34		x
396	ALL10	MLL	NA	NA	10.48		x
397	ALL10	T	NA	NA	8.17		x
398	ALL10	ER	NA	NA	8.70		x
399	ALL10	BAL	NA	NA	10.05		x
400	ALL10	T	NA	NA	5.72		x
401	ALL10	T	NA	NA	7.01		x
402	ALL10	HD	NA	NA	10.95		x
403	ALL10	ER	NA	NA	7.96		x
404	ALL10	BO	NA	NA	7.90		x
405	ALL10	BAL	NA	NA	10.76		x
406	ALL10	MLL	NA	NA	6.39		x
407	ALL10	BAL	NA	NA	6.31		x
408	ALL10	T	NA	NA	7.61		x
409	ALL10	HD	NA	NA	11.01		x
410	ALL10	T	NA	NA	8.09		x
411	ALL10	ER	NA	NA	8.67		x
412	ALL10	ER	NA	NA	8.10		x
413	ALL10	HD	NA	NA	10.15		x
414	ALL10	HD	NA	NA	11.34		x
415	ALL10	HD	NA	NA	12.10		x
416	ALL10	BO	NA	NA	9.35		x
417	ALL10	HD	NA	NA	11.40		x
418	ALL10	BO	NA	NA	7.77		x
419	ALL10	ER	NA	NA	9.58		x
420	ALL10	BO	NA	NA	11.13		x
421	ALL10	HD	NA	NA	11.40		x
422	ALL10	T	NA	NA	6.44		x

423	ALL10	T	NA	NA	7.67		x
424	ALL10	HD	NA	NA	11.54		x
425	ALL10	T	NA	NA	8.76		x
426	ALL10	ER	NA	NA	7.05		x
427	ALL10	HD	NA	NA	11.23		x
428	ALL10	T	NA	NA	9.18		x
429	ALL10	HD	NA	NA	10.99		x
430	ALL10	BO	NA	NA	8.61		x
431	ALL10	T	NA	NA	7.52		x
432	ALL10	HD	NA	NA	9.09		x
433	ALL10	BO	NA	NA	7.08		x
434	ALL10	ER	NA	NA	7.99		x
435	ALL10	T	NA	NA	7.45		x
436	ALL10	HD	NA	NA	9.51		x
437	ALL10	HD	NA	NA	11.98		x
438	ALL10	T	NA	NA	10.99		x
439	ALL10	HD	NA	NA	9.96		x
440	ALL10	ER	NA	NA	7.59		x
441	ALL10	BAL	NA	NA	9.64		x
442	ALL10	BO	NA	NA	10.98		x
443	ALL10	ER	NA	NA	8.75		x
444	ALL10	T	NA	NA	9.36		x
445	ALL10	BO	NA	NA	10.81		x
446	ALL10	HD	NA	NA	10.30		x
447	ALL10	HD	NA	NA	11.38		x
448	ALL10	HD	NA	NA	10.98		x
449	ALL10	BAL	NA	NA	9.64		x
450	ALL10	HD	NA	NA	10.25		x
451	ALL10	ER	NA	NA	6.64		x
452	ALL10	ER	NA	NA	7.42		x
453	ALL10	E2A	NA	NA	8.65		x
454	ALL10	T	NA	NA	5.58		x
455	ALL10	ER	NA	NA	5.94		x
456	ALL10	ER	NA	NA	10.17		x
457	ALL10	BO	NA	NA	6.97		x
458	ALL10	HD	NA	NA	10.04		x
459	ALL10	T	NA	NA	6.59		x
460	ALL10	ER	NA	NA	9.56		x
461	ALL10	HD	NA	NA	10.17		x
462	ALL10	BAL	NA	NA	8.25		x
463	ALL10	ER	NA	NA	6.02		x
464	ALL10	E2A	NA	NA	8.47		x
465	ALL10	BAL	NA	NA	8.99		x
466	ALL10	BAL	NA	NA	8.45		x
467	ALL10	T	NA	NA	11.66		x
468	ALL10	T	NA	NA	8.88		x
469	ALL10	E2A	NA	NA	8.72		x
470	ALL10	ER	NA	NA	6.59		x
471	ALL10	BO	NA	NA	7.83		x
472	ALL10	ER	NA	NA	7.65		x
473	ALL10	BO	NA	NA	6.00		x
474	ALL10	BO	NA	NA	6.99		x
475	ALL10	HD	NA	NA	10.63		x
476	ALL10	T	NA	NA	7.94		x
477	ALL10	ER	NA	NA	8.46		x
478	ALL10	BO	NA	NA	7.56		x
479	ALL10	T	NA	NA	6.66		x
480	ALL10	ER	NA	NA	9.76		x
481	ALL10	HD	NA	NA	11.44		x
482	ALL10	ER	NA	NA	5.79		x
483	ALL10	HD	NA	NA	9.85		x

484	ALL10	BA	NA	NA	9.26		x
485	ALL10	BA	NA	NA	11.11		x
486	ALL10	BA	NA	NA	10.24		x
487	ALL10	BA	NA	NA	10.14		x
488	ALL10	BA	NA	NA	11.39		x
489	ALL10	BA	NA	NA	9.61		x
490	other	T	0.148	Intermediate	7.25		
491	other	T	0.444	Intermediate	7.14		
492	other	T	0.060	Sensitive	4.36		
493	other	T	75.0	Intermediate	6.26		
494	other	T	0.387	Intermediate	5.28		
495	other	T	>250	Resistant	6.64		
496	other	T	0.305	Intermediate	7.14		
497	other	BO	0.054	Sensitive	9.39	x	
498	other	BO	12.6	Intermediate	5.05	x	
499	other	E2A	<0,008	Sensitive	6.33	x	
500	other	HD	0.052	Sensitive	10.86	x	
501	other	BA	0.048	Sensitive	7.91	x	
502	other	HD	0.050	Sensitive	12.12	x	
503	other	HD	133	Intermediate	12.32	x	
504	other	ER	>250	Resistant	10.18	x	
505	other	NA	0.061	Sensitive	6.63	x	
506	other	T	<0,008	Sensitive	6.60		
507	other	T	0.397	Intermediate	8.01		
508	other	T	0.278	Intermediate	6.97		
509	other	T	0.056	Sensitive	7.22		
510	other	T	0.158	Intermediate	4.75		
511	other	T	0.171	Intermediate	6.35		
512	other	T	>250	Resistant	4.69		

Genetic Subtype: HD=hyperdiploid, ER=ETV6-RUNX1+, BA=BCR-ABL1+, BAL= BCR-ABL1-Like, E2A=TCF3-PBX1+, MLL=MLL rearranged, BO= B-other, negative for aforementioned lesions, T=T-ALL, NA=not available

Prednisolone category: sensitive = ≤0.1 µg/ml, intermediate= >0.1-<150 µg/ml, resistant =≥150 µg/ml, see also reference 2 and 11.

Only BCP-ALL patients with *in vitro* prednisolone cytotoxicity data were used for limma-analysis, indicated by X in column 7, as we compare in vitro resistant to sensitive patients.
Only BCP-ALL and T-ALL patients of COALL97-03 and DCOG-ALL10 protocol, indicated by X in column 8, were used for survival analysis, as the analysis was stratified for treatment protocol.
Remaining patients belonged either to DCOG-ALL8 or -ALL9 protocols. EMP1 expression values of these patients were only used in Supplemental Figure S1.

Table S2.

BCP-ALL						
COALL-97/03 protocol						
			Univariate Analysis		Multivariate Analysis	
	Patients	Events	HR (95%-CI)	*p* -value	HR (95%-CI)	*p* -value
***EMP1* expression (mRNA)**						
< Median	67	9	1		1	
≥ Median	67	19	2.09(0.94-4.64)	0.07	1.81(0.77-4.25)	0.17
Age at diagnosis (years)						
Low <10	103	20	1		1	
High ≥10	31	8	1.23(0.54-2.79)	0.62	1.22(0.51-2.95)	0.66
WBC count (x 10^9/ L)						
Low < 50	100	16	1		1	
High ≥ 50	34	12	2.64(1.25-5.57)	0.01	2.66(1.12-6.35)	0.03
***In vitro* Prednisolone response**						
Good	54	8	1		1	
Intermediate	47	12	1.95(0.80-4.78)	0.14	1.57(0.62-3.94)	0.34
Poor	33	8	1.61(0.60-4.30)	0.34	1.38(0.47-4.05)	0.56

DCOG-ALL10 protocol						
			Univariate Analysis		Multivariate Analysis	
	Patients	Events	HR (95%-CI)	*p* -value	HR (95%-CI)	*p* -value
***EMP1* expression (mRNA)**						
< Median	119	7	1		1	
≥ Median	120	18	2.842(1.186-6.812)	0.02	3.172(1.123-8.958)	0.03
Age at diagnosis (years)						
Low <10	152	15	1		1	
High ≥10	87	10	1.857(0.813-4.243)	0.14	1.402(0.501-3.918)	0.52
WBC count (x 10^9/ L)						
Low < 50	188	21	1		1	
High ≥ 50	51	4	0.724(0.248-2.109)	0.55	0.563(0.176-1.802)	0.33
***In vivo* Prednisolone response**						
Good	223	23	1		1	
Poor	7	2	2.749(0.647-11.689)	0.17	0.864(0.160-4.651)	0.87

T-ALL						
COALL-97/03 protocol						
			Univariate Analysis		Multivariate Analysis	
	Patients	Events	HR (95%-CI)	*p* -value	HR (95%-CI)	*p* -value
***EMP1* expression (mRNA)**						
< Median	30	9	1		1	
≥ Median	30	11	1.19(0.49-2.86)	0.70	1.39(0.54-3.53)	0.49
Age at diagnosis (years)						
Low <10	34	11	1		1	
High ≥10	26	9	1.09(0.45-2.64)	0.85	1.13(0.46-2.80)	0.79
WBC count (x 10^9/ L)						
Low < 50	15	4	1		1	
High ≥ 50	45	16	1.45(0.49-4.34)	0.51	1.17(0.35-3.87)	0.80
***In vitro* Prednisolone response**						
Good	21	7	1		1	
Intermediate	22	9	1.37(0.51-3.68)	0.533	1.36(0.48-3.85)	0.56
Poor	17	4	0.72(0.21-2.48)	0.61	0.66(0.19-2.37)	0.53

DCOG-ALL10 protocol						
			Univariate Analysis		Multivariate Analysis	
	Patients	Events	HR (95%-CI)	*p* -value	HR (95%-CI)	*p* -value
***EMP1* expression (mRNA)**						
< Median	28	2	1		1	
≥ Median	28	8	4.40(0.93-20.73)	0.06	6.27(1.18-3.29)	0.03
Age at diagnosis (years)						
Low <10	29	5	1		1	
High ≥10	27	5	1.10(0.32-3.80)	0.88	1.80(0.43-7.45)	0.42
WBC count (x 10^9/ L)						
Low < 50	21	4	1		1	
High ≥ 50	34	6	0.85(0.24-3.01)	0.80	0.44(0.11-1.74)	0.24
***In vivo* Prednisolone response**						
Good	39	5	1		1	
Poor	16	5	2.54(0.74-8.79)	0.14	3.23(0.80-13.01)	0.10

Table S2. Univariate and multivariate analysis of EMP1 status, age, white blood cell count and *in vitro* and *in vivo* prednisolone resistance and prognosis. Univariate and multivariate analysis of the indicated prognostic factors were performed by a Cox regression model based on event-free survival and with riskgroup as stratum. *In vitro* prednisolone response data are only available of COALL-97/03 patients, in contrast *in vivo* prednisolone response data are only available of DCOG-ALL10 patients.

Table S3.

BCP-ALL						
COALL-97/03 protocol and DCOG-ALL10 cohort						
			Univariate Analysis		Multivariate Analysis	
	Patients	Events	HR (95%-CI)	*p* -value	HR (95%-CI)	*p* -value
***EMP1* expression (mRNA)**						
< Median	186	16	1		1	
≥ Median	187	37	2.42(1.34-4.35)	<0.01	2.41(1.32-4.39)	<0.01
Age at diagnosis (years)						
Low <10	255	35	1		1	
High ≥10	118	18	1.50(0.84-2.67)	0.17	0.89(0.485-1.635)	0.71
WBC count (x 10^9/ L)						
Low < 50	288	37	1		1	
High ≥ 50	85	16	1.58(0.88-2.83)	0.13	1.22(0.665-2.249	0.52
Subtype (risk)						
ER HD	227	17	1		1	
E2A BO	66	16	3.25(1.69-6.28)	<0.01	3.69(1.86-7.31)	<0.01
BA BAL MLL	67	20	3.84(1.96-7.54)	<0.01	3.35(1.61-6.98)	<0.01

Table S3. Univariate and multivariate analysis of EMP1 status, age, white blood cell count and subtypes in BCP-ALL. Univariate and multivariate analysis of the indicated prognostic factors were performed by a Cox regression model stratified for treatment protocol (COALL/DCOG) based on event-free survival. The percent of 5-year event-free survival was analyzed using Kaplan-Meier statistics. WBC=white blood cell count; ER=ETV6-RUNX1^{+}, HD=hyperdiploid, E2A=TCF3-PBX1+, BO=B-other, BA=BCR-ABL1^{+}, BAL=BCR-ABL1-like, MLL=MLL-rearranged. ER and HD belong to the low-risk group, E2A and BO to intermediate-risk group and BA, BAL and MLL to high-risk group.

THE NR4A ORPHAN NUCLEAR RECEPTORS DO NOT CONFER PREDNISOLONE RESISTANCE IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Resistance of leukemic cells to prednisolone is an obstacle for effective treatment of pediatric precursor B-cell acute lymphoblastic leukemia (BCP-ALL) at initial diagnosis and even more at relapse. In this study we demonstrate that in primary BCP-ALL cells obtained from 176 children *NR4A1* (Nur77), *NR4A2* (Nurr1) and *NR4A3* (Nor1) mRNA and (protein) levels were up to 3-fold ($p<0.007$) higher in *in vitro* prednisolone-resistant compared to sensitive cases. The NR4A family members are nuclear receptors which can antagonize the glucocorticoid receptor. We hypothesized that the NR4A family members may cause prednisolone resistance in BCP-ALL. Due to observed compensatory mechanisms between the NR4A members, we simultaneously silenced all three members in primary cells of BCP-ALL pediatric patients. Simultaneous knockdown did not sensitize to prednisolone whereas a modest significant decrease of 25.4±11.1% in cell viability was observed ($p=0.029$). In conclusion, although the NR4A family members are higher expressed in prednisolone-resistant BCP-ALL cells, these genes do not functionally contribute to prednisolone resistance. Targeting the NR4A receptors modestly reduces leukemic cell viability. Nevertheless, the requisite to target all three family members simultaneously due to compensatory mechanisms makes these genes unlikely candidates for a targeted drug approach to improve clinical outcome in children with BCP-ALL.

INTRODUCTION

Prednisolone is one of the principal chemotherapeutic drugs used to treat children with acute lymphoblastic leukemia (ALL). Resistance to prednisolone, both *in vitro* and *in vivo*, was shown to be of unfavorable prognostic value and has been used as a risk parameter in German COALL and BFM protocols as well as Dutch DCOG treatment protocols [1–3]. In addition, ALL cells often acquire resistance to prednisolone at the time the leukemia relapses [4]. Therapeutic intervention to overcome prednisolone resistance is therefore desirable.

Prednisolone serves as ligand for the cytosolic glucocorticoid receptor (GR, also known as NR3C1). Binding of prednisolone conformationally changes the GR dispersing inactivating cochaperones, leading to homodimerization (Figure 1). Hereafter, active GR orchestrates transcriptional regulation by binding to specific palindromic negative or positive glucocorticoid response elements (GRE) in the DNA, leading to gene repression or activation, respectively. Active GR can also sequester other transcription factors, such as AP-1 and NF-κB, thereby diminishing the transcriptional activation of AP-1 and NF-κB responsive genes.

To study prednisolone resistance in more detail, we generated gene expression profiles of *in vitro* prednisolone sensitive and resistant precursor B-ALL (BCP-ALL) cells of newly diagnosed patients. Limma analysis revealed increased expression of all *NR4A1*, *NR4A2* and *NR4A3* probesets in *in vitro* prednisolone resistant BCP-

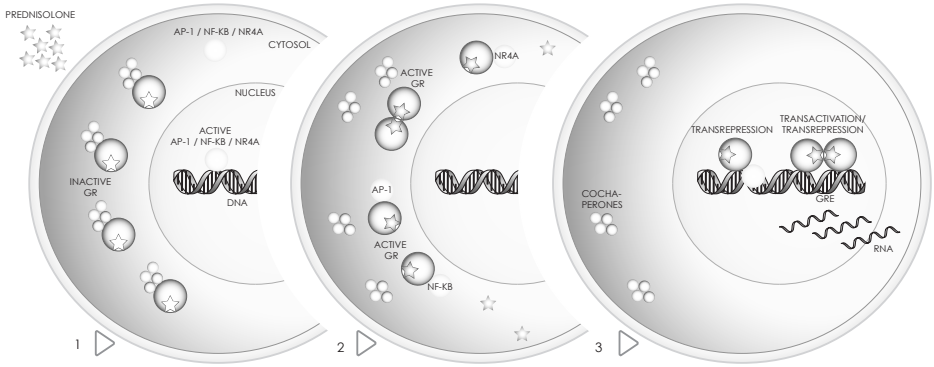

Figure 1. Prednisolone; mechanisms of action and counteraction by NR4A family.
Prednisolone binds the cytosolic glucocorticoid receptor (GR), which dissociates inactivating cochaperones leading to homodimerization. Hereafter, GR binds specific palindromic negative or positive glucocorticoid response elements (GRE) in the DNA leading to gene repression or activation, respectively. Active GR can also scavenge other transcription factors, such as AP-1 and NF-kB, inducing transrepression. NR4A family members can bind the GR as well, after which not only NR4A-dependent transcription but also GR-dependent transcription is diminished.

ALL cells (see results). Interestingly, *NR4A1* (protein name: Nur77), *NR4A2* (protein name: Nurr1), and *NR4A3* (protein name: Nor1) are orphan nuclear receptors which belong to the same steroid hormone receptor superfamily as GR[5]. In contrast to the GR, NR4A family receptors are constitutively active without need for ligand binding [6]. Nur77, Nurr1, and Nor1 bind the nerve growth factor-induced B and Nur DNA response element thereby transcriptionally regulating glucose and fatty acid metabolism, inflammation, central nervous system development, proliferation, differentiation and apoptosis[7]. A direct protein-protein interaction between GR and the NR4A family members has been demonstrated which was independent of GR homodimerization [8,9]. Remarkably, it has been shown that the direct interaction between NR4A family members and the GR represses GR-dependent transcription [8,9]. Based on this literature and our microarray data, we hypothesized that the upregulated expression of NR4A family members may be responsible for prednisolone resistance in ALL by direct repression of prednisolone induced transcription (Figure 1). In this study we aimed to silence the NR4A family members to evaluate the potential modulating effects on prednisolone resistance in primary BCP-ALL cells of pediatric patients.

MATERIALS AND METHODS

Processing of patients' leukemic cells

Bone marrow and peripheral blood samples were collected from children suspected to suffer from newly diagnosed ALL (before initial therapy) as approved by the institutional review board and after written informed consent was obtained. Mononuclear cells were isolated by lymphoprep density gradient centrifugation, as previously described[1]. If necessary, immunomagnetic beads were used to enrich for leukemic blasts, as only leukemic samples with ≥ 90% leukemic blasts were used in the present study. The subtype of each patient, i.e. hyperdiploid (>50 chromosomes), *ETV6-RUNX1*$^+$, *TCF3-PBX1*$^+$, MLL-rearrangement, *BCR-ABL1*$^+$, BCR-ABL1$^+$-like, or B-other (negative for aforementioned genomic lesions), was determined by means of FISH, PCR and by utilizing the 110-probeset classifier [10]. Mononuclear cells were isolated from bone marrow (nBM) and peripheral blood (nPB) of children who were suspected for a malignancy but who turned out to be negative for a hematological disorder. Cells were cultured in RPMI Dutch modification (Gibco) supplemented with 0.1% insulin-transferrin-sodium selenite (Sigma), 0.4 mM glutamine (Invitrogen), 0.25 µg/ml gentamycine (Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 0.125 µg/ml fungizone (Gibco) and 20% fetal calf serum (Integro) at 37°C in humidified air containing

5% CO_2. Cell viability and cell counts were determined by trypan blue exclusion staining assays (Miltenyi Biotec).

Gene Expression Microarrays

Total RNA was extracted from leukemic blasts (≥ 90% purity) by means of Trizol isolation (Invitrogen) according to the manufacturer's protocol. RNA quality and integrity was determined with the 2100 Bioanalyzer (Agilent). The One-Cycle cDNA Synthesis kit and the GeneChip IVT Labeling kit (Affymetrix) were used to synthesize cRNA. Processing and hybridization of cRNA to the Affymetrix U133 Plus 2.0 GeneChip oligonucleotide microarray was performed according to the manufacturer's protocol. Gene-expression values were calculated with the Affymetrix Microarray Suite version 5.0. Expression signals were scaled to the target intensity of 500 and log-transformed. Only arrays with a scaling factor <10 and GAPDH cRNA integrity (3'/5') <3 were used for subsequent analyses. Next, array data were normalized using the Affy R Package to calculate the Robust Multichip Average (RMA) and using the variance stabilization and normalization 2 (VSN2) package [11]. Differentially expressed genes between *in vitro* prednisolone resistant and sensitive BCP-ALL patients were identified with Limma R Package using subtype as a confounder in the statistical environment R, version 2.15.0. Correction for multiple tests was performed according to the false discovery rate (FDR) method [12]. All *NR4A1*, *NR4A2* and *NR4A3* probesets were statistically differentially expressed between *in vitro* prednisolone sensitive and resistant patients. Probesets 202340_x_at *NR4A1*, 204621_s_at *NR4A2*, 209959_at *NR4A3* were used for further analysis, as these probesets showed the most reliable differential expression between *in vitro* prednisolone sensitive and resistant patients.

