# Novel genomic determinants of apoptotic defects in acute lymphoblastic leukemia

Nieuwe genomische determinanten van apoptotische defecten in acute lymphoblastaire leukemie

Amy Holleman

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# Novel Genomic Determinants of Apoptotic Defects in Acute Lymphoblastic Leukemia

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**Amy Holleman**

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**Arma virumque cano, Troiae qui primus ab oris**
**Italiam, fato profugus, Laviniaque venit**
**litora, multum ille et terris iactatus et alto**
**vi superum saevae memorem Iunonis ob iram;**
**multa quoque et bello passus, dum conderet urbem...**

(Vergilius, Aeneïs I, 1-5)

**Vox audita perit, littera scripta manet.**
*The spoken word perishes, the written word remains.*

Voor Colin
en mijn ouders

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Chapter 1

# General introduction

Chapter 1

## 1.1 Hematopoiesis and leukemia.

All cells that circulate in the peripheral blood are derived from a common ancestor: the pluripotent stem cell in the bone marrow. During the process of blood cell formation (hematopoiesis), proliferation and differentiation of stem cells give rise to progenitor cells of the mixed myeloid and the lymphoid lineage. After further rounds of proliferation and differentiation, the myeloid lineage mainly generates monocytes (giving rise to macrophages) and granulocytes (neutrophils, basophils and eosinophils). Monocytes and granulocytes play critical roles in the body's main defense against pathogens. The lymphoid pathway mainly generates B and T lymphocytes. B lymphocytes differentiate further into plasma cells, which secrete immunoglobulins, required for elimination of pathogens. T lymphocytes play an important role in antigen-recognition and subsequent cell-mediated immunity.
In healthy individuals, a tight balance is maintained between proliferation, differentiation and release of the blood cells from the bone marrow. Leukemia is a malignant disease characterized by the uncontrolled proliferation of hematopoietic cells and the progressive accumulation of these cells within the bone marrow and secondary lymphoid tissues. The leukemic cells are thought to derive from clonal expansion of a single neoplastic cell, which fails to differentiate beyond the blast stage. Leukemia can be classified into acute and chronic leukemia. Acute leukemia progresses rapidly and if untreated, can be fatal within weeks or months. Chronic leukemia is seldom diagnosed in children, has a slower course over a much longer period and is fatal in months to years if untreated.

## 1.2 Acute leukemia.

Acute leukemia is the most common form of childhood cancer and the primary cause of cancer-related mortality in children. Acute leukemias that are characterized by the accumulation of malignant cells of the lymphoid lineage are called acute lymphoblastic leukemia (ALL) and leukemias that involve cells of the myeloid lineage are called acute myeloid leukemia (AML) or acute non-lymphoid leukemia (ANLL). ALL is subdivided into B-lineage and T-lineage ALL according to the presence or the absence of lineage-associated immunological markers. Adapted in the 1970’s the French-American-British (FAB) cell-classification system distinguishes among eight morphological subtypes of AML: FAB types M0-M7.[1]

**Clinical presentation**

The clinical symptoms of acute leukemia are directly attributable to the leukemic infiltration of the bone marrow, with resultant cytopenia: spontaneous bruises, purpura and hemorrhage due to thrombocytopenia, weakness, pallor and fatigue due to anemia, and fever, malaise and infections due to granulocytopenia. Clinical symptoms caused by organ infiltration are tender bones, enlargement of lymph nodes and abdominal discomfort caused by an enlarged liver and spleen.

**Age at diagnosis**

Leukemia is the most common cancer among children, representing approximately one third of cancer diagnoses among children younger than 18 years of age. ALL represents approximately 80% of all pediatric leukemias in children, whereas AML comprises 15-20%. ALL and AML and are diagnosed at an annual rate of 120 and 25 children per year in the Netherlands respectively.[2] There is a peak incidence of childhood ALL between ages of 2 to 8 years of age. After the age of 50 there is again a small but progressive increase in the frequency of ALL. The incidence of AML increases with age with a median age at diagnosis over 60 years.[3,4]

**Treatment outcome in acute leukemia**

In general, more than 98% of children with ALL achieve a first complete remission and 75-80% of these children stay in a long term continuous complete remission.[5-7] Bone marrow and/or extramedullary (e.g., central nervous system, testicular) relapses can occur during therapy or after completion of treatment. While the majority of children with recurrent ALL attain a second remission, the likelihood of cure is relatively poor (5-years event-free survival <50%) particularly for those with bone marrow relapse following short initial remission duration. Infants, defined as below 1 year of age, and adults with ALL have a much poorer prognosis, with a long-term event-free survival of only about 35% and 20-40% respectively.[8,9]