Reverse Phase Protein Array

Proteins from primary leukemic cells were isolated with protein lysis buffer containing 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton X-100, 10% Glycerol, 10 mM Sodium-pyrophosphate, 1 mM Sodium-orthovanadate, 10 mM Glycerolphosphate, DTT, Phenylmethylsulfonyl Fluoride, Aprotinin and Sodium-Fluoride. Protein concentration was quantified according to the BCA assay (Pierce). Lysates were spotted twice in triplicate on glass-backed nitrocellulose-coated array slides by the facility of Dr. E. F. Petricoin, George Mason University, Manassas, USA. Slides were stained with specific antibodies, incubated with a biotinylated secondary antibody and scanned using the NovaRay scanner. Protein levels were calculated relative to the total amount of protein per sample using MicroVigene Software. Antibodies used were: Nur77 antibody (Cell signal-

ling #3960), Nurr1 (Sigma #N6413), Nor1 (kindly provided by Prof C. de Vries, AMC, The Netherlands [13]) (Supplemental Table 1).

siRNA

siRNAs (Sigma) were designed utilizing rnaidesigner (Invitrogen) considering a maximum GC amount of 40-55% and tagged with an appropriate label (Supplemental Table 2). 2.0x10^6/ml leukemic patients' cells were transfected with all three siNR4As simultaneously, 0.1 µM/10^6 cells each, or with the same amount of siScrl, 0.3 µM/10^6 cells using the transfection reagent Dharmafect 4 (Thermo Scientific). Transfection efficiency was verified with the Accuri (BD Biosciences). Viability of transfected cells was assessed by a trypan blue exclusion assay. Cells were harvested for RNA and protein isolation after 72 hours of culture.

Quantitative RT-PCR

RNA was extracted using a Rneasy microkit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 1 µg RNA by 8 IU/µl MMLV (Promega), 20nM oligodT primers, 1µM random hexamer primers (Invitrogen), 200 µM dNTPs and 1 IU/µl RNAsin in MMLV-buffer (Promega). *NR4A1, NR4A2* and *NR4A3* mRNA levels were quantified by incorporation of SYBR Green (Thermo Scientific) by quantitative real-time (qRT) PCR (Applied Biosystems 7900HT). Primer sequences see Supplemental Table 3.

Western blot analysis

25 µg protein was loaded on Bio-Rad Mini-Protean TGX pre-cast anyKD and transferred to nitrocellulose membranes (Trans-Blot Turbo Mini Nitrocellulose Transfer Packs, Bio-Rad). Blots were blocked and probed with antibody according to Supplemental Table 1. Hereafter, protein levels were quantified using the Odyssey 3.0 application software (Li-COR).

In vitro MTT drug-resistance assay

Cytotoxicity of prednisolone (Bufa Pharmaceutical Products) in primary patients' cells was determined by the *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) drug-resistance assay after 4-days of exposure, as previously described [1]. Optical density values were measured on a Versamax (Molecular Devices) at λ=562 nm and λ=720nm. *In vitro* prednisolone sensitivity was defined by a concentration of prednisolone lethal to 50% of the cells (LC_{50}) below 0.1 µg/mL and prednisolone resistance was defined by a LC_{50} value above 150 µg/mL as shown previously to be predictive for clinical outcome in pediatric ALL[1]. Prednisolone response after siRNA treatment was determined

by the MTT drug-resistance assay after 3-days of exposure and prednisolone-response curves of NR4A-silenced cells were corrected for loss of cell viability caused by the silencing procedure itself.

Statistical Analysis

Spearman's rank correlation coefficient was calculated to compare microarray gene expression results to qRT-PCR results. Data from other experiments were compared with Mann-Whitney U tests. A p-value below 0.05 was considered statistically significant.

RESULTS

NR4A1, NR4A2 and NR4A3 mRNA expression levels are higher in prednisolone resistant leukemic cells

Microarray analysis of 176 primary BCP-ALL patients revealed that *NR4A1*, *NR4A2* and *NR4A3* mRNA levels were 3.0-fold (FDR=0.007), 2.6-fold (FDR=0.01) and 2.0-fold (FDR=0.01) respectively higher in leukemic cells of *in vitro* prednisolone resistant compared to sensitive patients (Microarray data are provided in Supplemental Table 4). Validation of microarray data by qRT-PCR indicated that the NR4A mRNA levels measured in 23 cases were significantly correlated (R_s=0.94, R_s=0.87 and R_s=0.91, respectively p<0.0001, Figure 2). *NR4A1*, *NR4A2* and *NR4A3* mRNA levels were 13.2-fold, 4.8-fold and 13.8-fold higher, respectively in BCP-ALL cells compared to normal bone marrow samples (p<0.05, Figure 2).

Nur77 and Nurr1 protein levels are higher in prednisolone resistant leukemic patients´ cells and are overall increased in leukemic cells compared to normal cells

Reverse phase protein array analysis revealed a 2.7-fold higher Nur77 (encoded by *NR4A1*) and 1.2-fold higher Nurr1 (encoded by *NR4A2*) protein level in *in vitro* prednisolone resistant compared to sensitive ALL patients' samples (Figure 3, p<0.01). Protein levels of Nor1 (encoded by *NR4A3*) did not differ (Figure 3). Nur77 and Nurr1 levels were significantly higher in BCP-ALL compared to normal BM, overall 5-fold (p<0.0001, Figure 3). A high expression of Nur77 was especially seen in hyperdiploid ALL patients (7.4-fold , p<0.0001), *BCR-ABL1*-like ALL patients (6.3-fold, p<0.0001), *ETV6-RUNX1*$^+$ ALL patients (3.9-fold, p<0.0001) and B-other ALL patients (3.3-fold p<0.0001) compared to normal bone marow samples. Notably, patients with the highest Nur77 expression were all *in vitro* intermediate resistant or resistant to prednisolone, with the exception of two patients (Figure 3).

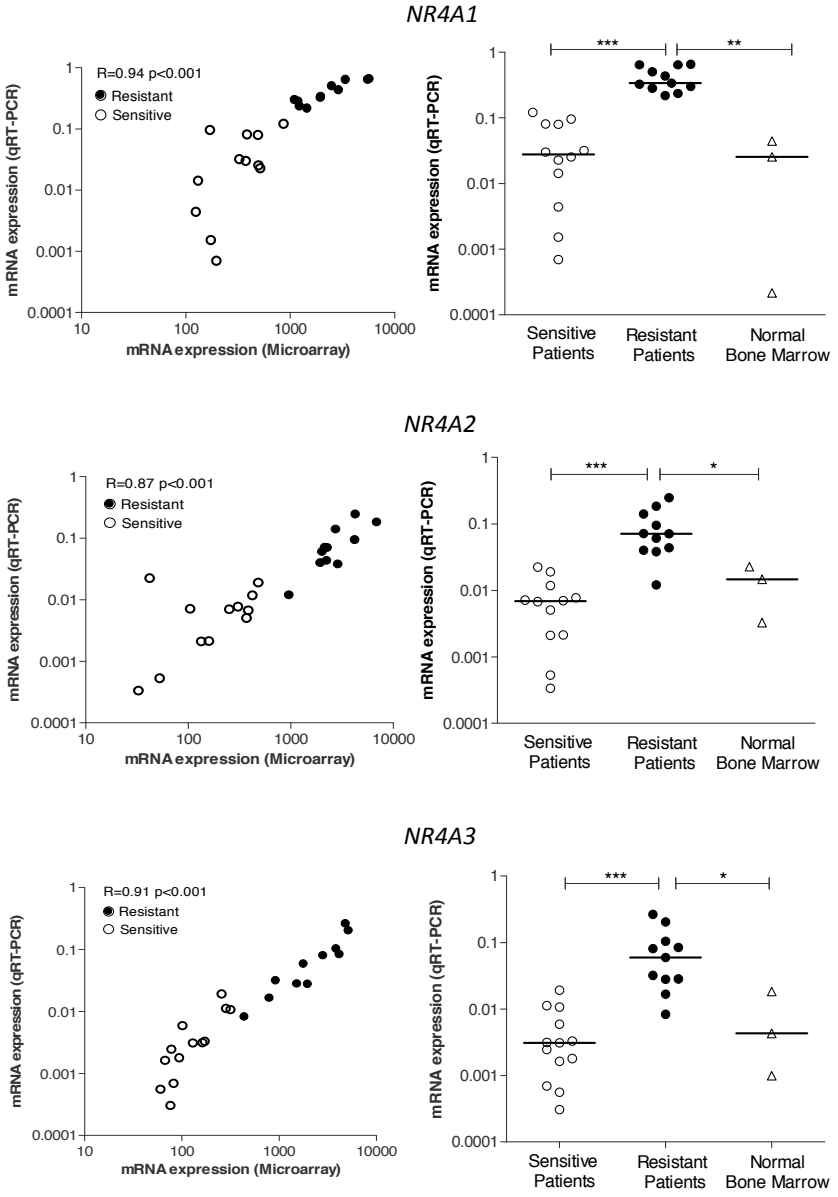

Figure 2. NR4A1, NR4A2 and NR4A3 mRNA expression is increased in prednisolone resistant leukemic patients´ cells.

NR4A1, NR4A2 and NR4A3 mRNA expression levels are higher in prednisolone resistant leukemic primary patient cell samples compared to sensitive samples as detected by microarrays. NR4A1, NR4A2 and NR4A3 mRNA levels detected by microarrays were correlated with those detected by qRT-PCR as shown for 12 *in vitro* prednisolone sensitive patients and 11 *in vitro* prednisolone resistant patients. qRT-PCR revealed that NR4A mRNA levels in 11 prednisolone resistant BCP-ALL cases were higher than those of 3 normal bone marrow samples (*p<0.05, **p<0.01, ***p<0.001)

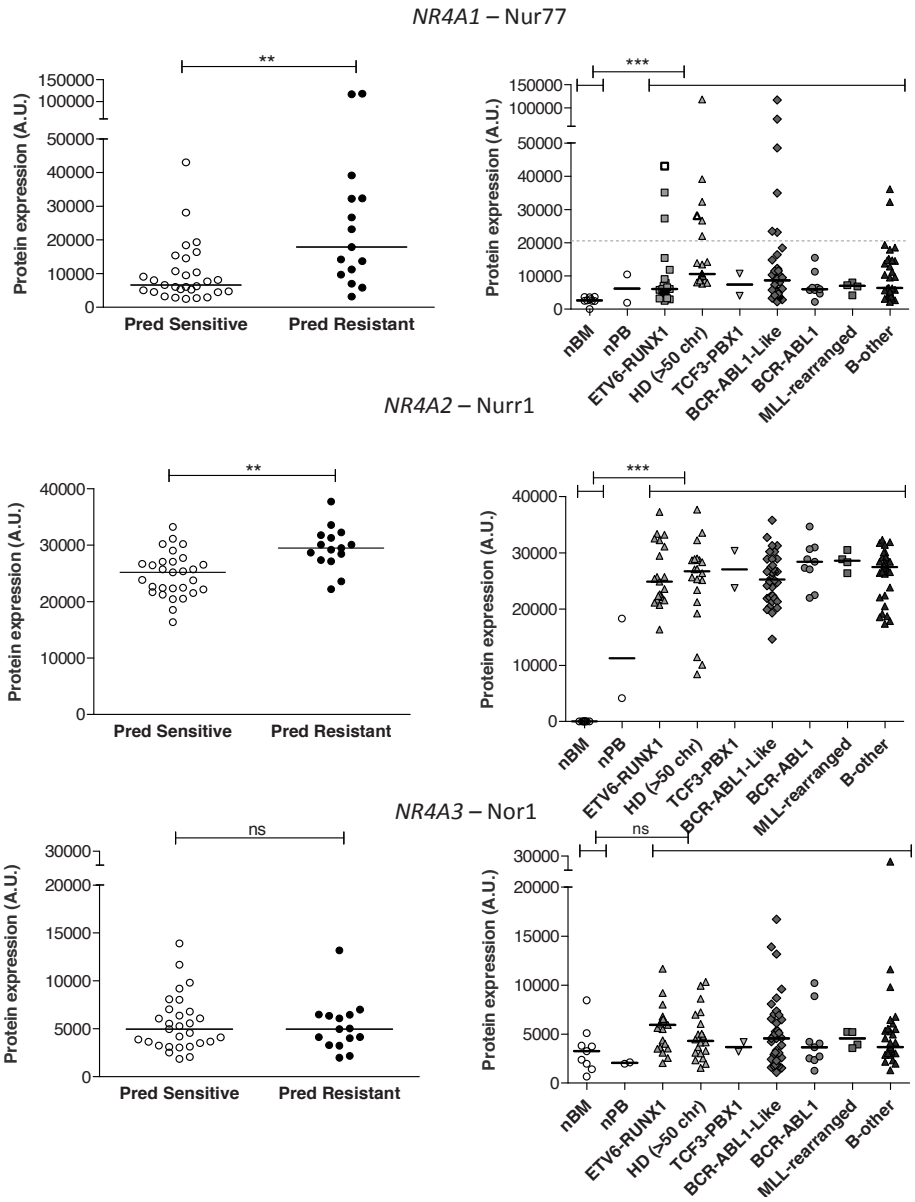

Figure 3. Nur77 and Nurr1 protein expression levels are higher in prednisolone resistant leukemic patients´ cells and are increased in leukemic cells compared to normal cells.
Protein levels of Nur77 (NR4A1 gene), Nurr1 (NR4A2 gene), Nor1 (NR4A3 gene) were analyzed by reverse phase protein arrays. Each dot represents a leukemic patient sample. Patient samples above the dashed line have high Nur77 expression levels and are intermediate resistant and resistant to prednisolone except for two sensitive patients who are indicated by white symbols. (**p<0.01, ***p<0.001). A.U. Arbitrary units normalized for total protein.

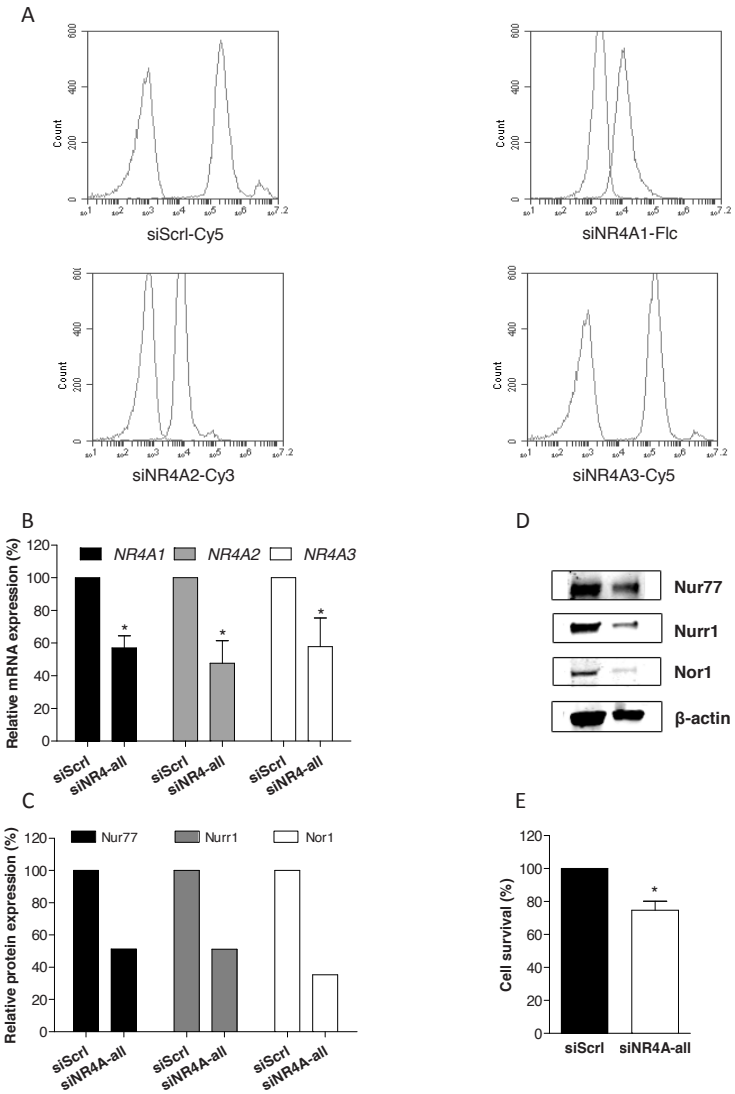

Figure 4. Knockdown of Nur77, Nurr1 and Nor1 in primary ALL cells affects cell survival.
Pediatric BCP-ALL cells were transfected simultaneously with siNR4A1-Flc, siNR4A2-Cy3 and siNR4A3-Cy5 (siNR4A-all), or with siScrl-Cy5. (A)Transfection efficiency at 72 hours after transfection was assessed by means of flow cytometry. (Mock; green line, siScrl; purple line, siNR4A-all; pink line) (B) NR4A1, NR4A2 and NR4A3 mRNA levels were determined 72 hours after transfection by qRT-PCR and calculated relative to siScrl after correcting for the housekeeping gene RPS20. (C) Western Blot analysis was used to determine Nur77, Nurr1 and Nor1 expression 72 hours after transfection relative to the housekeeping protein β-actin. (D) Protein expression levels were calculated with the Odyssey software and corrected for β-actin and are relative to the levels detected in siScrl treated cells. (E) Leukemic cell survival 72 hours after transfection was evaluated with a trypan blue exclusion assay. Data are presented as mean plus SEM of four different BCP-ALL patients' leukemic cell samples with the following subtype, two *ETV6-RUNX1*$^{+}$, one hyperdiploid and one sample with no known genetic aberration, i.e. B-other (*p<0.05).

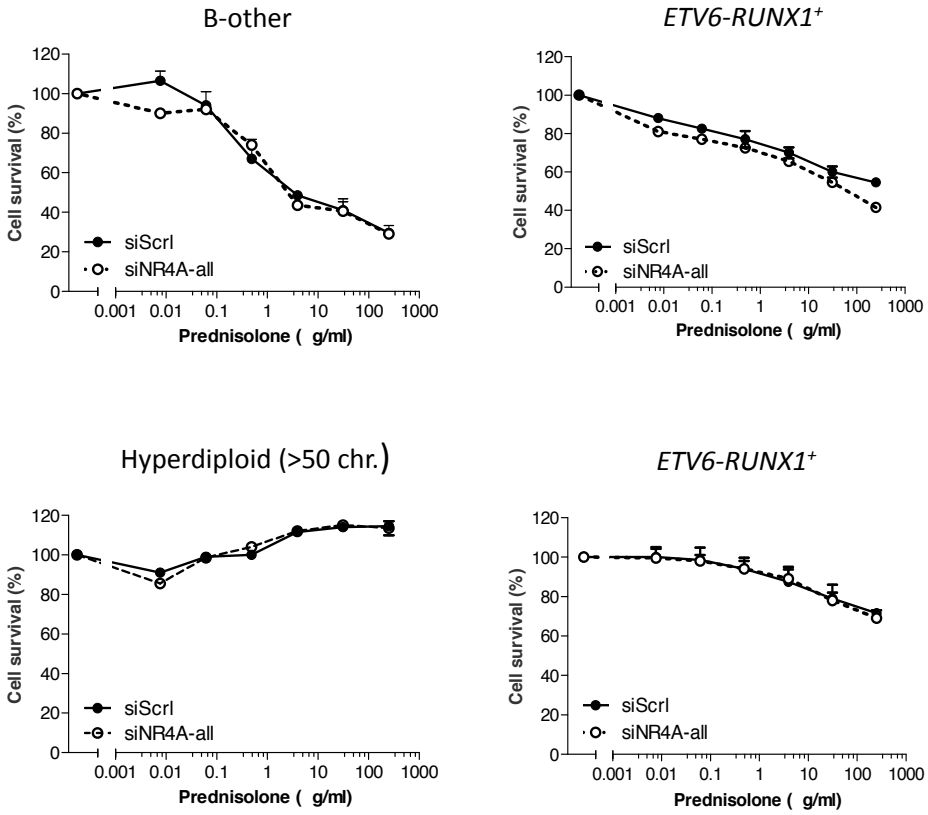

Figure 5. Knockdown of Nur77, Nurr1 and Nor1 in primary ALL cells does not sensitize to prednisolone.

Response to prednisolone was assessed in a three day MTT assay directly after transfection with either siScrl or co-transfection of siNR4A1, siNR4A2 and siNR4A3 (siNR4A-all). Sensitivity was corrected for cell death induced by NR4A1, NR4A2 and NR4A3 knockdown without co-exposure to prednisolone. Data are presented as mean plus SEM of a duplicate experiment.

Knockdown of Nur77, Nurr1 and Nor1 in primary BCP-ALL cells does not sensitize to prednisolone but does reduce cell viability

To test our hypothesis that increased expression of NR4A receptors antagonize the functionality of the GR and as such causes prednisolone resistance, we silenced the expression of these genes and assessed changes in the cytotoxicity of prednisolone. We observed that *NR4A1*, *NR4A2* and *NR4A3* expression levels are lower in a prednisolone resistant cell line than in patients' leukemic cells (Supplementary Figure 1). Moreover, knockdown of one *NR4A* led to an extreme upregulation in expression levels of the other *NR4A* family members (Supplementary Figure 1). We therefore simultaneously silenced all three NR4A family members in leukemic cells of *in vitro* prednisolone resistant BCP-ALL

patients who had high basal expression of *NRA41*, *NR4A2* and *NR4A3* mRNA. All primary patients' cells were 100% positive for the labeled siRNAs directed against the three NR4A-family members (Figure 4A). Co-transfection of all three siRNAs (siNR4A-all) reduced the mRNA levels by 57.1±14.5% for *NR4A1*, 47.7±27.5% for *NR4A2* and 57.8±35.0% for *NR4A3*, compared to the levels seen in equally dosed non-silencing scrambled control (siScrl) treated cells (n=4, $p<0.05$, Figure 4B). Nur77, Nurr1 and Nor1 protein levels were decreased to 51.4%, 51.2% and 35.4% respectively, compared to those observed in the siScrl cells (Figure 4C-D). Silencing of all three NR4As simultaneously decreased the viability of primary BCP-ALL patients' cells by 25.4±11.1% ($p<0.05$, Figure 4E). However, silencing of these three NR4As did not sensitize to prednisolone (Figure 5).

DISCUSSION

Resistance of leukemic cells to prednisolone is linked to an unfavorable clinical outcome in children with newly diagnosed ALL. Leukemic cells even become more resistant to prednisolone at the time of relapse [4,14]. Up to now the molecular mechanisms which denotes prednisolone resistance in leukemic cells are unclear. In this study we demonstrated increased *NR4A1* (Nur77), *NR4A2* (Nurr1) and *NR4A3* (Nor1) mRNA and protein levels in *in vitro* prednisolone resistant compared to sensitive patients. Nur77, Nurr1 and Nor1 directly bind the GR via their DNA binding domain, independent of GR homodimerization, and subsequently antagonize glucocorticoid induced transcriptional activation [8,9]. We hypothesized that upregulated NR4A family expression may be responsible for prednisolone resistance in ALL. Nur77, Nurr1 and Nor1 are highly homologues, the DNA binding and C-terminal ligand binding domains exhibit ~91-95% and ~60% homology, respectively whereas their N-terminal domains are highly divergent [15]. Nor1 can take over the function of Nur77, as *NR4A1*$^{-/-}$ mice appear normal due to compensation by *NR4A3* [16]. This compensation process most likely explains the upregulation of *NR4A2* and *NR4A3*, after *NR4A1* knockdown in BCP-ALL cells as shown in the present study (Supplemental Figure 1).

We therefore developed a system in which we could knockdown all three *NR4As* in primary pediatric ALL patients' cells. We were able to achieve ~50% knockdown of all three *NR4As* simultaneously. However, knockdown of the NR4A family did not result in prednisolone sensitization in primary ALL patient cell samples. Our results suggest that prednisolone resistance is not caused by impaired functioning of GR at the receptor level. In correspondence, previous studies showed that there was no correlation between baseline GR mRNA levels

and prednisolone resistance [17,18]. Prednisolone resistance has been correlated with aberrant function of apoptosis genes and increased glycolytic activity [19,20]. Impaired downregulation of anti-apoptotic MCL1 by prednisolone has been observed in prednisolone resistant patients [21,22]. Together these data suggest that resistance to prednisolone more likely is caused by mechanisms affecting the downstream intracellular signaling than by mechanisms affecting the effective GR receptor levels.

Although knockdown of the NR4A family did not result into prednisolone sensitization, we did see a decline in cell survival. Reported effects of the NR4A family on cell survival are context dependent. Nuclear export of Nur77 and Nor1 to the mitochondria activates pro-apoptotic Bcl-2 family members [23], which occurs in acute myeloid leukemia [24]. In contrast, Nur77, Nurr1 and Nor1 transcription regulation conveys an anti-apoptotic environment [25–27], as is the case in for example B cell lymphoma, melanoma, colon cancer [24,28], and as we show here, in BCP-ALL.

In conclusion, the NR4A family members are higher expressed in prednisolone resistant ALL cells, but do not functionally contribute to resistance in these cells. BCP-ALL is characterized by overall higher expression of the NR4A family compared to normal BM cells. Targeting of NR4A genes impairs leukemic cell survival, but compensatory mechanisms designate that all three NR4A members need to be targeted simultaneously to diminish cell survival. These data therefore show that NR4A genes are not suitable to reverse prednisolone resistance nor to induce cell death by targeted drugs.

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AUTHORSHIP CONTRIBUTIONS

IMA designed and performed research, analyzed and interpreted data, and wrote the paper; RVDD performed experiments; RP and MLDB designed, analyzed and interpreted data, and revised the paper.

REFERENCES

1. Boer, M. L. Den, Harms, D. O., Pieters, R., Kazemier, K. M., Gobel, U., Körholz, D., *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. *J Clin Onc* **21**, 3262–8 (2003).
2. Reiter, A., Schrappe, M., Ludwig, W. D., Hiddemann, W., Sauter, S., Henze, G., *et al.* Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86. *Blood* **84**, 3122–33 (1994).
3. Kaspers, G. J., Pieters, R., Zantwijk, C. H. Van, Wering, E. R. Van, Does-Van Den Berg, A. Van Der & Veerman, A. J. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. *Blood* **92**, 259–66 (1998).
4. Klumper, E., Pieters, R., Veerman, A. J., Huismans, D. R., Loonen, A. H., Hählen, K., *et al.* In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. *Blood* **86**, 3861–8 (1995).
5. Milbrandt, J. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. *Neuron* **1**, 183–8 (1988).
6. Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., *et al.* Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* **423**, 555–60 (2003).
7. Pearen, M. A. & Muscat, G. E. O. Minireview: Nuclear hormone receptor 4A signaling: implications for metabolic disease. *Molecular endocrinology (Baltimore, Md.)* **24**, 1891–903 (2010).
8. Martens, C., Bilodeau, S., Maira, M., Gauthier, Y. & Drouin, J. Protein-protein interactions and transcriptional antagonism between the subfamily of NGFI-B/Nur77 orphan nuclear receptors and glucocorticoid receptor. *Molecular endocrinology (Baltimore, Md.)* **19**, 885–97 (2005).
9. Strøm, B. O., Aden, P., Mathisen, G. H., Lømo, J., Davanger, S. & Paulsen, R. E. Transfection of chicken cerebellar granule neurons used to study glucocorticoid receptor regulation by nuclear receptor 4A (NR4A). *Journal of neuroscience methods* **193**, 39–46 (2010).
10. Boer, M. L. Den, Slegtenhorst, M. van, Menezes, R. X. De, Cheok, M. H., Buijs-Gladdines, J. G. C. A. M., Peters, S. T. C. J. M., *et al.* A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. *The lancet oncology* **10**, 125–34 (2009).
11. Huber, W., Heydebreck, A. von, Sültmann, H., Poustka, A. & Vingron, M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics (Oxford, England)* **18 Suppl 1**, S96–104 (2002).
12. Benjamini, Y. & Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser.* **57**, 289–300. (1995).
13. Bonta, P. I., Tiel, C. M. van, Vos, M., Pols, T. W. H., Thienen, J. V van, Ferreira, V., *et al.* Nuclear receptors Nur77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses. *Arteriosclerosis, thrombosis, and vascular biology* **26**, 2288–94 (2006).

14. Kaspers, G. J. L., Veerman, A. J. P., Pieters, R., Zantwijk, C. H. Van, Smets, L. A., Wering, E. R. Van, *et al.* In Vitro Cellular Drug Resistance and Prognosis in Newly Diagnosed Childhood Acute Lymphoblastic Leukemia. *Blood* **90**, 2723–2729 (1997).
15. Paulsen, R. E., Granås, K., Johnsen, H., Rolseth, V. & Sterri, S. Three related brain nuclear receptors, NGFI-B, Nurr1, and NOR-1, as transcriptional activators. *Journal of Molecular Neuroscience* **6**, 249–255 (1995).
16. Cheng, L. E., Chan, F. K., Cado, D. & Winoto, A. Functional redundancy of the Nur77 and Nor-1 orphan steroid receptors in T-cell apoptosis. *The EMBO journal* **16**, 1865–75 (1997).
17. Tissing, W. J. E., Meijerink, J. P. P., Boer, M. L. den, Brinkhof, B., Rossum, E. F. C. van, Wering, E. R. van, *et al.* Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. *Clinical cancer research* **11**, 6050–6 (2005).
18. Haarman, E. G., Kaspers, G. J. L., Pieters, R., Rottier, M. M. A. & Veerman, A. J. P. Glucocorticoid receptor alpha, beta and gamma expression vs in vitro glucocorticod resistance in childhood leukemia. *Leukemia* **18**, 530–7 (2004).
19. Holleman, A., Cheok, M. H., Boer, M. L. den, Yang, W., Veerman, A. J. P., Kazemier, K. M., *et al.* Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. *New Engl J Med* **351**, 533–542 (2004).
20. Holleman, A. The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia. *Blood* **107**, 769–776 (2006).
21. Bonapace, L., Bornhauser, B. C., Schmitz, M., Cario, G., Ziegler, U., Niggli, F. K., *et al.* Induction of autophagy-dependent necroptosis is required for childhood acute lymphoblastic leukemia cells to overcome glucocorticoid resistance. *J Clin Invest* **120**, 1310–23 (2010).
22. Ariës, I. M., Hansen, B. R., Koch, T., Evans, W. E., Pieters, R. & Boer, M. L. Den Prednisolone Resistance in Pediatric Acute Lymphoblastic Leukemia Can Be Synergistically Overcome by Inhibition of Anti-Apoptotic MCL1 and Glycolysis [Abstract]. *Blood* **120**, 3528 (2012).
23. Thompson, J. & Winoto, A. During negative selection, Nur77 family proteins translocate to mitochondria where they associate with Bcl-2 and expose its proapoptotic BH3 domain. *The Journal of experimental medicine* **205**, 1029–36 (2008).
24. Mullican, S. E., Zhang, S., Konopleva, M., Ruvolo, V., Andreeff, M., Milbrandt, J., *et al.* Abrogation of nuclear receptors Nr4a3 and Nr4a1 leads to development of acute myeloid leukemia. *Nature medicine* **13**, 730–5 (2007).
25. Brás, A., Albar, J. P., Leonardo, E., Buitrago, G. G. de & Martínez-A, C. Ceramide-induced cell death is independent of the Fas/Fas ligand pathway and is prevented by Nur77 overexpression in A20 B cells. *Cell death and differentiation* **7**, 262–71 (2000).
26. Wu, H., Lin, Y., Li, W., Sun, Z., Gao, W., Zhang, H., *et al.* Regulation of Nur77 expression by -catenin and its mitogenic effect in colon cancer cells. *The FASEB Journal* **25**, 192–205 (2010).
27. Ke, N., Claassen, G., Yu, D.-H., Albers, A., Fan, W., Tan, P., *et al.* Nuclear hormone receptor NR4A2 is involved in cell transformation and apoptosis. *Cancer research* **64**, 8208–12 (2004).
28. Ramirez-Herrick, A. M., Mullican, S. E., Sheehan, A. M. & Conneely, O. M. Reduced NR4A gene dosage leads to mixed myelodysplastic/myeloproliferative neoplasms in mice. *Blood* **117**, 2681–90 (2011).

Supplemental Table 1. Antibodies RPPA and Western Blot

Antigen	Manufacturer (#)	Blocking, Dilution, Conditions
β-Actin	Abcam #ab8226	5% BSA or 5% milk in TBST, 1:20000, 1 hour room temperature
Nur77	Cell Signaling #3960	5% BSA in TBST, 1:500 , 2 hour room temperature
Nurr1	Sigma #N6413	5% milk in TBST, 1:1000, overnight 4 °C
Nor1	Provided by Prof C. de Vries [11]	5% BSA in TBST, 1:1000, overnight 4 °C
Secondary antibodies	Li-COR, #926-32210, #926-32211, #926-32220	1:5000 – 1:10000 in same solution as primary antibody, 1 hour room temperature

Supplemental Table 2. siRNA sequences

siRNA	Sense	Antisense	Label
siNR4A1	GAGGGAGAGAGCUAUUCCAUGCCUA	UAGGCAUGGAAUAGCUCUCUCCCUC	5' Fluorescein
siNR4A2	CCCUGGAAAUAACUGAGCACUUUGA	UCAAAGUGCUCAGUUAUUUCCAGGG	5' Cy3
siNR4A3	ACUCAACACCCAGAGAUCUUGAUUA	UAAUCAAGAUCUCUGGGUGUUGAGU	5'Cy5
siScrl	GGACAAGGCCAUGCAAUUGGUACAA	UUGUACCAAUUGCAUGGCCUUGUCC	5' Cy5

Supplemental Table 3. Primer sequences

Gene	Forward (5' – 3')	Reverse (5' – 3')
NR4A1	GGG GAG GGA GAG AGC TAT	TTA GCC AGG CAG ATG TAC TT
NR4A2	GCG AAC CCT GAC TAT CAA	AGG AGA AGG CAG AAA TGT C
NR4A3	ACG TCG AAA CCG ATG TCA GTA	GGC TGA GAA GGT TCC TGT TGT A
RPS20	AAG GGC TGA GGA TTT TTG	CGT TGC GGC TTG TTA G

Supplemental Table 4.

NR4A1, NR4A2, NR4A3 microarray expression and patient characteristics of 176 pediatric BCP-ALL patients.

Patient	Treatment	Genetic	LC50	Score	NR4A1	NR4A2	NR4A3
Number	Protocol	Subtype	Prednisolone µg/ml	Prednisolone	202340_x_at	204621_s_at	209959_at
1	COALL	HD	0,106	2	11,30	11,20	9,59
2	COALL	HD	2,287	2	11,47	11,18	8,13
3	COALL	BAL	0,337	2	11,30	11,13	9,97
4	COALL	BO	0,334	2	11,08	11,52	9,61
5	COALL	BAL	250,000	3	10,95	10,84	8,25
6	COALL	TA	0,042	1	10,09	10,48	9,87
7	COALL	BAL	250,000	3	10,62	11,70	12,34
8	COALL	BO	0,225	2	11,77	11,36	10,06
9	COALL	HD	14,844	2	10,01	10,63	10,01
10	COALL	MLL	0,283	2	10,55	11,33	11,17
11	COALL	HD	0,450	2	11,89	12,18	11,26
12	COALL	TA	0,020	1	9,36	8,48	8,35
13	COALL	HD	250,000	3	10,65	10,90	9,65
14	COALL	BAL	250,000	3	9,08	9,77	7,39
15	COALL	BAL	250,000	3	12,47	12,74	12,01
16	COALL	BAL	195,313	3	10,46	10,27	9,48
17	COALL	TA	228,966	3	11,37	9,92	10,49
18	COALL	BO	154,018	3	10,10	11,06	8,76
19	COALL	BAL	250,000	3	10,83	9,85	8,96
20	COALL	TA	250,000	3	10,82	9,97	10,88
21	COALL	BO	250,000	3	10,49	10,97	9,62
22	COALL	BAL	250,000	3	11,33	10,91	8,55
23	COALL	BO	250,000	3	11,72	11,12	11,90
24	COALL	TA	0,031	1	9,32	8,91	8,49
25	COALL	BO	0,040	1	10,12	8,05	8,35
26	COALL	TA	0,068	1	10,94	10,43	10,75
27	COALL	BAL	27,107	2	10,30	9,76	10,18
28	COALL	BO	0,014	1	9,59	8,84	11,53
29	COALL	HD	250,000	3	11,28	11,48	10,93
30	COALL	BO	0,031	1	11,18	9,57	7,03
31	COALL	TA	250,000	3	12,43	11,41	12,22
32	COALL	TA	250,000	3	9,43	9,41	8,86
33	COALL	BO	17,578	2	10,97	8,93	7,32
34	COALL	TA	0,008	1	9,22	8,71	7,70
35	COALL	BAL	0,324	2	11,66	11,26	10,46
36	COALL	E2A	0,030	1	10,88	9,27	10,20
37	COALL	BAL	0,061	1	9,25	8,96	8,78
38	COALL	HD	0,026	1	9,00	9,51	7,65
39	COALL	BA	0,032	1	11,46	12,07	8,67
40	COALL	BO	0,024	1	9,39	10,36	7,61
41	COALL	TA	0,040	1	10,47	10,50	8,89
42	COALL	BO	0,243	2	10,69	9,87	10,53
43	COALL	TA	0,057	1	10,51	10,34	11,12
44	COALL	HD	0,280	2	9,50	9,58	8,42
45	COALL	BO	0,028	1	8,35	8,91	7,02

46	COALL	TA	0,384	2	9,70	9,30	9,62
47	COALL	BAL	0,275	2	10,12	9,63	8,13
48	COALL	TA	0,045	1	10,06	9,23	9,24
49	COALL	BAL	0,553	2	11,39	11,59	10,64
50	COALL	TA	0,302	2	8,94	8,94	7,87
51	COALL	BAL	0,583	2	11,46	11,88	9,92
52	COALL	TA	0,247	2	9,77	9,67	9,69
53	COALL	TA	0,091	1	8,94	7,06	6,55
54	COALL	TA	0,397	2	9,61	7,78	9,56
55	COALL	BO	250,000	3	11,49	12,05	10,79
56	COALL	HD	0,306	2	10,49	11,00	9,97
57	COALL	HD	0,208	2	8,08	8,33	8,29
58	COALL	NA	0,271	2	10,39	10,28	9,12
59	COALL	HD	0,421	2	10,89	11,25	7,87
60	COALL	HD	0,417	2	10,07	9,68	9,13
61	COALL	BO	250,000	3	10,53	9,87	8,61
62	COALL	TA	0,050	1	8,59	7,97	8,15
63	COALL	HD	0,253	2	11,08	9,99	8,29
64	COALL	TA	0,427	2	11,65	9,73	9,44
65	COALL	BA	3,621	2	7,87	9,06	6,94
66	COALL	BO	3,871	2	11,31	10,17	9,18
67	COALL	BO	0,074	1	8,63	9,52	8,58
68	COALL	BO	0,179	2	11,68	11,66	11,47
69	COALL	TA	154,891	3	8,92	8,80	8,40
70	COALL	TA	0,133	2	9,28	8,98	8,15
71	COALL	BAL	0,039	1	7,14	8,20	6,28
72	COALL	HD	250,000	3	10,92	12,03	11,45
73	COALL	BO	0,023	1	8,02	8,29	9,01
74	COALL	TA	0,008	1	8,08	8,49	7,22
75	COALL	HD	0,034	1	10,23	10,07	7,95
76	COALL	TA	250,000	3	10,30	9,59	10,24
77	COALL	BO	250,000	3	10,26	11,14	10,56
78	COALL	BO	0,009	1	8,53	6,82	8,50
79	COALL	HD	0,025	1	11,77	11,56	10,76
80	COALL	HD	0,058	1	10,11	9,06	8,55
81	COALL	BO	0,008	1	10,92	11,92	11,87
82	COALL	HD	0,008	1	10,39	9,78	7,88
83	COALL	TA	0,346	2	10,17	10,44	10,30
84	COALL	HD	0,199	2	10,49	10,40	7,27
85	COALL	HD	0,049	1	9,75	10,70	7,02
86	COALL	HD	0,059	1	11,63	11,44	9,98
87	COALL	HD	250,000	3	9,30	9,09	7,95
88	COALL	HD	0,040	1	10,67	9,84	8,38
89	COALL	TA	0,053	1	10,28	9,25	10,32
90	COALL	BAL	0,338	2	10,67	11,10	10,75
91	COALL	HD	0,057	1	10,49	9,67	9,10
92	COALL	BAL	0,034	1	11,35	11,45	8,88
93	COALL	BO	250,000	3	10,21	10,91	9,83
94	COALL	TA	0,026	1	9,75	7,31	7,34
95	COALL	BO	0,046	1	7,41	6,70	7,01