Although a complete remission is achieved in up to 80-90% of children with AML, the long term event-free survival for AML is only 60%.[10-12] The relatively unfavorable prognosis of children with AML is caused by a high proportion of relapses after initial achievement of complete remission (30-40%).[13] Although a second complete remission is induced in approximately 70% of the children with recurrent AML, only 30-35% of these children stay in a long term continuous complete remission.[14]

### 1.3 Risk factors in acute leukemia.

A variety of clinical and biological parameters has been associated with response to treatment in childhood acute leukemia. These risk factors are summarized in Table 1.

**Table 1: Risk factors in childhood acute leukemia**

| Prognostic factor | Favorable feature | Unfavorable feature |
|---|---|---|
| Age at diagnosis | ≥ 1 years, <10 years | <1 years, ≥ 10 years |
| White blood cell count | low, e.g. <50 × $10^9$ cells/L | high, e.g. ≥ 50 × $10^9$ cells/L |
| Immunophenotype (ALL) | common ALL, pre-B-ALL | pro-B ALL, T-ALL |
| FAB classification (AML) | M1 auer$^+$, M2, M3, M4eo | M0, M6, M7 |
| Genetic abnormalities (B-ALL) | hyperdiploid>50<br>t(12;21) | hypodiploid<45<br>t(9;22), 11q23 rearranged |
| Genetic abnormalities (T-ALL) | overexpression *HOX11*<br>t(11;19) | overexpression *TAL1*, *LYL1* |
| Genetic abnormalities (AML) | t(8;21), t(15;17), inv(16) | t(1;22), t(6;9), inv(3), del(5q), del(7q), monosomy 5, monosomy 7, trisomy 8, complex karyotypes |
| Early response to treatment | <1000 blasts/µl in PB after 1 week of systemic induction with PRED and a single intrathecal dose of MTX | ≥1000 blasts/µl in PB after 1 week of systemic induction with PRED and a single intrathecal dose of MTX |
| Response to induction therapy (ALL) | <5% blasts in the BM<br>detectable MRD | ≥5% blasts in the BM<br>no detectable MRD |
| *In vitro* drug resistance | $LC_{50}$ PRED ≤ 0.100 µg/ml<br>$LC_{50}$ VCR ≤ 0.391 µg/ml<br>$LC_{50}$ ASP ≤ 0.033 IU/ml<br>$LC_{50}$ DNR ≤ 0.075 µg/ml | $LC_{50}$ PRED ≥ 150 µg/ml<br>$LC_{50}$ VCR ≥ 1.758 µg/ml<br>$LC_{50}$ ASP ≥ 0.912 IU/ml<br>$LC_{50}$ DNR ≥ 0.144 µg/ml |

*Abbreviations*: auer$^+$=auer rods present, B-ALL=B-lineage ALL, T-ALL=T-lineage ALL, PRED=prednisolone, VCR=vincristine, ASP=L-asparaginase and DNR=daunorubicin, MTX=methotrexate, PB=peripheral blood, BM=bone marrow, MRD=minimal residual disease (persisting leukemic involvement of the BM on completion of induction therapy).

#### White blood cell count and age

To date, white blood cell count and age at the time of initial diagnosis are the two most important factors predictive of outcome in B-lineage ALL, although they are not prognostic in T-lineage ALL.[13,15,16] *In vitro* studies in ALL and AML cells demonstrated that drug uptake as well as drug-induced apoptosis decreased with increasing cell density.[17,18] However, the underlying mechanisms that account for the adverse outcomes associated with elevated white blood cell count are currently unknown. The impact of age on clinical outcome in acute leukemia may be explained by its association and genetic abnormalities; hyperdiploidy (>50 chromosomes) is predominantly found in 1- to 10-year old patients, t(12;21)/[*TEL-AML1*] in 2- to 5-year-

old patients and *MLL* rearrangements in infants.[19,20] Furthermore, age is associated with *in vitro* responsiveness to single drugs and *in vivo* response to induction treatment.[20,21] Compared to younger patients with ALL, adolescents (10-21 years of age) and adults have a higher incidence of unfavorable (high white blood cell count, T-cell immunophenotype and t(9;22)/[*BCR-ABL*]) and a lower incidence of favorable clinical and biologic features (hyperdiploidy (>50 chromosomes) and t(12;21)/[*TEL-AML1*]).[15,22-24] Children with T-lineage ALL were reported to have a worse prognosis compared with children with B-lineage ALL to the presence of numerous adverse presenting features, such as older age, high white blood cell count and *in vitro* resistance to a variety of drugs.[20,21,25-27] The poor prognosis associated with T-lineage ALL and mature B-ALL has progressively improved by risk-adjusted intensified protocols.[28,29]