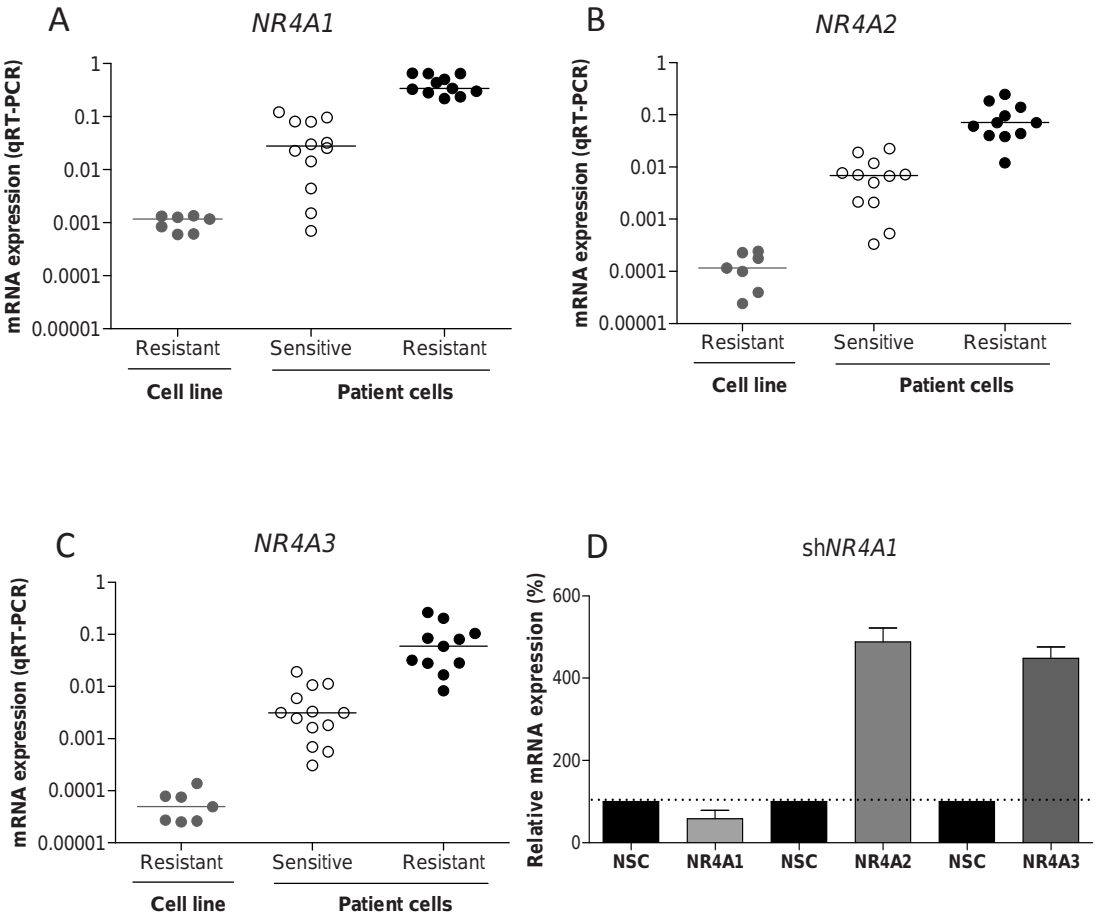
96	COALL	HD	210,938	3	11,69	11,76	11,13
97	COALL	TA	0,025	1	11,63	10,86	12,34
98	COALL	BO	2,767	2	10,82	11,14	9,91
99	COALL	BO	250,000	3	7,26	7,52	6,86
100	COALL	BO	0,429	2	9,12	9,67	9,70
101	COALL	BO	2,197	2	9,78	10,77	7,13
102	COALL	HD	0,129	2	9,31	9,55	6,86
103	COALL	BAL	172,176	3	9,37	9,77	8,08
104	COALL	TA	0,008	1	7,61	8,53	6,67
105	COALL	TA	0,025	1	11,27	9,35	11,33
106	COALL	HD	0,015	1	10,87	10,83	11,22
107	COALL	BAL	0,106	2	10,20	10,57	7,68
108	COALL	HD	0,011	1	11,60	11,59	9,59
109	COALL	E2A	250,000	3	9,84	9,69	10,16
110	COALL	BO	0,008	1	8,97	8,73	10,52
111	COALL	HD	0,050	1	9,92	10,46	9,24
112	COALL	BAL	0,027	1	9,37	9,51	8,22
113	COALL	BAL	0,054	1	10,42	10,10	6,91
114	COALL	HD	206,250	3	10,90	11,78	10,47
115	COALL	TA	0,465	2	10,76	9,04	10,87
116	COALL	TA	250,000	3	10,93	9,90	12,32
117	COALL	BO	0,045	1	7,68	9,82	7,77
118	COALL	BAL	2,608	2	11,55	11,23	11,60
119	COALL	HD	0,186	2	10,57	10,90	8,02
120	COALL	TA	0,049	1	11,35	11,84	12,26
121	COALL	HD	0,028	1	9,06	9,61	7,16
122	COALL	HD	0,049	1	10,82	11,76	8,73
123	COALL	BO	0,042	1	9,99	10,50	6,45
124	COALL	HD	0,038	1	10,12	10,75	8,37
125	COALL	BO	250,000	3	11,22	11,20	12,08
126	COALL	HD	250,000	3	11,71	9,92	10,09
127	COALL	BAL	0,409	2	10,72	11,05	12,01
128	COALL	HD	250,000	3	11,12	11,32	8,47
129	COALL	TA	0,131	2	9,24	8,86	8,65
130	COALL	TA	0,054	1	9,01	8,72	8,00
131	COALL	TA	0,173	2	9,13	9,04	9,45
132	COALL	TA	0,032	1	8,96	8,59	7,43
133	COALL	TA	0,396	2	11,23	9,66	10,80
134	COALL	TA	0,175	2	7,19	6,84	5,97
135	DCOG	E2A	0,008	1	9,68	6,95	8,31
136	DCOG	HD	0,052	1	10,47	10,74	8,38
137	DCOG	TA	0,040	1	11,40	8,58	9,81
138	DCOG	TA	0,060	1	8,70	7,84	6,29
139	DCOG	BO	0,054	1	8,01	8,12	7,53
140	DCOG	MLL	250,000	3	10,01	8,49	7,91
141	DCOG	BO	0,036	1	8,58	7,30	8,08
142	DCOG	HD	3,626	2	7,66	7,60	5,86
143	DCOG	TA	0,219	2	7,44	5,72	6,36
144	DCOG	HD	0,357	2	9,14	8,76	7,08
145	DCOG	TA	0,090	1	7,15	6,94	6,05

146	DCOG	TA	250,000	3	8,87	7,63	7,49
147	DCOG	TA	250,000	3	11,58	11,85	12,41
148	DCOG	BO	250,000	3	8,74	8,26	6,59
149	DCOG	TA	0,159	2	7,74	7,28	6,30
150	DCOG	TA	250,000	3	12,40	12,04	13,31
151	DCOG	TA	0,008	1	8,27	7,48	6,44
152	DCOG	HD	0,208	2	11,31	11,41	10,04
153	DCOG	TA	0,061	1	7,04	5,03	6,25
154	DCOG	BA	0,048	1	10,77	10,29	10,80
155	DCOG	BAL	250,000	3	12,47	13,08	12,89
156	DCOG	BAL	2,846	2	12,00	12,11	10,26
157	DCOG	HD	0,330	2	10,58	9,77	7,29
158	DCOG	E2A	0,008	1	6,96	5,40	5,91
159	DCOG	HD	0,008	1	10,41	10,93	8,52
160	DCOG	HD	250,000	3	10,38	10,46	9,03
161	DCOG	HD	0,151	2	9,69	9,25	7,75
162	DCOG	E2A	0,087	1	9,47	10,21	8,02
163	DCOG	TA	0,102	2	10,50	9,40	9,39
164	DCOG	TA	250,000	3	10,13	8,49	8,34
165	DCOG	TA	0,047	1	10,39	9,54	8,94
166	DCOG	HD	0,050	1	11,14	11,60	10,15
167	DCOG	HD	132,523	2	11,21	11,18	9,20
168	DCOG	HD	0,041	1	8,55	8,25	6,07
169	DCOG	TA	250,000	3	11,14	11,21	10,61
170	DCOG	NA	0,061	1	11,34	7,88	9,13
171	DCOG	BO	0,008	1	8,30	7,03	6,52
172	DCOG	TA	0,041	1	10,87	10,37	11,21
173	DCOG	HD	0,427	2	9,05	8,97	7,58
174	DCOG	TA	0,343	2	11,26	9,74	9,84
175	DCOG	BO	12,600	2	7,87	6,75	6,77
176	DCOG	HD	2,158	2	10,04	9,34	9,28

Genetic Subtype: HD=hyperdiploid, TA=ETV6-RUNX1+, BA=BCR-ABL1+, BAL= BCR-ABLLike, E2A=TCF3-PBX1+, MLL=MLL rearranged, BO= B-other, negative for aforementioned lesions

Prednisolone score: 1 sensitive = ≤0.1 µg/ml, 2 intermediate=<0.1-<150 µg/ml, 3 resistant =≥150 µg/ml

Supplemental Figure 1.



Supplemental Figure 1.
Knockdown of *NR4A1* induces strong upregulation of *NR4A2* and *NR4A3*.
A-C. *NR4A1*, *NR4A2* and *NR4A3* mRNA expression was evaluated by qRT-PCR in 12 *in vitro* prednisolone sensitive patients, 11 *in vitro* prednisolone resistant patients' and several timepoints of an *in vitro* prednisolone resistant BCP-ALL cell line Reh (*ETV6-RUNX1*$^{+}$). **D.** mRNA expression of *NR4A1*, *NR4A2* and *NR4A3* was assessed 96 hours after lentiviral sh*NR4A1* knockdown, corrected for RPS20 expression and relative to a non-silencing control short hairpin (NSC). Data are presented as mean plus SEM of two independent experiments.

TOWARDS PERSONALIZED THERAPY IN PEDIATRIC ACUTE LYMPHOBLASTIC LEUKEMIA; RAS MUTATIONS AND PREDNISOLONE RESISTANCE

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Submitted

ABSTRACT

The 5-year event-free survival of pediatric precursor-B acute lymphoblastic leukemia (BCP-ALL) has currently reached 80-90%. Targeted drugs are warranted to cure the remaining 10-20%. Prednisolone has been shown a pivotal drug and resistance remains an adverse prognostic factor in BCP-ALL. In this study we demonstrate that prednisolone downregulated the activity of the RAS-RAF-MEK pathway and STAT6 in prednisolone sensitive patients cells, but was not capable to do so in prednisolone resistant patients. In addition, we identified activating RAS-pathway-mutations (NRAS, KRAS, PTPN11 and FLT3) in 10 out of 26 patients (38%) of which 6 cases harboured ≥ 2 RAS-mutations predominantly associated with presence of minor clones (<30%). Exposure of primary patients' ALL samples to Trametinib (MEK-inhibitor), Sorafinib (BRAF-inhibitor), AS1517499 (STAT6-inhibitor) or Crizotinib (cMET-inhibitor) as single agents did not affect leukemic cell survival. However, Trametinib was impressively potent when combined with prednisolone eradicating all leukemic cells of the NRAS/KRAS-mutated BCP-ALL patients. In one NRAS/KRAS-mutated case Sorafenib but not Trametinib sensitized to prednisolone significantly. In conclusion, our data implicate that BCP-ALL patients should be screened for RAS-activating-mutations as these patients may benefit from treatment with Trametinib/Sorafenib combined with prednisolone. We furthermore advise to perform *in vitro* inhibitor assays in RAS-mutated BCP-ALL patients' cells to elucidate which inhibitor may be the most effective in individual patients.

INTRODUCTION

Optimization of treatment protocols and improved risk stratification have enhanced event-free survival rates in pediatric precursor-B acute lymphoblastic leukemia (BCP-ALL) up to 80-90% [1]. Remarkably, these results are obtained without changing the core chemotherapeutic drugs that have been used for decades, including prednisolone, L-asparaginase and vincristine. To cure the remaining 20% of patients and to reduce long-term side effects in survivors, more personalized targeted therapy is warranted. The remarkable improvement of survival in *BCR-ABL1*-positive pediatric BCP-ALL with the addition of the ABL1 tyrosine kinase inhibitor dasatinib and imatinib [2–4] to current treatment protocols is a key-example and encourages further investigation of targeted therapy. Optimized high-throughput screening has brought tailored therapy a step closer. Recently, high-throughput sequencing in 187 high-risk BCP-ALL cases identified a high frequency of recurrent alterations in crucial signaling pathways, including B-cell development/differentiation (68% of cases), the TP53/RB tumor suppressor pathway (54%), RAS signaling (50%), and Janus kinases (11%) [5]. These results are remarkable, although the clinical and biologic significance has yet to be investigated.

Novel targeted drugs will most likely serve as adjuvants to current chemotherapy regimens. Prednisolone has been shown to be the most pivotal in treating pediatric BCP-ALL, as *in vivo* and *in vitro* response to prednisolone is an important predictor for long-term clinical outcome[6]. Moreover, relapsed leukemic cells gain prednisolone resistance [7]. Hence, to improve clinical outcome drugs need to be found which reverse resistance to prednisolone. Prednisolone is a glucocorticoid that binds the glucocorticoid receptor, which acts as a transcription factor regulating the expression of numerous genes eventually leading to apoptosis. Eight hours of prednisolone exposure of sensitive BCP-ALL cells already results in differential expression of 51 genes [8]. In this study we hypothesized that prednisolone resistance in BCP-ALL may be caused by dysregulation of prednisolone responsive survival proteins. We aimed to target these unresponsive proteins *in vitro* with targeted inhibitors to increase sensitivity to prednisolone leading to cytotoxicity of otherwise resistant leukemic cells.

MATERIALS AND METHODS

Processing of patients' leukemic cells

Bone marrow samples were collected from children with newly diagnosed ALL after written consent as approved by the institutional review board. Mononuclear cells were isolated by lymphoprep density gradient centrifugation, as previously described [9]. Only leukemic samples with ≥ 90% leukemic blasts were used in the present study. If applicable, enrichment of leukemic blasts was achieved with immunomagnetic beads. Each patient was examined for the following genomic lesions, i.e. hyperdiploid (>50 chromosomes), *ETV6-RUNX1*$^{+}$, *TCF3-PBX1*$^{+}$, *MLL*-rearrangement, *BCR-ABL1*$^{+}$ and *BCR-ABL1*$^{+}$-like by means of FISH, PCR and by utilizing the 110-probeset classifier [10]. Patients negative for aforementioned genomic abberations or signature were named B-other. Cells were cultured in RPMI Dutch modification (Gibco) supplemented with 0.1% insulin-transferrin-sodium selenite (Sigma), 0.4 mM glutamine (Invitrogen), 0.25 µg/ml gentamycine (Gibco), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 0.125 µg/ml fungizone (Gibco) and 20% fetal calf serum (Integro) at 37°C in humidified air containing 5% CO_2.

Reverse Phase Protein Array

Proteins were isolated from 1) unexposed primary BCP-ALL cells obtained at initial diagnosis 2) normal mononuclear cells obtained from non-leukemic pediatric bone marrow samples and 3) primary BCP-ALL cells that were exposed for 48h to 0 µg/ml, 1 µg/ml or 250 µg/ml prednisolone. Proteins were isolated with protein lysis buffer containing 25 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 1% Triton X-100, 10% glycerol, 10 mM sodium-pyrophosphate, 1 mM sodium-orthovanadate, 10 mM glycerolphosphate, DTT, phenylmethylsulfonyl fluoride, aprotinin and sodium-fluoride. Protein concentration was quantified by means of the BCA assay (Pierce). Hereafter, lysates were spotted twice in triplicate on glass-backed nitrocellulose-coated array slides by the facility of Dr. E. F. Petricoin, George Mason University, Manassas, USA. Slides were subsequently stained with indicated antibodies, incubated with a biotinylated secondary antibody and scanned using the NovaRay scanner. The MicroVigene Software was used to calculate protein levels relative to the total amount of protein per sample. Antibodies used were: phospho-STAT6(Y641) (Cell signaling (CS) #9361), phospho-MET(Y1234-1235) (CS #3126), phospho RAS-GRF1(S916) (CS #3321), phospho-ARAF(S299) (CS #4431), phospho-BRAF(S455) (CS #2696), phospho-CRAF(S338) (CS #9427), phospho-MEK1/2(S217-221) (CS #9121), phospho-AKT(S473) (CS #9271), phospho-NFκB(S536) (CS #3031), phospho-p38MAPK(T180-Y182) (CS #9211), phospho-SAPK-JNK(T183-Y185) (CS #9251), phospho-JAK2(Y1007) (CS

#3771), phospho-TYK2(Y1054/55) (CS #9321), phospho-STAT5(Y694) (CS #9351), phospho-P70S6K(T389) (CS #9208), phospho-CREB(S133) (CS #9191) and phospho-PLCgamma2(Y759) (CS #3874).

Western Blot

Proteins were isolated (see reverse phase protein array for method) from primary patients' cells treated for 4 days with the indicated inhibitor. There were only enough leukemic cells of one patient (patient D) for extensive western blotting studies. Protein samples (total 25 µg) were loaded on pre-cast gels and transferred to nitrocellulose membranes (Bio-Rad). Blots were blocked and probed with the following antibodies; phospho-MEK1/2(S217-221) (CS #9121), phospho-ERK1/2(Thr202/Tyr204) (CS #9101), phospho-AKT(S473) (CS #9271), phospho-BRAF(S455) (CS #2696), and β-Actin (Abcam, ab6276). Hereafter, protein levels were quantified using the Odyssey 3.0 application software (Li-COR).

Ion Torrent deep sequencing

DNA was extracted from leukemic blasts (≥ 90% purity) by means of Trizol isolation according to the manufacturer's protocol (Invitrogen). DNA concentration was determined by the Quant-it picogreen method (Invitrogen). Deep sequencing was performed on the Ion PGM using the Ion AmpliSeq Library Kit 2.0, the Ion AmpliSeq Cancer Panel Primer Pool and Ion Xpress Barcode adapters 1-32 (Life Technologies). The multiplexed PCR covered several hotspot mutations in BRAF, NRAS, HRAS, KRAS, PTPN11, FLT3 and cMET, as was reported in the Cosmic database (Supplemental Table 1). A maximum of 16 indexed samples were pooled in equimolar fashion and sequenced on an Ion Torrent 318B chip using the 200bp sequencing chemistry according to manufacturer's protocol. Sequences were analyzed using the Torrent_Suite 3.4.2 software (variant caller v3.4.51874). Variants were annotated using an in-house developed pipeline using the Ensembl databases (www.ensembl.org).

MTT assay

Cytotoxicity of prednisolone (Bufa Pharmaceutical Products) in primary patients' cells (as indicated in Table 1) was determined by the *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) drug-resistance assay after 4-days of exposure, as previously described[9]. Optical density values were measured on the Versamax (Molecular Devices) at λ=562 nm and λ=720nm. *In vitro* prednisolone sensitivity was defined by a concentration of prednisolone lethal to 50% of the cells (LC_{50}) below 0.1 µg/mL and prednisolone resistance was defined by a LC_{50} value above 150 µg/mL as shown previously to be predictive for clinical outcome in

pediatric ALL [9,11,12]. Cytotoxicity to Trametinib, Sorafenib, Crizotinib (Selleckchem) and AS1517499 (Axon Medchem) in primary patients' cells was determined by the MTT assay after 4-days of exposure. These inhibitors were dissolved in 100% DMSO and were tested in a serial dilution ranging between 0.0002 and 20 μM. Cytotoxicity of these inhibitors together with prednisolone was determined after 4 days by the MTT assay. Prednisolone dose-response curves were corrected for loss of cell viability caused by the inhibitors and the solvent itself.

Statistical Analysis

Prednisolone-induced changes in protein expression were analyzed with a Kruskal-Wallis test. A T-test was used to compare data obtained in resistant and sensitive patients, and to test the prednisolone sensitizing effects of inhibitors on cell viability compared to vehicle control. The dose-response curves of prednisolone in combination with an inhibitor was analyzed by two-way ANOVA, testing the interaction between inhibitor*prednisolone. A p-value below 0.05 was considered statistically significant.

RESULTS

Prednisolone-responsive survival proteins are dysregulated in prednisolone resistant primary patients' cells

To study our hypothesis that prednisolone resistance is caused by dysregulation of prednisolone-responsive survival proteins, we analyzed phosphorylation levels of 17 key-signaling proteins in leukemic cells obtained from *in vitro* prednisolone sensitive and resistant ALL patients (Table 1, column 2). We observed that basal phosphorylation levels of these 17 proteins did not differ between prednisolone resistant and sensitive patients' cells (Supplemental Figure 1A). Exposure to prednisolone for 48h induced discrepant phosphorylation changes in 6 out of 17 proteins between prednisolone resistant and sensitive cases. Prednisolone downregulated the activity of the RAS-RAF-MEK pathway in prednisolone sensitive patients cells but not in resistant patients (Figure 1A-D). Phosphorylation levels of RAS, ARAF, BRAF, MEK1/2 and also STAT6 were 2.5-fold, 8.1-fold, 2.3-fold, 2.9-fold and 3.1-fold ($p<0.01$), respectively higher in prednisolone resistant compared to sensitive patients' cells after prednisolone exposure (Figure 1A-E). In contrast, a 3.8-fold induction of cMET phosphorylation was observed in resistant patients' cells after prednisolone exposure, whereas no change was observed in sensitive cells (Figure 1F). In summary, we observed a dysregulation of RAS-RAF-

MEK, STAT6 and cMET phosphorylation upon prednisolone exposure specifically in leukemic cells of prednisolone resistant patients.

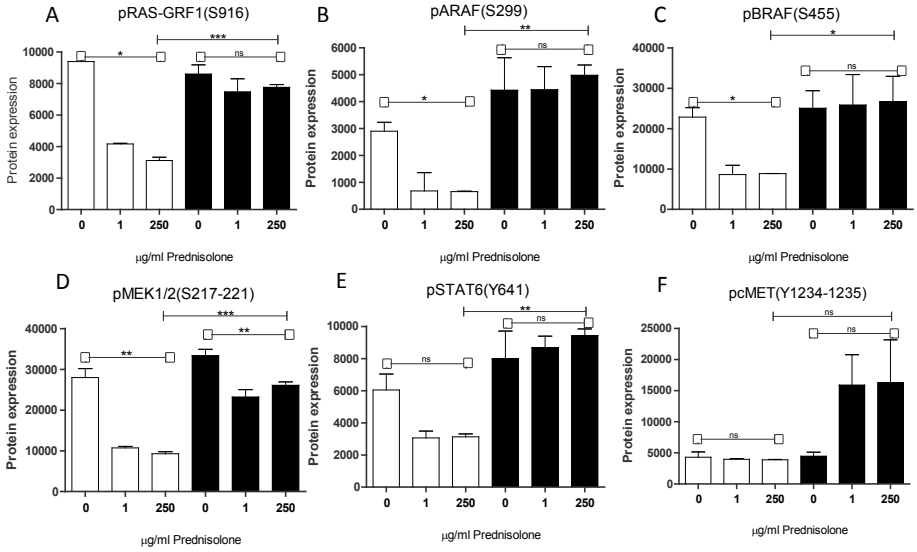

Figure 1. Prednisolone mediated dysregulation of pivotal survival proteins is impaired in prednisolone resistant primary patient cells.

(A-F) Protein phosphorylation was analyzed by means of reverse phase protein array in three pediatric BCP-ALL cases with *in vitro* prednisolone sensitive and three cases with *in vitro* resistant leukemic cells. Samples were exposed for 48h to 0 µg/ml, 1 µg/ml prednisolone or 250 µg/ml prednisolone. Data are presented as mean plus SEM of three independent patients' samples. (A Kruskal-Wallis test was used to compare 0, 1, 250 µg/ml data points indicated by ⊓–⊓ and a T-test was used to compare data between sensitive and resistant patients indicated by |–| *p<0.05, **p<0.01, ***p<0.001, ns=not significant, A.U. Arbitrary units.)

RAS pathway mutations in pediatric BCP-ALL and *in vitro* prednisolone resistance

The finding that prednisolone is incapable of downregulating the activity of RAS-RAF-MEK pathway in prednisolone resistant patients, prompted us to investigate the presence of RAS-pathway activating mutations. These mutations are known to induce constitutive RAS signaling which results in a survival advantage of cancer cells [13]. We investigated hotspot regions of mutations in the protein coding domains of BRAF, NRAS, HRAS, KRAS, PTPN11 and FLT3 as documented in the COSMIC database (Supplemental Table 1) with Ion Torrent deep-sequencing (~1000x read depth) in 26 pediatric BCP-ALL patients with known genetic subtype and *in vitro* prednisolone response (Table 1). In addition, we examined in previously described

hotspot regions the presence of mutations in cMET (Supplemental Table 1) but no cMET mutations were found. 10 out of 26 patients (38%, Table 1) carried activating NRAS and/or KRAS mutations or additional mutations in the FLT3 receptor or regulatory PTPN11 (SHP2) known to trigger RAS-MEK pathways in hematopoietic cells [14,15]. Single heterozygous NRAS/KRAS-mutations were found in four cases with one dominant clone, representing 50-100% of cells (Table 1, patient 3, 5, 6 and 8). Two or more RAS-pathway mutations were found in six cases (Table 1, patient 1, 2, 4, 7, 9 and 10). Of interest, these mutations were often found in subclones as small as 5%. We identified 9 activating point mutations in NRAS, of which 6 in exon 1 (4 in codon 12 and 2 in codon 13) and 3 in exon 2 (2 in codon 61 and 1 in codon 64). In KRAS we found 8 activating point mutations in exon 1 (codon 12 and 13) and none in exon 2 (codon 61). The activating mutations found in PTPN11 comprised codon 69 and FLT3 codon 835. We did not find mutations in BRAF and HRAS. Interestingly, we detected more often RAS activating mutations in prednisolone resistant compared to sensitive patients (54% vs. 23% Table 2). This difference was not statistically different (Fisher's exact test: p=0.2), this might be due to limited sample size (n=13 vs. n=13). Furthermore, the mutations did not present in one specific genetic subtype (Table 2) nor did the two patients that relapsed in this small cohort harbour a RAS-mutation (Table 1). Collectively, we have found a high frequency (38%) of RAS-mutations in BCP-ALL cells taken at initial diagnosis, 60% consisted of several subclones and the presence of these mutations seems associated with prednisolone resistance of leukemic cells.

Inhibitors against MEK1/2, BRAF, STAT6, cMET do not reduce viability of patients' leukemic cells

We studied the effect of specific inhibitors directed against MEK1/2 (Trametinib), BRAF (Sorafenib), STAT6 (AS1517499) and cMET (Crizotinib) (Supplemental Table 2) in five *in vitro* prednisolone resistant BCP-ALL patients' samples (Table 1, column 3) expressing high levels of the targeted proteins in leukemic cells compared to normal mononuclear bone marrow cells (Figure 2A). Trametinib, Sorafenib, AS1517499 or Crizotinib did not induce substantial cell death in prednisolone resistant leukemic patients' cells (Fig. 2B-E). Western blot analysis verified that Trametinib specifically decreased phospho-ERK and Sorafenib decreased phospho-BRAF (Figure 2F). Western blot analysis also verified that AS1517499 specifically decreased phospho-AKT and Crizotinib decreased phospho-MEK1/2 and phospho-AKT, which are known downstream targets of STAT6[16–18] and cMET[19,20], respectively (Figure 2F).

Table 1. Patients' characteristics and mutations.

Patient Number	Pred exposure Protein Study	Inhibitor Study	Genetic Subtype	LC50 Prednisolone µg/ml	Category Prednisolone	Treatment Protocol	Risk-group	Non-response	Relapse	Death	EFS	Mutated Gene	Genomic Variant	Protein Variant	Coverage Depth	Heterozygous Frequency	% Mutated cells
1			BO	>250	Resistant	COALL03	HR	0	0	0	4.54	NRAS	T/C	Q61R	1163	13.59	27.18
												NRAS	C/T	G13D	1534	2.48	4.96
												FLT3	A/C	D835E	2042	12.10	24.2
												PTPN11	G/A	E69K	1913	6.53	13.06
2			BO	>250	Resistant	COALL03	HR-S	0	0	0	3.75	NRAS	C/A	G13V	1570	43.89	87.78
												NRAS	C/T	G12D	1562	5.12	10.24
3			BO	>250	Resistant	COALL03	HR-S	0	0	0	6.34	KRAS	C/T	G12D	1073	45.29	90.58
4		X Patient A	BO	>250	Resistant	ALL10	MR	0	0	0	4.48	KRAS	C/T	G13D	613	13.7	27.4
												NRAS	C/T	G12D	1109	6.85	13.7
												NRAS	T/C	Q61R	1012	2.47	4.94
5			BAL	>250	Resistant	COALL97	HR-S	0	0	0	12.36	KRAS	C/T	G12D	666	35.14	70.28
6		X Patient B	ER	>250	Resistant	COALL03	LR-I	0	0	0	7.45	KRAS	C/G	G12R	1010	51.58	103.16
7	X	X Patient C	ER	154	Resistant	COALL03	LR-I	0	0	0	4.79	NRAS	C/A	G12V	1523	35.00	70.00
												KRAS	C/T	G12S	616	7.95	15.9
8			BAL	0.06	Sensitive	COALL03	HR-S	0	0	0	5.83	KRAS	C/T	G12S	624	27.56	55.12
9	X		ER	0.04	Sensitive	COALL03	HR-S	0	0	0	5.48	KRAS	C/A	G12V	1096	29.56	59.12
												NRAS	A/C	Y64D	1231	10.07	20.14
10			ER	0.06	Sensitive	COALL03	LR-S	0	0	0	5.48	KRAS	C/T	G12S	532	12.41	24.82
												NRAS	C/T	G12S	1306	2.83	5.66
11	X		BO	>250	Resistant	COALL03	HR-S	0	0	0	5.05	wildtype					
12			BAL	>250	Resistant	ALL10	MR	0	0	0	4.08	wildtype					
13			BAL	195	Resistant	COALL03	HR-S	0	0	0	8.84	wildtype					
14		X Patient D	ER	>250	Resistant	ALL9	/	0	0	0	/	wildtype					
15			ER	>250	Resistant	COALL03	LR-S	0	0	0	5.99	wildtype					
16	X	X Patient E	ER	>250	Resistant	ALL10	MR	0	0	0	4.78	wildtype					
17	X		BO	0.07	Sensitive	COALL03	HR-S	0	0	0	4.84	wildtype					
18			BO	0.05	Sensitive	COALL03	LR-R	0	0	0	3.87	wildtype					
19			BO	0.01	Sensitive	ALL10	MR	0	0	0	2.86	wildtype					
20			BO	0.04	Sensitive	COALL03	HR-S	0	1	0	2.23	wildtype					
21			BAL	0.03	Sensitive	COALL03	HR-S	0	0	0	4.00	wildtype					
22			BAL	0.04	Sensitive	COALL03	HR-S	0	0	0	4.64	wildtype					
23	X		BAL	0.05	Sensitive	COALL03	HR-S	0	1	0	1.08	wildtype					
24			ER	0.01	Sensitive	COALL03	LR-R	0	0	0	5.96	wildtype					
25			ER	0.05	Sensitive	COALL03	LR-R	0	0	0	4.93	wildtype					
26			ER	0.01	Sensitive	ALL10	SR	0	0	0	4.33	wildtype					

Subtype: BO=B-other (negative for hyperdiploidy, ETV6-RUNX1+, BCR-ABL1+, BCR-ABL1-Like, TCF3-PBX1+, MLL rearranged), ER=ETV6-RUNX1+, BAL=BCR-ABL1-like ; LC50 prednisolone (µg/ml): Prednisolone concentration (µg/ml) that killed 50% of leukemic cells ; Category Prednisolone: sensitive ≤0.1 µg/ml, resistant ≥150 µg/ml [9] ; Riskgroup: HR=high risk, MR=medium risk, LR=low risk, S=standard protocol, I=Intensified protocol, R=Reduced protocol ; EFS=Event-free survival (years) ; Non-response, relapse, death: 0=no event, 1=event ; Coverage depth: Number of reads.

Table 2. Frequency of RAS mutations in relation to prednisolone sensitivity.

In vitro prednisolone response	RAS-mutations			
	B-other	*BCR-ABL1*-like	*ETV6-RUNX1+*	Total
Sensitive	0% (0/4 patients)	25% (1/4 patients)	40% (2/5 patients)	23% (3/13 patients)
Resistant	80% (4/5 patients)	33% (1/3 patients)	40% (2/5 patients)	54% (7/13 patients)
Total	44% (4/9 patients)	29% (2/7patients)	40% (4/10 patients)	38% (10/26 patients)

Trametinib (MEK inhibitor) and Sorafenib (BRAF inhibitor) restore prednisolone sensitivity in RAS mutated patients

We next evaluated the prednisolone sensitizing properties of these inhibitors in primary BCP-ALL patients' cells (Figure 3, Supplemental Figure 2, Tabel 1). Remarkably, we observed a complete sensitization to prednisolone after Trametinib treatment in patient 2 and 3 (Figure 3B1-C2 $p<0.01$). 100% of the leukemic cells of patient 2 harboured a G12R mutated KRAS and 70% and 16% of leukemic cells of patient 3 had a G12V NRAS and G12S KRAS mutation, respectively (Table 1). Trametinib was not effective in patient 1 of which 27% of the cells harboured a G13D KRAS activating mutation, and 14% and 5% of leukemic cells harboured G12D and Q61R NRAS mutations, respectively (Figure 3A1 and Table 1). Interestingly, this patient was the only patient in which Sorafenib sensitized cells to prednisolone up to 61% compared to prednisolone and vehicle control (Figure A1-A2, $p=0.001$). Trametinib also had prednisolone sensitizing effects in the RAS-wildtype patients, although less pronounced compared to the effects seen in RAS-mutated cases. (Figure 3D1-E2, $p=0.01$).

These highly prednisolone sensitizing effects were not seen for AS1517499 (STAT6 inhibitor) and Crizotinib (cMET inhibitor). The STAT6 inhibitor AS1517499 modestly sensitized 1 out of 5 patient cell samples ($p=0.01$, Figure 3C1, Supplemental Figure 2). However, in 3 out of 5 patient cell samples inhibition of STAT6 had an opposite effect and even moderately increased prednisolone resistance (Figure 3, Supplemental Figure 2). Crizotinib modestly sensitized leukemic cells in 2 out of 5 patient cell samples ($p<0.05$, Figure 3D1-E1, Supplemental Figure 2). There was no specific association between the prednisolone sensitizing effects of these inhibitors and RAS-mutation status. Overall, Trametinib and Sorafenib were impressively potent when combined with prednisolone eradicating especially leukemic cells of RAS-mutated patients.

DISCUSSION

Prednisolone is able to target a wide range of genes eventually leading to apoptosis of leukemic cells [8]. To our knowledge this is the first study showing

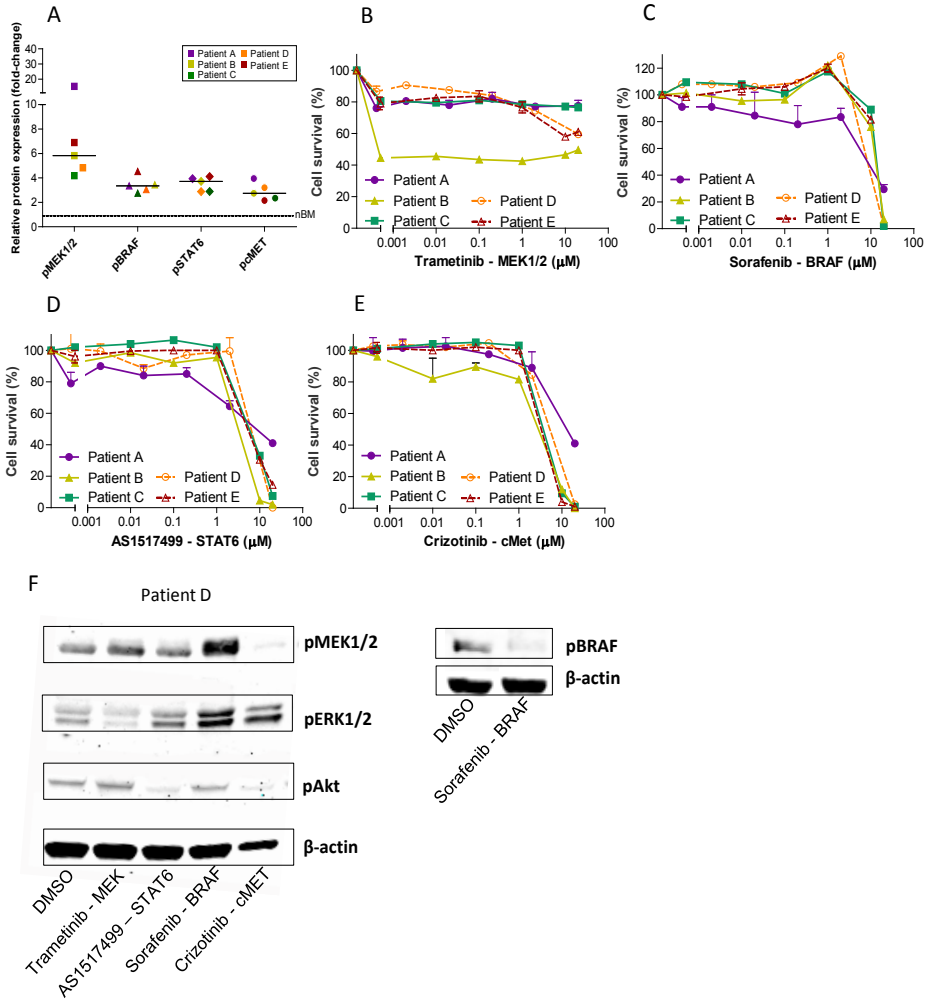

Figure 2. Inhibitors against MEK, BRAF, STAT6, cMET do not reduce viability of patients' leukemic cells.

(A) Protein phosphorylation levels of MEK1/2, BRAF, STAT6 and cMET was analyzed in leukemic cells of five prednisolone resistant BCP-ALL samples by reverse phase protein array. Phosphorylated protein levels in patients were compared to the levels observed in normal bone marrow mononuclear cells (dashed line set at 1). (B-E) Five prednisolone resistant BCP-ALL patients were treated for 4-days with the indicated inhibitor in a concentration range between 0.0002-20 μM, whereafter leukemic cell survival was analyzed by MTT-assay and corrected for effects caused by vehicle itself. (F) MEK1/2(S217-221), ERK1/2(Thr202/Tyr204), AKT(S473) and BRAF(S455) phosphorylation of patient D treated for 4 days with 5μM of the indicated inhibitors or 5μM of the vehicle DMSO was analyzed by western blot. β-actin expression was used as a loading control. Data are presented as mean plus SEM.

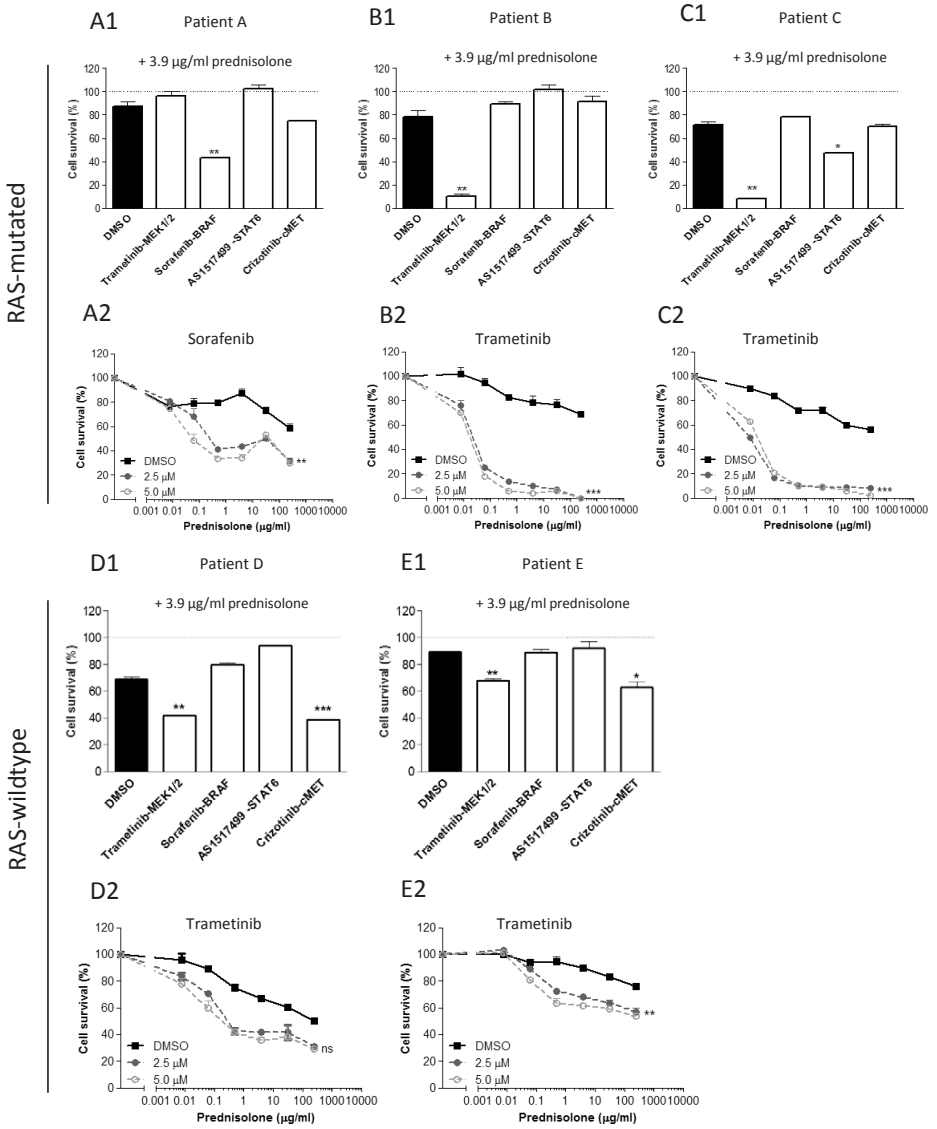

Figure 3. Trametinib (MEK inhibitor) and Sorafenib (BRAF inhibitor) restore prednisolone sensitivity in RAS-mutated patients

(A1-E1) Sensitivity to 3.9 µg/ml prednisolone of five distinct pediatric BCP-ALL patients' cell samples co-incubated with 2.5 µM Trametinib (MEK1/2 inhibitor), Sorafenib (BRAF inhibitor), AS1517499 (STAT6 inhibitor), Crizotinib (cMET inhibitor) or vehicle (DMSO) control measured in a 4-day MTT assay. Data are presented as mean plus SEM of a duplicate experiment (T-test *p<0.05, **p<0.01, ***p<0.001). **(A2-E2)** Dose-response curve of prednisolone combined with the inhibitor giving the most prominent effect in A1-E1. Graphs represent 2.5 or 5.0 µM Sorafenib (BRAF inhibitor), Trametinib (MEK inhibitor) or vehicle control (DMSO). Data are presented as mean plus SEM of a duplicate experiment (two-way ANOVA, interaction inhibitor*prednisolone *p<0.05, **p<0.01, ***p<0.001). Sensitivity was corrected for cell death induced by the inhibitor/vehicle itself in the absence of prednisolone to facilitate assessment of pure prednisolone sensitizing effects. The upper three patients have RAS-mutations, the lower two patients are RAS-wildtype.

that prednisolone downregulates the RAS-RAF-MEK pathway and STAT6 protein phosphorylation in prednisolone sensitive patients' ALL cells which is blocked in prednisolone resistant ALL cells, whereas an active upregulation of the proto-oncogene cMET was observed in prednisolone resistant cells. Inhibitors directed against either MEK1/2, BRAF, STAT6 or cMET did not induce substantial cell death in prednisolone resistant ALL patients' cells despite the presence of relative high targeted phosphorylated protein levels. This suggests that the expression level of phosphorylated proteins is not sufficient to guide the choice of an inhibitor for targeted treatment. Remarkably, highly resistant leukemic patients' cells harbouring KRAS/NRAS mutations were completely sensitized to prednisolone in the presence of the MEK1/2 inhibitor Trametinib or the BRAF inhibitor Sorafenib. These sensitizing effects were not seen for AS1517499 (STAT6 inhibitor) and Crizotinib (cMET inhibitor). Collectively, these results suggest that MEK/BRAF inhibitors are mainly indicated for RAS-mutated BCP-ALL cases and only when used in combination with prednisolone.

The synergy between prednisolone and MEK/BRAF inhibitors in RAS-mutated patients might be explained by the following three mechanisms: 1) The MEK/BRAF inhibitors mostly target RAS-mutant signaling [21,22]. The limited effect of MEK/BRAF inhibitors as single agents on viability of BCP-ALL cells might be explained by recent findings that targeted RAS-mutant signaling resensitizes to tyrosine kinase signaling via wildtype RAS [21,22]. Prednisolone targets wildtype RAS as is evident by the presence of a glucocorticoid response element in RAS [23] and our finding that prednisolone decreases RAS phosphorylation levels in prednisolone sensitive cells. The synergy between MEK/BRAF inhibitors and prednisolone might therefore be explained by a prednisolone-mediated decrease of RAS-wildtype signaling and MEK/BRAF targeted decrease of RAS-mutant signaling. 2) RAS mutations might inhibit glucocorticoid-dependent inhibition of the survival protein AP1, as was shown in lung and epithelial cell lines [24,25]. Targeting the mutated RAS-pathway genes may enable prednisolone to inhibit AP1, thereby reducing cell viability. 3) It is known that RAS activating mutations generate an anti-apoptotic environment by decreasing pro-apoptotic BIM levels [26] , increasing anti-apoptotic BCL2 [27] and MCL1 [28] levels. We previously showed that prednisolone downregulates MCL1 in sensitive but not in prednisolone resistant BCP-ALL cells [29]. Targeting MEK/BRAF may therefore synergistically act with prednisolone on the balance between pro- and anti-apoptotic proteins.

In this study we observed a relative high frequency of RAS-pathway mutated patients (38%) and these mutations seem to be overrepresented in prednisolone resistant compared to sensitive patients. Prednisolone resistance has recently been associated with RAS mutations in major clones of infant ALL [30]. Remarkably,

deep-sequencing of BCP-ALL cases in the present study revealed multiple minor clones in 60% of mutated cases. Different HRAS and NRAS activating mutations as well as mutations in genes inducing the RAS-pathway (FLT3 and PTPN11) were found. In contrast, other studies hardly detected concomitant mutations [30–32] and only one study in high-risk BCP-ALL leukemias identified multiple NRAS/KRAS mutations in 3% of patients [5]. Our finding that RAS-mutations are not mutually exclusive in most leukemic patients, not only encourages for future mutation analysis by means of deep-sequencing instead of sanger-sequencing, but also signifies the importance of these mutations in leukemia. This is further supported by our discovery of co-occurrence of minor clones with different RAS-pathway mutations and the finding that RAS mutations predominating at relapse could be retrospectively demonstrated in minor clones at initial diagnosis of the corresponding patients [33,34].

Collectively, our results indicate that AS1517499 (STAT6 inhibitor) and Crizotinib (cMET inhibitor) are not suitable as therapeutic options to resensitize leukemic patients' cells to prednisolone. Potential therapeutic effectiveness of MEK/BRAF inhibitors is mainly indicated for NRAS/KRAS mutated cases when combined with prednisolone. Potential inhibitors of choice in BCP-ALL are Trametinib (reviewed in [35]) and Sorafenib, since both inhibitors have been approved by the FDA for use in other cancers (Supplemental Table 2). In addition, Sorafenib was shown to be well tolerated and effective in FLT3 internal tandem duplicate acute myeloid leukemia[36–38]. Our data indicate that Trametinib and Sorafenib should not be given as a monotherapy in NRAS/KRAS mutated patients, but are mainly effective in combination with prednisolone. Furthermore, we advise that leukemic cells of children in which RAS-pathway mutations have been detected should be first *in vitro* tested for sensitivity to Trametinib and Sorafenib inhibitors (combined with prednisolone) to guide the choice which inhibitor is most benificial for the patiënt.

In conclusion, RAS mutations are frequently found in leukemic cells of children with BCP-ALL. Targeting RAS-signaling with Trametinib and Sorafenib could serve as a novel therapeutic option to modulate prednisolone resistance and may provide a way to further improve the clinical outcome of childhood BCP-ALL.

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AUTHORSHIP CONTRIBUTIONS

IMA designed and performed experiments, analyzed and interpreted data, and wrote the paper; ERD performed experiments; MJK , EC, EEV, JJM and HNC contributed to the Ion Torrent mutation analysis and interpretation of mutation data; RP and MLDB designed research, interpreted data, and revised the paper.

REFERENCES

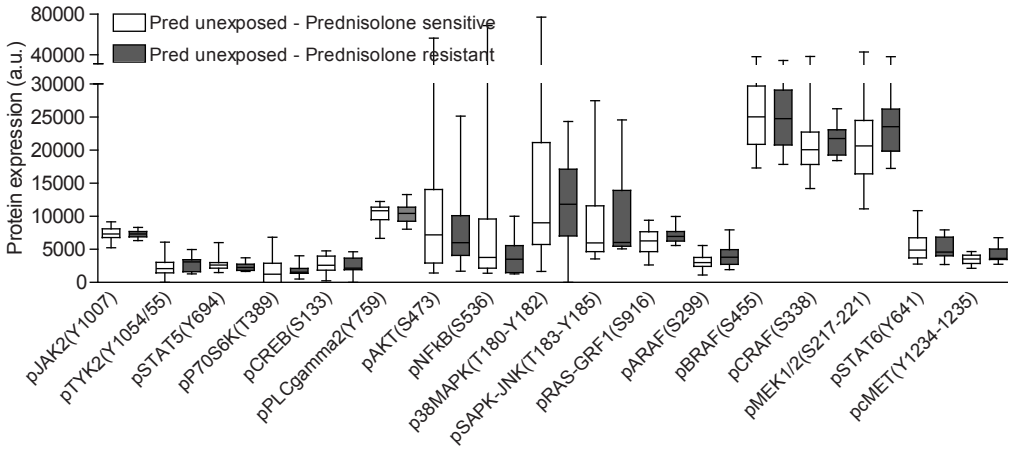
1. Pui C-H, Mullighan CG, Evans WE, Relling M V. Pediatric acute lymphoblastic leukemia: where are we going and how do we get there? Blood. 2012 Aug 9;120(6):1165–74.
2. Zwaan CM, Rizzari C, Mechinaud F, Lancaster DL, Lehrnbecher T, van der Velden VHJ, et al. Dasatinib in Children and Adolescents With Relapsed or Refractory Leukemia: Results of the CA180-018 Phase I Dose-Escalation Study of the Innovative Therapies for Children With Cancer Consortium. Journal of clinical oncology. 2013 May 28;31(19):2460–8.
3. Schultz KR, Bowman WP, Aledo A, Slayton WB, Sather H, Devidas M, et al. Improved early event-free survival with imatinib in Philadelphia chromosome-positive acute lymphoblastic leukemia: a children's oncology group study. Journal of clinical oncology. 2009 Nov 1;27(31):5175–81.
4. Biondi A, Schrappe M, De Lorenzo P, Castor A, Lucchini G, Gandemer V, et al. Imatinib after induction for treatment of children and adolescents with Philadelphia-chromosome-positive acute lymphoblastic leukaemia (EsPhALL): a randomised, open-label, intergroup study. The lancet oncology. 2012 Sep;13(9):936–45.
5. Zhang J, Mullighan CG, Harvey RC, Wu G, Chen X, Edmonson M, et al. Key pathways are frequently mutated in high-risk childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood. 2011 Sep 15;118(11):3080–7.
6. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood. 1998 Jul 1;92(1):259–66.
7. Klumper E, Pieters R, Veerman AJ, Huismans DR, Loonen AH, Hählen K, et al. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. Blood. 1995 Nov 15;86(10):3861–8.
8. Tissing WJE, den Boer ML, Meijerink JPP, Menezes RX, Swagemakers S, van der Spek PJ, et al. Genomewide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. Blood. 2007 May 1;109(9):3929–35.
9. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Körholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. Journal of clinical oncology. 2003 Sep 1;21(17):3262–8.
10. Den Boer ML, van Slegtenhorst M, De Menezes RX, Cheok MH, Buijs-Gladdines JGCAM, Peters STCJM, et al. A subtype of childhood acute lymphoblastic leukaemia with poor treatment outcome: a genome-wide classification study. The lancet oncology. 2009 Feb;10(2):125–34.
11. Kaspers GJL, Veerman AJP, Pieters R, Van Zantwijk CH, Smets LA, Van Wering ER, et al. In Vitro Cellular Drug Resistance and Prognosis in Newly Diagnosed Childhood Acute Lymphoblastic Leukemia. Blood. 1997 Oct;90(7):2723–9.
12. Pieters R, Huismans DR, Loonen AH, Hählen K, van der Does-van den Berg A, van Wering ER, et al. Relation of

cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet. 1991 Aug 17;338(8764):399–403.
13. Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, et al. Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget. 2011 Mar;2(3):135–64.
14. Neel BG, Gu H, Pao L. The "Shp"ing news: SH2 domain-containing tyrosine phosphatases in cell signaling. Trends in biochemical sciences. 2003 Jun;28(6):284–93.
15. Rosen DB, Minden MD, Kornblau SM, Cohen A, Gayko U, Putta S, et al. Functional characterization of FLT3 receptor signaling deregulation in acute myeloid leukemia by single cell network profiling (SCNP). PloS one. 2010 Jan;5(10):e13543.
16. Wurster AL, Rodgers VL, White MF, Rothstein TL, Grusby MJ. Interleukin-4-mediated protection of primary B cells from apoptosis through Stat6-dependent up-regulation of Bcl-xL. The Journal of biological chemistry. 2002 Jul 26;277(30):27169–75.
17. Galka E, Thompson J, Poritz L, Fitzpatrick L, Zhang W, Koltun W. Alterations in pro-survival proteins Bcl-2 and AKT contribute to apoptosis in Stat6-deficient cell lines. Journal of Surgical Research. 2004 Oct;121(2):310.
18. Thieu VT, Nguyen ET, McCarthy BP, Bruns HA, Kapur R, Chang C-H, et al. IL-4-stimulated NF-kappaB activity is required for Stat6 DNA binding. Journal of leukocyte biology. 2007 Aug 1;82(2):370–9.
19. Ma PC, Maulik G, Christensen J, Salgia R. c-Met: structure, functions and potential for therapeutic inhibition. Cancer metastasis reviews. 2003 Dec;22(4):309–25.
20. Organ SL, Tsao M-S. An overview of the c-MET signaling pathway. Therapeutic advances in medical oncology. 2011 Nov;3(1 Suppl):S7–S19.
21. Young A, Lou D, McCormick F. Oncogenic and wild-type Ras play divergent roles in the regulation of mitogen-activated protein kinase signaling. Cancer discovery. American Association for Cancer Research; 2013 Jan 1;3(1):112–23.
22. Hayes TK, Der CJ. Mutant and wild-type Ras: co-conspirators in cancer. Cancer discovery. American Association for Cancer Research; 2013 Jan 1;3(1):24–6.
23. Zachos G, Zoumpourlis V, Sekeris C, Spandidos D. Binding of the glucocorticoid and estrogen-receptors to the human h-ras oncogene sequences. International journal of oncology. 1995 Mar;6(3):595–600.
24. Johnson TA, Li J, Beer DG. Influence of the glucocorticoid receptor on c-fos inducibility in activated ras-containing mouse lung cells. Molecular carcinogenesis. 1996 Oct;17(2):70–7.
25. Boudreau F, Zannoni S, Pelletier N, Bardati T, Yu SJ, Asselin C. Negative regulation of glucocorticoid-dependent induction of c-fos by ras in intestinal epithelial cells. Molecular and cellular biochemistry. 1999 May;195(1-2):99–111.
26. Weston CR, Balmanno K, Chalmers C, Hadfield K, Molton SA, Ley R, et al. Activation of ERK1/2 by deltaRaf-1:ER* represses Bim expression independently of the JNK or PI3K pathways. Oncogene. 2003 Mar 6;22(9):1281–93.
27. Sánchez-García I, Martín-Zanca D. Regulation of Bcl-2 gene expression by BCR-ABL is mediated by Ras.

Journal of molecular biology. 1997 Mar 28;267(2):225–8.
28. Boisvert-Adamo K, Longmate W, Abel E V, Aplin AE. Mcl-1 is required for melanoma cell resistance to anoikis. Molecular cancer research : MCR. 2009 Apr;7(4):549–56.
29. Ariës IM, Hansen BR, Koch T, Evans WE, Pieters R, Den Boer ML. Prednisolone Resistance in Pediatric Acute Lymphoblastic Leukemia Can Be Synergistically Overcome by Inhibition of Anti-Apoptotic MCL1 and Glycolysis [Abstract]. Blood. 2012;120(21):3528.
30. Driessen EMC, van Roon EHJ, Spijkers-Hagelstein JAP, Schneider P, de Lorenzo P, Valsecchi MG, et al. Frequencies and prognostic impact of RAS mutations in MLL-rearranged acute lymphoblastic leukemia in infants. Haematologica. 2013 Jun;98(6):937–44.
31. Yamamoto T, Isomura M, Xu Y, Liang J, Yagasaki H, Kamachi Y, et al. PTPN11, RAS and FLT3 mutations in childhood acute lymphoblastic leukemia. Leukemia research. 2006 Sep;30(9):1085–9.
32. Paulsson K, Horvat A, Strömbeck B, Nilsson F, Heldrup J, Behrendtz M, et al. Mutations of FLT3, NRAS, KRAS, and PTPN11 are frequent and possibly mutually exclusive in high hyperdiploid childhood acute lymphoblastic leukemia. Genes, chromosomes & cancer. 2008 Jan;47(1):26–33.
33. Case M, Matheson E, Minto L, Hassan R, Harrison CJ, Bown N, et al. Mutation of genes affecting the RAS pathway is common in childhood acute lymphoblastic leukemia. Cancer research. 2008 Aug 15;68(16):6803–9.
34. Mullighan CG, Zhang J, Kasper LH, Lerach S, Payne-Turner D, Phillips LA, et al. CREBBP mutations in relapsed acute lymphoblastic leukaemia. Nature. 2011 Mar 10;471(7337):235–9.
35. Wright CJM, McCormack PL. Trametinib: first global approval. Drugs. 2013 Jul;73(11):1245–54.
36. Ravandi F, Alattar ML, Grunwald MR, Rudek MA, Rajkhowa T, Richie MA, et al. Phase 2 study of azacytidine plus sorafenib in patients with acute myeloid leukemia and FLT-3 internal tandem duplication mutation. Blood. 2013 Jun 6;121(23):4655–62.
37. Inaba H, Rubnitz JE, Coustan-Smith E, Li L, Furmanski BD, Mascara GP, et al. Phase I pharmacokinetic and pharmacodynamic study of the multikinase inhibitor sorafenib in combination with clofarabine and cytarabine in pediatric relapsed/refractory leukemia. Journal of clinical oncology. 2011 Aug 20;29(24):3293–300.
38. Widemann BC, Kim A, Fox E, Baruchel S, Adamson PC, Ingle AM, et al. A phase I trial and pharmacokinetic study of sorafenib in children with refractory solid tumors or leukemias: a Children's Oncology Group Phase I Consortium report. Clinical Cancer Research. 2012 Nov 1;18(21):6011–22.

A

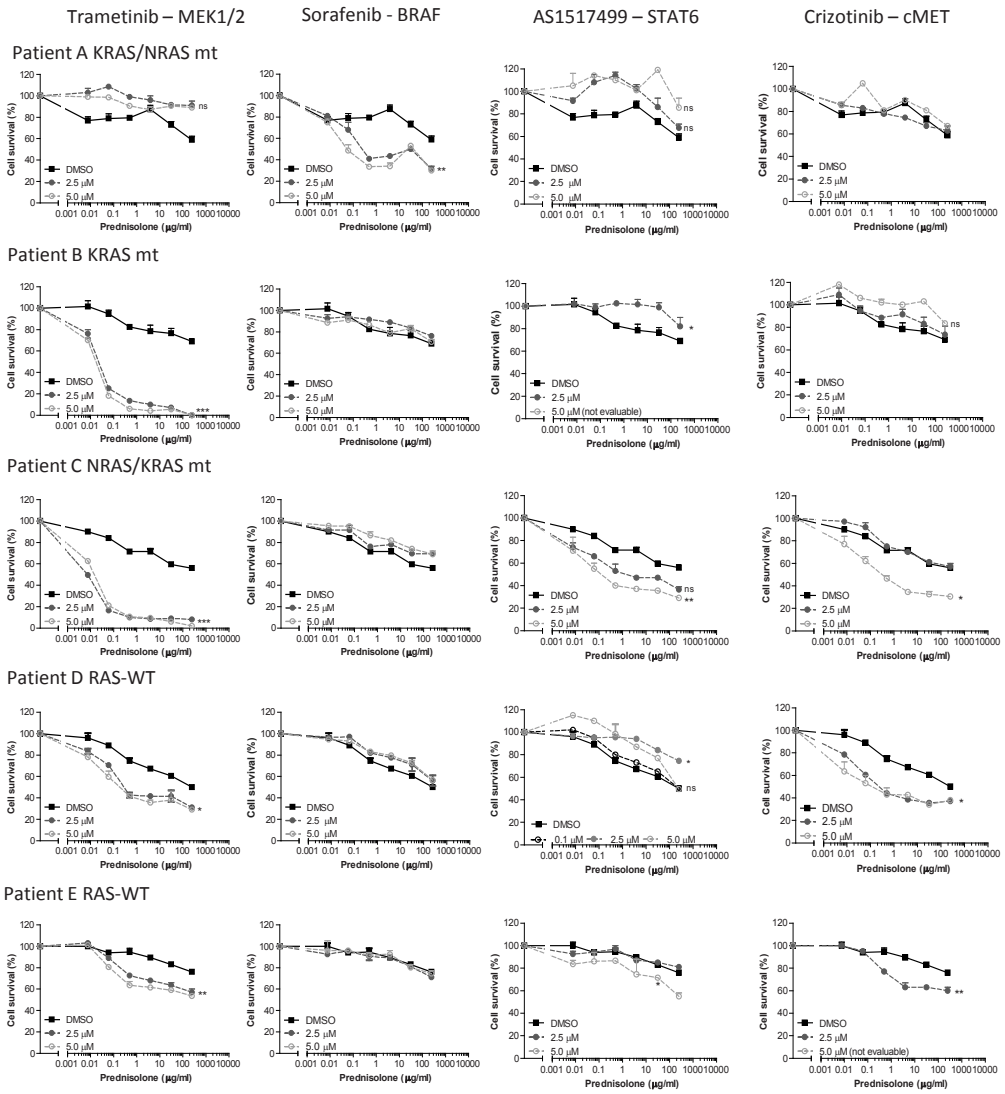
Supplemental Figure 1.



Supplemental Figure 1. Prednisolone mediated dysregulation of pivotal survival proteins is impaired in prednisolone resistant primary patients' cells.

(A) Phosphorylation levels of 17 proteins were analyzed by means of reverse phase protein array (relative to total protein) of 31 *in vitro* prednisolone sensitive and 15 prednisolone resistant unexposed samples taken from BCP-ALL patients' at initial diagnosis. P-value was not significant for all proteins. Data are presented as mean plus SEM of three patients (T-test, *p<0.05, **p<0.01, ***p<0.00 , a.u. Arbitrary Units)

Supplemental Figure 2.



Supplemental Figure 2. ***Trametinib (MEK inhibitor) and Sorafenib (BRAF inhibitor) restored prednisolone sensitivity in RAS-mutated patients*** Dose-response curves of five pediatric BCP-ALL patients' cell samples exposed to prednisolone together with 2.5 or 5.0 µM of inhibitor or vehicle (DMSO). Data are presented as mean plus SEM of a duplicate experiment (repeated measurement two-way ANOVA, *p<0.05, **p<0.01, ***p<0.001). To facilitate assessment of cellular sensitization to prednisolone by the inhibitors, cell survival was corrected for the cell death induced by the inhibitor. The upper three patients have RAS-mutations, the lower two patients are RAS-wildtype (see also Table 1).

Supplemental Table 1.

Gene	Cosmic hotspot codons examined
BRAF	444, 464, 466, 469, 471, 581, 587, 592, 594, 595, 596, 597, 599, 600, 601 and 605
NRAS	12, 13, 18, 61 and 64
HRAS	12, 13 and 61
KRAS	12,13,19,22,59,61 and 146
PTPN11	60, 61, 69, 72, 73,76, 502 and 503
FLT3	451,572, 592,597, 599, 601, 602, 603, 834, 835, 836 and 842
cMET	168, 375, 1010,1112, 1248,1253 and 1268

Supplemental Table 2.

Inhibitor	Main Target	FDA approval
Trametinib	MEK1-2	FDA approved for melanoma
Sorafenib	BRAF	FDA approved for renal cell carcinoma and hepatocellular carcinoma
AS1517499	STAT6	No clinical trial data
Crizotinib	cMET	FDA approved for non-small lung carcinoma

DISCUSSION AND PERSPECTIVES

DISCUSSION AND PERSPECTIVES

Better risk stratification and continuous optimization of treatment protocols, without changing the primary chemotherapeutic drugs that have been used for decades, have improved pediatric precursor-B acute lymphoblastic (BCP-ALL) leukemia event-free survival rates up to 80-90% [1]. Targeted drugs adjuvating current chemotherapy regimens are warranted to cure the remaining 10-20%. Sixty years after the first discovery of its anti-leukemic competences, prednisolone is still the primary drug used in the treatment of BCP-ALL and T-ALL. *In vivo* and *in vitro* resistance against prednisolone remains an adverse prognostic factor in pediatric BCP-ALL [2,3]. Moreover, relapsed leukemic cells acquire prednisolone resistance disproportionally to other chemotherapeutic drugs [4]. To eventually cure the remaining 10%-20% of ALL patients it is essential to unravel prednisolone resistance and find targeted drugs that modulate prednisolone resistance.

PREDNISOLONE INDUCED APOPTOSIS: MULTIFACTORIAL APPROACH

Recent investigations indicated that prednisolone resistant cells have defects in drug-induced apoptosis mechanisms, such as caspase-3 activation and PARP [5]. Additionally, it was discovered that the pro-apoptotic BCL-2-interacting mediator of cell death (BIM) expression is increased upon prednisolone treatment in sensitive, but not in resistant cells [6,7]. In fact, knockdown of BIM induced prednisolone resistance in a sensitive cell line [8]. Microarray analysis of *in vitro* prednisolone resistant and sensitive patients' BCP-ALL cells showed that specifically anti-apoptotic MCL1 and DAPK1 are associated with prednisolone resistance [9]. Indeed, down-regulation of MCL1 in prednisolone resistant MLL-rearranged leukemia cell lines by RNA interference led to prednisolone sensitization [10]. In addition, rapamycin, a mTOR inhibitor, was shown to sensitize leukemic cell lines to prednisolone by downregulating MCL1 [11]. Analysis of apoptotic protein expression levels in prednisolone sensitive and resistant primary patient BCP-ALL samples after *in vitro* exposure to prednisolone pointed to MCL1 as the key apoptotic protein associated with prednisolone resistance **(Chapter 2)**. Locked-nucleic-acid (LNA) oligonucleotides were therefore developed, which were shown to specifically knockdown MCL1 and sensitized leukemic cell lines to prednisolone **(Chapter 2).** LNAs are currently investigated in phase I early clinical trials (www.ClinicalTrials.gov, NCT01186328, NCT01337518, NCT00466583), as they are conformationally structured to prevent most of the current hurdles in siRNA treatment [12]. MCL1 LNA antisense may therefore offer a more specific way of

silencing MCL1 than the current BCL-2 family inhibitors R-(-)-gossypol (AT101) and obatoclax (GX-15-070), which aspecifically block several members of the BCL-2 family [13,14]. However, silencing of MCL1 also promoted the glycolytic route in cells thereby providing a rescue mechanism for these cells **(Chapter 2)**. These results might be explained by the finding that MCL1 was recently shown to facilitate ATP production by activating the respiratory complexes in mitochondria [15]. Knockdown of MCL1 may therefore impair oxidative phosphorylation and forces cells to produce ATP by glycolysis to ensure survival. We previously observed that prednisolone resistance is associated with increased glucose consumption in leukemic cells. Inhibition of glycolysis with 2-DG sensitized both leukemic cell lines and primary BCP-ALL cells to prednisolone [16]. Co-exposure of leukemic cell lines with MCL1 LNA and 2-DG synergistically inhibited leukemic cell survival and promoted sensitization to prednisolone **(Chapter 2).** In addition to LNAs, 2-DG is also currently studied in phase I clinical trials (www.ClinicalTrials.gov, NCT00096707).

Overall, MCL1 is most likely the key apoptotic protein associated with prednisolone resistance. Treatment with MCL1 LNA antisense and 2-DG provides a promising approach to decrease leukemic cell survival and to sensitize ALL patients' cells to prednisolone. Most importantly, different mechanisms of survival and resistance co-exist and therefore warrant investigation of multifactorial approaches to sensitize ALL cells to prednisolone in clinics.

PREDNISOLONE RESISTANCE AND MICROENVIRONMENT INTERACTION

Prednisolone binds the glucocorticoid receptor, which in turn transcriptionally regulates the expression of numerous genes eventually leading to apoptosis. Eight hours of prednisolone exposure of sensitive BCP-ALL cells already results in differential expression of 51 genes [17]. To examine the gene expression pattern that might cause prednisolone resistance in more detail, microarray gene expression profiles of 256 children with newly diagnosed prednisolone sensitive or resistant BCP-ALL or T-ALL were analyzed. Epithelial membrane protein 1 (EMP1) was identified as one of the most discriminative genes **(Chapter 3)**. EMP1 is a small hydrophobic four-transmembrane glycoprotein [18]. Knockdown of EMP1 not only reduced leukemic BCP-ALL and T-ALL cell viability and sensitized to prednisolone, but also abrogated migration and adhesion of leukemic cells to mesenchymal stromal cells (MSCs) **(Chapter 3)**. Only recently, the bone marrow microenvironment was demonstrated to be an important contributor to

resistance of leukemic cells to drugs [19,20]. Chemokines secreted by MSCs, such as stem cell factor 1 and CXCL12, can for example stimulate leukemic cell homing and survival [21,22]. MSCs can rescue cells from L-asparaginase by secreting asparagine [20] or induce leukemic drug resistance through upregulation of the voltage-dependent channel hERG1 [19]. MSC-based sheltering of ALL cells may also provide a physical barrier for chemotherapeutics. Indeed, MSCs potentially protect leukemic cells against prednisolone-induced apoptosis **(Chapter 3)**. Moreover, silencing of EMP1 reduced in part the protection by MSCs **(Chapter 3)**.

Overall, EMP1 was shown to play an important pathobiological role in ALL and the development of an EMP1 inhibitory small compound may serve as a potential new therapeutic option for ALL. These findings have shed light to an entire new dimension of drug resistance research and potentiate the importance to study drug resistance in relation to the microenvironment in more depth. The current set-up of *in vitro* assays used to determine drug resistance in leukemic cells does not consider the influence of the microenvironment on drug resistance. Most certainly, improved *in vitro* bone marrow models and xenograft mouse models are necessary to study the microenvironment and leukemic interaction and importance for cellular drug resistance in more detail. In addition, a new high throughput technique should be developed that enables detection of microenvironment induced drug resistance in patients' leukemic cells.

GLUCOCORTICOID RECEPTOR ANTAGONIZING MECHANISMS

Analysis of mRNA and protein array profiles obtained from BCP-ALL cells of newly diagnosed patients, revealed an increased mRNA and protein expression of the NR4A transcription factor family (*NR4A1, NR4A2* and *NR4A3*) in prednisolone resistant compared to sensitive patients **(Chapter 4)**. Interestingly, *NR4A1* (protein name: Nur77), *NR4A2* (protein name: Nurr1), and *NR4A3* (protein name: Nor1) are orphan nuclear receptors which belong to the same steroid hormone receptor superfamily as the glucocorticoid receptor (GR) [23]. A direct protein-protein interaction between GR and the NR4A family members exists, which represses GR-dependent transcription [24,25]. Although there is a strong association between NR4A expression and prednisolone resistance, functional studies in primary patients' cells did not indicate that NR4A genes are causally related to resistance in primary patients´ cells **(Chapter 4)**. The role of other GR binding transcription factors, such as AP-1 and NF-κB [26] in prednisolone resistance are contradictory as well. While some studies showed that overexpression of NF-κB or AP-1 might

contribute to prednisolone resistance [10,27–29], others did not [30]. These results suggest that impaired functioning of GR at the receptor level most likely does not cause prednisolone resistance. In line with this are previous studies showing that there was no correlation between baseline GR mRNA levels [31,32], polymorphisms or mutations in the GR gene [31] or mRNA expression levels of chochaperones [33] and prednisolone resistance.

Overall, NR4A genes are not suitable targets to reverse prednisolone resistance. Most importantly, resistance to prednisolone is more likely caused by mechanisms affecting the downstream intracellular signaling than by mechanisms affecting the effective prednisolone or GR receptor levels.

PREDNISOLONE RESISTANCE MUTATIONS AND PERSONALIZED THERAPY

In addition to apoptotic molecules, also several pro-survival mechanisms have been associated with the failure of prednisolone to induce apoptosis. Inhibition of the important survival proteins JNK and ERK [34], PI3 kinase and AKT [35,36] and Src kinase[37] sensitized ALL cell lines to prednisolone. Examination of survival protein levels in prednisolone sensitive and resistant BCP-ALL patients' samples after exposure to prednisolone revealed that prednisolone downregulated the activity of the RAS-RAF-MEK pathway and STAT6 in prednisolone sensitive patients cells, but was not capable to do so in prednisolone resistant patients **(Chapter 5)**. In addition, an active upregulation of the proto-oncogene cMET in prednisolone resistant cells became evident that was not present in prednisolone sensitive cells. Other studies also indicated that prednisolone targets the MAPK pathway [17,38], although a direct link between RAS, RAF, STAT6 and cMET protein phosphorylation and prednisolone resistance has not been made before. We also identified a higher frequency of RAS activating mutations in prednisolone resistant compared to sensitive patients **(Chapter 5)**. An association between RAS-mutations in major clones and prednisolone resistance was recently shown in infant ALL [39]. We detected a RAS mutation frequency of 38% in newly diagnosed BCP-ALL patients and in 60% of the mutated patients multiple minor clones with different HRAS and NRAS activating mutations and mutations in genes inducing the RAS-pathway (FLT3 and PTPN11) were identified, signifying the importance of these mutations in leukemia **(Chapter 5)**. In contrast, other studies hardly detected co-existence of different mutations in the same patients [39–41] and only one study in high-risk BCP-ALL leukemias identified multiple NRAS/KRAS mutations in 3% of patients [42]. Remarkably, highly resistant leukemic patients cells

harbouring KRAS/NRAS mutations were completely sensitized to prednisolone in the presence of the MEK1/2 inhibitor Trametinib **(Chapter 5)**. In another KRAS/NRAS mutated patient Sorafenib (BRAF inhibitor) but not Trametinib sensitized to prednisolone significantly **(Chapter 5)**. These highly sensitizing effects were not seen for AS1517499 (STAT6 inhibitor) and Crizotinib (cMET inhibitor) **(Chapter 5)**.

Overall, BCP-ALL patients should be screened for RAS mutations, as these patients could benefit from treatment with Trametinib or Sorafenib combined with prednisolone. Most importantly, our results highlight the heterogeneous aspect of prednisolone resistance and encourages for further investigation of mutation-based prednisolone resistance combined with personalized targeted therapy. Leukemia often consists of multiple subclones with sometimes different genetic abnormalities, as we showed for RAS mutations. These minor clones can be responsible for later relapses [43]. It has also been shown that relapsed leukemic cells gain prednisolone resistance [4]. Fundamental in future research will therefore be investigations of prednisolone resistance in relapsed leukemias and how to treat (minor) subclones more effectively, preferably at the time of initial diagnosis of ALL.

In conclusion, this thesis indicates the multifactoriality of prednisolone resistance in pediatric ALL. Our results point out that MCL1 LNAs , 2-DG, an EMP1 inhibitor, Trametinib (MEK inhibitor) and Sorafenib (BRAF inhibitor) may be of therapeutic value to reverse prednisolone resistance in childhood ALL. We also show that the causes of resistance differ between patients supporting a more personalized approach to improve outcome for those children who are not cured with current regimens.

REFERENCES

1. Pui C-H, Mullighan CG, Evans WE, Relling M V. Pediatric acute lymphoblastic leukemia: where are we going and how do we get there? Blood. 2012 Aug 9;120(6):1165–74.
2. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood. 1998 Jul 1;92(1):259–66.
3. Den Boer ML, Harms DO, Pieters R, Kazemier KM, Gobel U, Körholz D, et al. Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. Journal of clinical oncology. 2003 Sep 1;21(17):3262–8.
4. Klumper E, Pieters R, Veerman AJ, Huismans DR, Loonen AH, Hählen K, et al. In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia. Blood. 1995 Nov 15;86(10):3861–8.
5. Holleman A, den Boer ML, Kazemier KM, Janka-Schaub GE, Pieters R. Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukemia. Blood. 2003 Dec 15;102(13):4541–6.
6. Abrams MT, Robertson NM, Yoon K, Wickstrom E. Inhibition of glucocorticoid-induced apoptosis by targeting the major splice variants of BIM mRNA with small interfering RNA and short hairpin RNA. J Biol Chem. 2004 Dec 31;279(53):55809–17.
7. Lu J, Quearry B, Harada H. p38-MAP kinase activation followed by BIM induction is essential for glucocorticoid-induced apoptosis in lymphoblastic leukemia cells. FEBS letters. 2006 Jun 12;580(14):3539–44.
8. Ploner C, Rainer J, Niederegger H, Eduardoff M, Villunger A, Geley S, et al. The BCL2 rheostat in glucocorticoid-induced apoptosis of acute lymphoblastic leukemia. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK. 2008 Feb;22(2):370–7.
9. Holleman A, den Boer ML, de Menezes RX, Cheok MH, Cheng C, Kazemier KM, et al. The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia. Blood. 2006 Jan 15;107(2):769–76.
10. Stam RW, Den Boer ML, Schneider P, de Boer J, Hagelstein J, Valsecchi MG, et al. Association of high-level MCL-1 expression with in vitro and in vivo prednisone resistance in MLL-rearranged infant acute lymphoblastic leukemia. Blood. 2010 Feb 4;115(5):1018–25.
11. Wei G, Twomey D, Lamb J, Schlis K, Agarwal J, Stam RW, et al. Gene expression-based chemical genomics identifies rapamycin as a modulator of MCL1 and glucocorticoid resistance. Cancer cell. 2006 Oct;10(4):331–42.
12. Koch T, Rosenbohm C, Hansen HF, Hansen B, Straarup EM, Kauppinen S. Locked Nucleic Acid: Properties and Therapeutic Aspects. In Therapeutic Oligonucleotides. Kurreck J, editor. Cambridge: Royal Society of Chemistry; 2008. p. 103–41.

13. Etxebarria A, Landeta O, Antonsson B, Basañez G. Regulation of anti-apoptotic MCL-1 function by gossypol: mechanistic insights from in vitro reconstituted systems. Biochem Pharmacol. 2008 Dec 1;76(11):1563–76.
14. Nguyen M, Marcellus RC, Roulston A, Watson M, Serfass L, Murthy Madiraju SR, et al. Small molecule obatoclax (GX15-070) antagonizes MCL-1 and overcomes MCL-1-mediated resistance to apoptosis. PNAS. 2007 Dec 4;104(49):19512–7.
15. Perciavalle RM, Stewart DP, Koss B, Lynch J, Milasta S, Bathina M, et al. Anti-apoptotic MCL-1 localizes to the mitochondrial matrix and couples mitochondrial fusion to respiration. Nature cell biology. 2012 Jun;14(6):575–83.
16. Hulleman E, Kazemier KM, Holleman A, VanderWeele DJ, Rudin CM, Broekhuis MJC, et al. Inhibition of glycolysis modulates prednisolone resistance in acute lymphoblastic leukemia cells. Blood. 2009 Feb 26;113(9):2014–21.
17. Tissing WJE, den Boer ML, Meijerink JPP, Menezes RX, Swagemakers S, van der Spek PJ, et al. Genomewide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells. Blood. 2007 May 1;109(9):3929–35.
18. Taylor V, Welcher AA, Program AE, Suter U. Epithelial membrane protein-1, peripheral myelin protein 22, and lens membrane protein 20 define a novel gene family. The Journal of biological chemistry. 1995 Dec 1;270(48):28824–33.
19. Pillozzi S, Masselli M, De Lorenzo E, Accordi B, Cilia E, Crociani O, et al. Chemotherapy resistance in acute lymphoblastic leukemia requires hERG1 channels and is overcome by hERG1 blockers. Blood. 2011 Jan 20;117(3):902–14.
20. Iwamoto S, Mihara K, Downing JR, Pui C-H, Campana D. Mesenchymal cells regulate the response of acute lymphoblastic leukemia cells to asparaginase. The Journal of clinical investigation. 2007 Apr;117(4):1049–57.
21. Sison EAR, Brown P. The bone marrow microenvironment and leukemia: biology and therapeutic targeting. Expert review of hematology. 2011 Jun;4(3):271–83.
22. Masson K, Rönnstrand L. Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3. Cellular signalling. 2009 Dec;21(12):1717–26.
23. Milbrandt J. Nerve growth factor induces a gene homologous to the glucocorticoid receptor gene. Neuron. 1988 May;1(3):183–8.
24. Martens C, Bilodeau S, Maira M, Gauthier Y, Drouin J. Protein-protein interactions and transcriptional antagonism between the subfamily of NGFI-B/Nur77 orphan nuclear receptors and glucocorticoid receptor. Molecular endocrinology (Baltimore, Md). 2005 Apr;19(4):885–97.
25. Strøm BO, Aden P, Mathisen GH, Lømo J, Davanger S, Paulsen RE. Transfection of chicken cerebellar granule neurons used to study glucocorticoid receptor regulation by nuclear receptor 4A (NR4A). Journal of neuroscience methods. 2010 Oct 30;193(1):39–46.
26. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for

gene repression. Endocrine reviews. 2003 Aug;24(4):488–522.
27. Nicholson L, Hall AG, Redfern CP, Irving J. NFkappaB modulators in a model of glucocorticoid resistant, childhood acute lymphoblastic leukemia. Leukemia research. Elsevier Ltd; 2010 Oct;34(10):1366–73.
28. Schulze-osthoff K. Critical role of nuclear factor-κB and stress-activated protein kinases in steroid unresponsiveness. The FASEB Journal. 2002;16(13):1832–4.
29. Holleman A, Cheok MH, den Boer ML, Yang W, Veerman AJP, Kazemier KM, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. The New England journal of medicine. Mass Medical Soc; 2004;351(6):533–42.
30. Bailey S, Hall a G, Pearson a D, Redfern CP. The role of AP-1 in glucocorticoid resistance in leukaemia. Leukemia. 2001 Mar;15(3):391–7.
31. Tissing WJE, Meijerink JPP, den Boer ML, Brinkhof B, van Rossum EFC, van Wering ER, et al. Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia. Clinical cancer research. 2005 Aug 15;11(16):6050–6.
32. Haarman EG, Kaspers GJL, Pieters R, Rottier MMA, Veerman AJP. Glucocorticoid receptor alpha, beta and gamma expression vs in vitro glucocorticod resistance in childhood leukemia. Leukemia. 2004 Mar;18(3):530–7.
33. Tissing WJE, Meijerink JPP, den Boer ML, Brinkhof B, Pieters R. mRNA expression levels of (co)chaperone molecules of the glucocorticoid receptor are not involved in glucocorticoid resistance in pediatric ALL. Leukemia. 2005 May;19(5):727–33.
34. Miller AL, Garza AS, Johnson BH, Thompson EB. Pathway interactions between MAPKs, mTOR, PKA, and the glucocorticoid receptor in lymphoid cells. Cancer cell international. 2007 Jan;7:3.
35. Spijkers-Hagelstein JAP, Pinhanços SM, Schneider P, Pieters R, Stam RW. Chemical genomic screening identifies LY294002 as a modulator of glucocorticoid resistance in MLL-rearranged infant all. Leukemia. 2013 Aug 20;
36. Fuka G, Kantner H-P, Grausenburger R, Inthal A, Bauer E, Krapf G, et al. Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts. Leukemia. 2012 May;26(5):927–33.
37. Spijkers-Hagelstein JAP, Mimoso Pinhanços S, Schneider P, Pieters R, Stam RW. Src kinase-induced phosphorylation of annexin A2 mediates glucocorticoid resistance in MLL-rearranged infant acute lymphoblastic leukemia. Leukemia. 2013 Apr;27(5):1063–71.
38. Miller AL, Garza AS, Johnson BH, Thompson EB. Pathway interactions between MAPKs, mTOR, PKA, and the glucocorticoid receptor in lymphoid cells. Cancer cell international. 2007 Jan;7:3.
39. Driessen EMC, van Roon EHJ, Spijkers-Hagelstein JAP, Schneider P, de Lorenzo P, Valsecchi MG, et al. Frequencies and prognostic impact of RAS mutations in MLL-rearranged acute lymphoblastic leukemia in infants. Haematologica. 2013 Jun;98(6):937–44.
40. Yamamoto T, Isomura M, Xu Y, Liang J, Yagasaki H, Kamachi Y, et al.

PTPN11, RAS and FLT3 mutations in childhood acute lymphoblastic leukemia. Leukemia research. 2006 Sep;30(9):1085–9.

41. Paulsson K, Horvat A, Strömbeck B, Nilsson F, Heldrup J, Behrendtz M, et al. Mutations of FLT3, NRAS, KRAS, and PTPN11 are frequent and possibly mutually exclusive in high hyperdiploid childhood acute lymphoblastic leukemia. Genes, chromosomes & cancer. 2008 Jan;47(1):26–33.
42. Zhang J, Mullighan CG, Harvey RC, Wu G, Chen X, Edmonson M, et al. Key pathways are frequently mutated in high-risk childhood acute lymphoblastic leukemia: a report from the Children's Oncology Group. Blood. 2011 Sep 15;118(11):3080–7.
43. Case M, Matheson E, Minto L, Hassan R, Harrison CJ, Bown N, et al. Mutation of genes affecting the RAS pathway is common in childhood acute lymphoblastic leukemia. Cancer research. 2008 Aug 15;68(16):6803–9.

SUMMARY

SUMMARY

20% of ALL patients relapse on or after current treatment strategies. Treatment failure has been associated with cellular resistance to prednisolone, the leading drug in multi-drug treatment of ALL. The molecular mechanisms behind prednisolone resistance in pediatric ALL are poorly defined. This thesis aimed to find new druggable targets that may modulate prednisolone resistance.

Recent investigations indicated that prednisolone resistant cells have defects in drug-induced apoptosis mechanisms. Analysis of apoptotic protein expression levels **(Chapter 2)** in prednisolone sensitive and resistant primary patient precursor-B acute lymphoblastic leukemia (BCP-ALL) samples after *in vitro* exposure to prednisolone pointed to MCL1 as the key apoptotic protein associated with prednisolone resistance. Clinically relevant locked-nucleic-acid (LNA) oligonucleotides were developed, which were shown to specifically silence the expression of MCL1 and induced apoptosis of leukemic cell lines. However, silencing of MCL1 appeared to increase glycolysis of leukemic cells thereby rescuing these cells from prednisolone-induced apoptosis. Targeting both MCL1 with MCL1 LNA and glycolysis with 2-deoxyglucose (2-DG) synergistically inhibited leukemic cell survival and in part mutually sensitized leukemic cells to prednisolone. Our results indicate that MCL1 LNA antisense and 2-DG provide a promising therapeutic approach to decrease leukemic cell survival and sensitize ALL patients' cells to prednisolone. These findings also illustrate that investigation of multifactorial and combined ways to sensitize leukemic cells to prednisolone are warranted.

To examine prednisolone resistance in more detail, we generated microarray gene expression profiles of 256 children with newly diagnosed ALL. Epithelial membrane protein 1 (EMP1) was identified as one of the most discriminative genes for prednisolone resistance **(Chapter 3)**. EMP1 is a small hydrophobic four-transmembrane glycoprotein of which the biological function is largely unknown. We discovered that knockdown of EMP1 in leukemic cell lines increased apoptosis and induced cell cycle arrest leading to large reductions in leukemic cell survival. Moreover, EMP1 knockdown sensitized three out of six cell lines to prednisolone. Silencing of EMP1 also abrogated migration and adhesion of leukemic cell lines to mesenchymal stromal cells. Notably, we also discovered that EMP1 contributes to MSC-mediated prednisolone resistance, as prednisolone resistance triggered during leukemic MSC coculture was partially reversed after EMP1 knockdown. Pathway analysis indicated that EMP1 signals through Src kinase family phosphorylation to activate amongst others JNK, STAT3, STAT5, CREB and NF-κB. Finally, we discovered that high expression of *EMP1* is a novel poor prognostic factor in BCP-ALL. Overall, our results indicate that EMP1 plays

an important pathobiological role in ALL. The development of an EMP1 inhibitory small compound may serve as a potential new therapeutic option for ALL. Most importantly, first insights in the role of the microenvironment in drug resistance have become evident which encourages further investigations.

Next to microarray gene expression profiles, we also generated protein array profiles of *in vitro* prednisolone sensitive and resistant BCP-ALL cells of 123 newly diagnosed patients. An increased mRNA and protein expression of the NR4A transcription factor family (*NR4A1, NR4A2* and *NR4A3*) was detected in prednisolone resistant patients compared to sensitive patients **(Chapter 4)**. The NR4A family are orphan nuclear receptors, which antagonize the glucocorticoid receptor. We hypothesized that upregulated NR4A family expression is responsible for prednisolone resistance in BCP-ALL. Simultaneous siRNA mediated knockdown of Nur77 (*NR4A1*), Nurr1 (*NR4A2*) and Nor1 (*NR4A3*) protein in pediatric leukemic patients' ALL cells did, however, not sensitize these cells to prednisolone. We observed a significant, albeit modest, decrease in leukemic cell survival. Overall our data implicate that the NR4A genes are not suitable targets to reverse prednisolone resistance. These data also suggest that resistance to prednisolone is more likely caused by mechanisms affecting the downstream intracellular signaling than by mechanisms affecting the effective GR receptor levels.

In addition to apoptotic molecules, also several pro-survival mechanisms have been associated with prednisolone resistance which was therefore examined further in **Chapter 5**. Although basal protein expression in resistant and sensitive patients appeared similar, differences became strikingly evident after exposure to prednisolone. Remarkably, prednisolone downregulated the activity of the RAS-RAF-MEK pathway and STAT6 in prednisolone sensitive patients cells, but was not capable to do so in prednisolone resistant cases. In addition, an active upregulation of the proto-oncogene cMET was found in prednisolone resistant compared to prednisolone sensitive cells. The finding that prednisolone is incapable of downregulating the activity of RAS-RAF-MEK pathway in prednisolone resistant patients, prompted us to investigate the presence of RAS-pathway activating mutations. We discovered activating NRAS and KRAS mutations in 38% of patients. In 60% of mutated patients we even found multiple subclones with different mutations. We also detected a tendency of more RAS activating mutations in prednisolone resistant compared to sensitive patients. Inhibitors against MEK (Trametinib), BRAF (Sorafenib), STAT6 (AS1517499), or cMET (Crizotinib) did not induce substantial cell death *in vitro* in BCP-ALL patient samples. However, Trametinib was impressively potent when combined with prednisolone eradicating especially all NRAS/KRAS-mutated leukemic cells. In one NRAS/

KRAS-mutated case Sorafenib but not Trametinib sensitized the leukemic cells to prednisolone significantly. Our data implicate that BCP-ALL patients should be screened for RAS-mutations as these patients could benefit from treatment with Trametinib/Sorafenib combined with prednisolone. We furthermore advise analysis of the *in vitro* response of RAS mutated BCP-ALL patients' cells to Trametinib and Sorafenib. Our results illustrate the heterogeneity among causes of prednisolone resistance and encourages the investigation of RAS-activating mutations with high read-depth to select patients who may be eligible for more targeted drugs.

Overall, the results in this thesis point out that MCL1 LNAs , 2-DG, an EMP1 inhibitor, Trametinib (MEK inhibitor) and Sorafenib (BRAF inhibitor) belong to the therapeutic options to reverse prednisolone resistance which may contribute to cure the remaining 10-20% of ALL patients. Future investigations should focus on multifactorial approaches to completely sensitize ALL cells to prednisolone and on further in depth investigations of microenvironment induced prednisolone resistance and personalized mutation-based therapies to revert prednisolone resistance.

SAMENVATTING

SAMENVATTING

Van kinderen met acute lymfatische leukemie (ALL) recidiveert 20% tijdens of na de huidige behandelingsstrategieën. Het falen van de huidige behandeling is geassocieerd met cellulaire resistentie tegen prednisolon, het voornaamste medicijn dat gebruikt wordt in de huidige combinatie-chemotherapie. De moleculaire mechanismen betrokken bij prednisolon resistentie in kinder ALL zijn niet volledig bekend. Het doel van dit proefschrift was om nieuwe aanknopingspunten te vinden waarmee we prednisolon resistentie kunnen opheffen.

Recente bevindingen tonen aan dat prednisolon resistente cellen een defect hebben in medicijn-geïnduceerde celdood mechanismen. Wij hebben daarom celdood eiwitten in leukemiecellen van kinderen met ALL geanalyseerd **(Hoofdstuk 2)**. Hieruit bleek dat een hoge eiwit expressie van MCL1 waarschijnlijk prednisolon resistentie veroorzaakt. We hebben daartoe klinisch relevante locked-nucleic-acid (LNA) oligonucleotiden ontwikkeld die de expressie van MCL1 omlaag bracht. De verlaging van MCL1 induceerde celdood en maakte de leukemiecellen gevoeliger voor prednisolon. Echter niet alle leukemiecellen konden hiermee gedood worden. We hebben ontdekt dat deze cellen zichzelf weer gedeeltelijk konden redden door hun energieaanmaak te verhogen. Toen we daarom tegelijkertijd de MCL1 eiwit expressie verlaagde met MCL1 LNAs en de energieaanmaak verlaagde met 2-deoxyglucose (2-DG) doodde dit gezamenlijk de leukemiecellen en werden ze gevoeliger voor prednisolon. Onze resultaten laten daarmee zien dat MCL1 LNA en 2-DG nieuwe therapeutische mogelijkheden kunnen bieden. Deze bevindingen laten ook zien dat meer onderzoek nodig is naar multifactoriële oorzaken van prednisolon resistentie en combinatietherapie om leukemiecellen gevoeliger te maken voor prednisolon.

Om prednisolon resistentie verder te bestuderen hebben we gekeken naar de expressie van duizenden genen in leukemiecellen van 256 kinderen met nieuw gediagnosticeerde ALL. Met behulp van deze informatie hebben we epitheliaal membraan proteïne 1 (EMP1) geïdentificeerd als een mogelijk betrokken gen bij prednisolon resistentie **(Hoofdstuk 3)**. EMP1 is een eiwit dat op het celmembraan zit en waarvan de biologische functie niet bekend is. We hebben ontdekt dat het uitzetten van EMP1 expressie de leukemiecellen doodt en ze gevoeliger maakt voor prednisolon. Het uitzetten van EMP1 inhibeerde tevens de beweging en aanhechting van leukemiecellen aan mesenchymale stroma cellen (MSCs). Mesenchymale stroma cellen zijn belangrijke cellen in het beenmerg die ervoor kunnen zorgen dat leukemiecellen overleven. We hebben ontdekt dat deze MSCs leukemiecellen resistenter kunnen maken voor prednisolon en dat het uitzetten van EMP1 dit verhinderd. Verder hebben we ontdekt dat kinderen met

een hoge expressie van EMP1 een slechtere overleving hebben dan kinderen met een lage expressie van EMP1. Samengevat laten onze resultaten zien dat EMP1 een belangrijke pathobiologische rol speelt in leukemie. Het ontwikkelen van een EMP1 inhiberend medicijn zou kunnen dienen als een mogelijke nieuwe therapeutische behandelingsoptie voor kinderen met leukemie. Verder laten onze resultaten eerste inzichten zien in de rol van het micromilieu in medicijn resistentie dat verder onderzoek naar dit fenomeen aanmoedigt.

Naast het bestuderen van duizenden genen, hebben we ook zo'n 250 eiwitten bestudeerd van 123 nieuw gediagnosticeerde kinderen met ALL. We hebben daarbij een verhoogde gen en eiwit expressie gevonden van de NR4A transcriptiefactor familie (*NR4A1*, *NR4A2* en *NR4A3*) in prednisolon resistente patiënten vergeleken met prednisolon sensitieve patiënten **(Hoofdstuk 4)**. De NR4A familie zijn eiwitten die de werking van prednisolon tegen kunnen werken. Het tegelijkertijd uitzetten van het Nur77 (*NR4A1*), Nurr1 (*NR4A2*) en Nor1 (*NR4A3*) eiwit in leukemiecellen van kinderen maakte deze cellen echter niet gevoeliger voor prednisolon. We zagen wel een significant maar bescheiden verlaging van celoverleving. Samengevat impliceren onze resultaten dat de NR4A genen geen geschikte targets zijn om prednisolon resistentie op te heffen. Deze data suggereren ook dat resistentie voor prednisolon waarschijnlijk veroorzaakt wordt door mechanismen die voornamelijk de ondergelegen intracellulaire signalering beïnvloeden dan door mechanismen die het effectieve actieve prenisolon receptor niveau beïnvloeden.

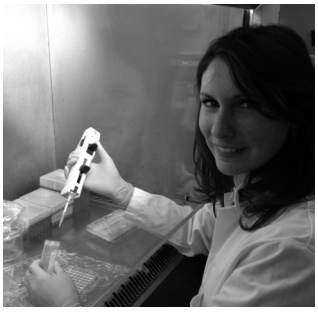
Naast celdood mechanismen zijn ook andere overlevings mechanismen geassocieerd met prednisolon resistentie, deze zijn verder onderzocht in **Hoofdstuk 5**. We zagen dat prednisolon de activiteit van het RAS-overlevingspad en STAT6 verlaagde in prednisolon sensitieve ALL cellen van kinderen, maar niet in staat was dit te doen in de resistente cellen. Tevens vonden we een actieve opregulatie van het proto-oncogen cMET in prednisolon resistente leukemiecellen vergeleken met sensitieve leukemiecellen. De bevinding dat prednisolon niet in staat is de activiteit van het RAS-overlevingspad te verlagen in prednisolon resistente patiënten, spoorde ons aan om de aanwezigheid van RAS-pad activerende afwijkingen te onderzoeken. We hebben afwijkingen in NRAS en KRAS gevonden in 38% van de patiënten. In 60% van deze patiënten vonden we afwijkingen in kleine groepen cellen (subklonen). Medicijnen tegen MEK (Trametinib), BRAF (Sorafenib), STAT6 (AS1517499) of cMET (Crizotinib) waren niet in staat de leukemiecellen *in vitro* te doden. Echter, Trametinib was indrukwekkend potent in combinatie met prednisolon en alle afwijkende NRAS/KRAS leukemiecellen werden hiermee volledig gedood. In één patient met NRAS/KRAS-afwijkende leukemiecellen sensitiseerde Sorafenib, maar niet Trametinib

de cellen voor prednisolon. Onze data impliceert dat leukemiepatiënten gescreend zouden moeten worden op RAS-afwijkingen, daar deze patiënten wellicht voordeel kunnen hebben bij behandeling met Trametinib/Sorafenib in combinatie met prednisolon. Onze resultaten illustreren de heterogeniteit van prednisolon resistentie en moedigen aan om RAS-activerende afwijkingen verder te onderzoeken om zodoende patiënten te selecteren die geschikt zijn voor deze leukemiecel-specifieke medicijnen.

Samengevat, dit proefschrift toont aan dat MCL1 LNAs, 2-DG, een EMP1 inhibitor, Trametinib en Sorafenib behoren tot de mogelijke therapeutische opties om prednisolon resistentie op te heffen en zouden zo kunnen bijdragen aan de genezing van de resterende 10-20% kinderen met ALL. Toekomstig onderzoek zou zich moeten richten op een multifactoriële benadering om ALL cellen volledig gevoelig te maken voor prednisolon, op verder diepteonderzoek naar de invloed van het micromilieu op prednisolon resistentie en op individuele afwijking-gebaseerde therapieën om prednisolon resistentie tegen te gaan.

ABOUT THE AUTHOR

CURRICULUM VITAE - ENGLISH



Ingrid Ariës was born on April 8th 1986 in Maastricht, The Netherlands. She passed her Gymnasium exam at Trichter College Maastricht in 2004. Her thesis was on "The effects of chloorhexidinedigluconaat and triclosan on *Streptococcus Mutans*". In the same year she started with the Bachelor Molecular Life Sciences at Maastricht University and graduated *Cum Laude* in 2007. Hereafter, she started the Master Clinical Molecular Sciences at Maastricht University. During her 6 month junior internship she studied the effects of exogenous galectin-1 on tumour angiogenesis at the department of Pathology, Research School for Growth and Development Maastricht, which resulted in an authorship. She obtained an Erasmus Exchange Grant and Dutch Cancer Society Grant to continue this study during her 9-month senior internship in Milan Italy. At the Istituto Europeo di Oncologia Milano Italy, department of Hematology/Oncology, she studied the role of galectin-1 during tumour vasculogenesis in galectin-1 knockout mice. She obtained her Masters of Science degree *Cum Laude* in 2009. She moved to Rotterdam in September 2009 to start her PhD thesis in the research group of Prof. Dr. Rob Pieters and Dr. M.L. den Boer at the department of Pediatric Oncology/ Hematology, Sophia Children's Hospital – Erasmus MC. The research performed during this period is presented in this thesis. In October 2013 she started a new research project under the supervision of Prof.dr. Rob Pieters and Dr. M.L. den Boer named clonal evolution and personalized targeted therapy in pediatric acute lymphoblastic leukemia. She will move to Boston, USA, in the summer of 2014. In the researchgroup of Dr. A. Gutierrez, Children's Hospital Boston-Harvard, she will investigate chemotherapy/apoptosis resistance in pediatric T-cell acute lymphoblastic leukemia.

CURRICULUM VITAE - NEDERLANDS

Ingrid Ariës is geboren op 8 april 1986 te Maastricht. In 2004 behaalde ze haar Gymnasium diploma aan het Trichter College te Maastricht. Voor haar eindthesis onderzocht ze de effecten van chloorhexidinedigluconaat en triclosan op de Streptococcus Mutans. In datzelfde jaar startte ze met de bachelor opleiding Molecular Life Sciences aan de Universiteit Maastricht en in 2007 slaagde ze *Cum Laude*. Hierna begon ze met de Master Clinical Molecular Sciences aan de Universiteit Maastricht. Tijdens haar 6 maanden durende junior stage onderzocht ze de effecten van exogeen galectine-1 op tumor angiogenese (Afdeling Pathologie, Research School for Growth and Development Maastricht), dat een auteurschap opleverde. Ze behaalde een Erasmus Exchange Beurs en KWF studenten Beurs om dit onderzoek te vervolgen tijdens haar 9 maanden durende senior stage in Milaan Italië. Aan de Istituto Europeo di Oncologia Milano Italy, afdeling Hematologie/Oncologie bestudeerde ze de rol van galectin-1 tijdens tumour vasculogensis in galectin-1 knockout muizen. Ze behaalde haar Masterof Science diploma *Cum Laude* in 2009. In september 2009 verhuisde ze naar Rotterdam om te beginnen aan haar promotieproject in de onderzoeksgroep van Prof.dr. Rob Pieters and Dr. M.L. den Boer op de afdeling Hematologie/Oncologie , Sophia kinderziekenhuis – Erasmus MC. Het onderzoek uitgevoerd tijdens deze periode is beschreven in dit proefschrift. In Oktober 2013 is ze begonnen aan een nieuw project onder de supervisie van Prof.dr. Rob Pieters en Dr. M.L. den Boer genaamd klonale evolutie en op maat gemaakte therapie in acute lymfatische kinderleukemie. In de zomer van 2014 zal ze verhuizen naar Boston, Verenigde Staten. In de onderzoeksgroep van Dr. A. Gutierrez, Kinderziekenhuis Boston-Harvard, zal ze chemotherapie/apoptosis resistentie in T-cell acute lymfatische kinderleukemie onderzoeken.

LIST OF PUBLICATIONS

Ariës IM, van den Dungen ESR, Koudijs MJ, Cuppen E, Voest EE, Molenaar JJ, Caron HN, Pieters R, den Boer ML. Towards personalized targeted therapy in pediatric acute lymphoblastic leukemia; RAS mutations and prednisolone resistance. (Submitted).

Ariës IM, Jerchel IS, van den Dungen ESR, van den Berk LCJ, Boer JM, Horstmann MA, Escherich G, Pieters R, den Boer ML. EMP1, a novel poor prognostic factor in pediatric leukemia regulates prednisolone resistance, cell proliferation, migration and adhesion. (Submitted).

Ariës IM, Hansen BR, Koch T, van den Dungen ESR, Evans WE, Pieters R, Den Boer ML. The synergism of MCL1 and glycolysis on pediatric acute lymphoblastic leukemia cell survival and prednisolone resistance. **Haematologica**. 2013 Dec;98(12):1905-11

Ariës IM, van den Dungen ESR, Pieters R, den Boer ML. The NR4A orphan nuclear receptors do not confer prednisolone resistance in pediatric acute lymphoblastic leukemia. **Leukemia**. 2013 Sep 23. doi: 10.1038/leu.2013.275. [Epub ahead of print]

Akbari Moqadam F, Lange-Turenhout EAM, **Ariës IM**, Pieters R, den Boer ML. MiR-125b, miR-100 and miR-99a co-regulate vincristine resistance in childhood acute lymphoblastic leukemia. **Leukemia research**. 2013 Oct;37(10):1315–21.

Arabi A, Ullah K, Branca RMM, Johansson J, Bandarra D, Haneklaus M, Fu J, **Ariës I,** et al. Proteomic screen reveals Fbw7 as a modulator of the NF-κB pathway. **Nature communications**. 2012 Jan;3:976.

Thijssen VL, Barkan B, Shoji H, **Ariës IM**, Mathieu V, Deltour L, Hackeng TM, Kiss R, Kloog Y, Poirier F, Griffioen Aw. Tumor cells secrete galectin-1 to enhance endothelial cell activity. **Cancer Research**. 2010 Aug 1;70(15):6216-24

PHD PORTFOLIO

PHD PORTFOLIO

Summary of PhD Training and Teaching Activities

Name: Ingrid M.A.A. Ariës
Erasmus Mc department: Pediatric Oncology/Hematology
Research School: Molecular Medicine
PhD period: Sept 2009 - Sept 2013
Promotor: Prof.dr. R. Pieters
Co-promotor: Dr. M.L. den Boer

Seminars/Courses/Workshops	Year	ECTS
Biobase knowledge Library and ExPlain Analysis, Postgraduate School Molecular Medicine Erasmuc Medical Center, Rotterdam, The Netherlands, Oct 2009	2009	0.4
Biomedical Research Techniques VIII, Postgraduate School Molecular Medicine, Erasmuc Medical Center, Rotterdam, The Netherlands, Oct 2009	2009	1.5
Basic and Translational Oncology, Postgraduate School Molecular Medicine, Erasmuc Medical Center, Rotterdam, The Netherlands, Nov 2009	2009	1.8
Basic Oncology Course, Dutch Oncology Association, Seminar Estate Avegoor, Ellecom, The Netherlands, March 2010	2010	2
Basic Course on "R", Postgraduate School Molecular Medicine, Erasmuc Medical Center, Rotterdam, The Netherlands, June 2010	2010	1.4
Analysis of Microarray Gene Expression Data, Center for Human and Clinical Genetics Leiden University Medical Center, The Netherlands, June 2010	2010	2
Photoshop CS3 Workshop, Postgraduate School Molecular Medicine Erasmuc Medical Center, Rotterdam, The Netherlands, June 2010	2010	0.3
Statistics for Genomics Data-Analysis , Department of Epidemiology and Biostatistics, VU University Medical Center, Amsterdam, The Netherlands, Nov 2010	2010	2
The Ensembl Workshop, Postgraduate School Molecular Medicine, Erasmuc Medical Center, Rotterdam, The Netherlands, Sept 2010	2010	0.6
English Biomedical Writing and Communication, Postgraduate School Molecular Medicine Erasmuc Medical Center, Rotterdam, The Netherlands, Dec 2011 - March 2012	2011/2012	4
Classical Methods for Data Analysis (CC02), Netherlands Institute for Health Sciences, Rotterdam, The Netherlands, Sept - Oct 2012	2012	5.7
Indesign CS5 Workshop, Postgraduate School Molecular Medicine, Erasmuc Medical Center, Rotterdam, The Netherlands, June 2013	2013	0.15

PHD PORTFOLIO - CONTINUED

International and National Conferences and Presentations	Year	ECTS
Erasmus Postgraduate school Molecular Medicine Day 2011, Rotterdam, The Netherlands, Feb 2011, poster presentation	2011	0.6
American Association for Cancer Research (AACR) Annual Meeting 2012, Chicago, Illinois, USA, April 2012, poster presentation	2012	1.6
The International BFM Study group (IBFM) Annual Meeting 2012, Santiago, Chili, April 2012, poster presentation, 1st price poster award	2012	1.6
European Hematology Association (EHA) Annual Meeting 2012, Amsterdam, The Netherlands, June 2012, poster presentation	2012	1.6
International Society of Paediatric Oncology (SIOP) Annual Meeting 2012, London, United Kingdom, Oct 2012, oral presentation	2012	2
American Society of Hematology (ASH) Annual Meeting 2012, Atlanta Georgia, USA, Dec 2012, poster presentation	2012	1.6
Erasmus MC Daniel den Hoed Day 2013, Rotterdam The Netherlands, Oct 2013, oral and poster presentation	2013	2
American Society of Hematology (ASH) Annual Meeting 2013, New Orleans, Louisiana, USA, Dec 2013, oral and poster presentation, abstract achievement award	2013	2
Other Presentations		
Research Meetings Laboratory of Pediatrics and Pediatric Oncology	2009-2013	4

Reviewing	Year	ECTS
Reviewer for Leukemia Research and Treatment, a peer-reviewed open access journal that publishes original research articles, review articles, and clinical studies related to all aspects of leukemia.	2012	3

Teaching	Year	ECTS
Supervising Ms. Isabel Jerchel during her final year MSc Molecular Medicine, Rotterdam University, The Netherlands, Master Thesis 12-month Internship on "Unraveling prednisolone resistance in Acute Lymphoblastic Leukemia"	2011-2012	10
Supervising Ms. Giulia Pellizari during part of her final year MSc Medicina Molecolare, University of Trieste, Italy, Master Thesis Internship on "The role of TPMT and ITPA in Thiopurine resistance"	2012	4
Supervising Rotterdam University Medicine Students during their Minor Pediatric Oncology/Hematology Laboratory Techniques	2012 and 2013	4

Total	**60**

DANKWOORD

DANKWOORD

"Appreciation is a wonderful thing. It makes what is excellent in others belong to us as well." - Voltaire

Na 10.000 uren in het lab, 9000 MTTs, 4500 lentivirale knockdown experimenten, 200 microarrays, 1 RSI pipetteerduim, 1 gebroken pols, 20 presentaties, 5 internationale congressen, 6 lasergame wedstrijden, 4 kerstpartijtjes en de zomerse lab-day out, 100 gezellige etentjes, bioscoopavondjes of stapavondjes en veel nieuwe vrienden verder is het na 4 jaar dan eindelijk zover, ik ga promoveren yeahhh!! Natuurlijk was dit allemaal nooit gelukt zonder de hulp van mijn promotor, co-promoter, collega's, vrienden en familie die ik ontzettend dankbaar ben.

"Never, never, never give up." - Winston Churchill

Allereerst bedank ik alle patiënten en hun ouders. Jullie maken het mogelijk om de behandeling en overlevingskans van acute lymfatische leukemie nog verder te verbeteren!

"The mediocre teacher tells. The good teacher explains. The superior teacher demonstrates. The great teacher inspires." - William Arthur Ward

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"Alone we can do so little; together we can do so much" - Helen Keller

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"A friend is one of the nicest things you can have,
and one of the best things you can be." - Douglas Pagels

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"Together is my favourite place to be."

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