**Genetic abnormalities**

Genetic abnormalities in acute leukemia include chromosomal gains and losses, chromosomal translocations, enhanced expression of proto-oncogenes or decreased expression/function of tumor supressor genes. Genetic abnormalities are identified in the leukemic cells of 60-75% of children with ALL and 50-60% children with AML.[8] Frequencies of the individual genetic abnormalities and corresponding treatment outcomes are provided in Table 2.

Genetic abnormalities associated with a relatively favorable outcome in B-lineage ALL are t(12;21)/[*TEL-AML1*] and hyperdiploidy (>50 chromosomes). In contrast, hypodiploidy (<45 chromosomes), mixed-lineage leukemia (*MLL*) rearrangements (especially t(4;11)/[*MLL-AF4*]) and t(9;22)/[*BCR-ABL*] are associated with poor prognosis. The presence of t(1;19)/[*E2A-PBX1*] was considered a poor prognostic factor, but its significance disappeared with the use of intensified chemotherapy for this form of ALL. Within T-lineage ALL, patients with t(11;19)/[*MLL-ENL*] or overexpression of *HOX11* have a favorable prognosis compared to patients with overexpression of *TAL1* and *LYL1*. The prognostic significance of the *HOX11L2* subtype largely depends on the type of treatment administered.

Genetic abnormalities associated with a relatively favorable outcome in AML are t(8;21)/[*AML-ETO*], t(15;17)/[*PML-RARα*], and inv(16)/[*CBFβ-MYH11*], whereas monosomy 5/del(5q), monosomy 7/del(7q), inv(3)/t(3;3), trisomy 8, t(1;22)/[*RBM15-MKL1*], t(6;9)/[*DEK-CAN*], and a complex karyotype (>3 chromosomal abnormalities) define an AML group associated with a particularly poor prognosis.

**Table 2: Frequency of genetic abnormalities and estimated 5-year event-free survival (EFS) in childhood acute leukemia**

| Genetic abnormality | Genes involved | Freq. (%) | 5-yr EFS (%) | Reference |
|---|---|---|---|---|
| **B-lineage ALL:** | | | | |
| Hyperdiploid >50 | - | 25-30 | 75-90 | 30-34 |
| Hypodiploid <45 | - | 1-9 | 25-42 | 30-33,35 |
| t(1;19) | *E2A-PBX1* | 5-8 | 70-85 | 30-34,36 |
| t(4;11) | *MLL-AF4* | 2-5 | 10-35 | 32,37-40 |
| t(9;22) | *BCR-ABL* | 2-5 | 17-40 | 30,31,34,41-43 |
| t(12;21) | *TEL-AML1* | 16-27 | 85-95 | 32,33,37,44-46 |
| **T-lineage ALL:** | | | | |
| t(11;19) | *MLL-ENL* | 5-8 | 85-95 | 33,47 |
| t(7;10), t(10;14), del(10q24) | *HOX11* | 1-33 | 80-92 | 31,47-51 |
| t(5;14) | *HOX11L2* | 2-24 | 30-60 | 31,47-49,52 |
| t(1;14), *TAL1* recombination | *TAL1* | 12-26 | 30-43 | 31,33,47,53 |
| t(7;19), unknown abnormality | LYL1 | 2-22 | 30-40 | 31,33,47 |
| **AML:** | | | | |
| t(1;22) | *RBM15-MKL1* | 1-3 | <50 | 34,43,54 |
| t(6;9) | *DEK-CAN* | ~1 | unknown | 34,43,54 |
| inv(3)/t(3;3) | *EVI1* | <1 | unknown | 34 |
| inv(16)/t(16;16) | *CBFβ-MYH11* | 5-12 | 47-76 | 34,54-57 |
| monosomy 5/del(5q) | - | 1-2 | unknown | 54 |
| monosomy 7/del(7q) | - | 2-11 | 0-50 | 54,55,57 |
| trisomy 8 | - | 1-23 | unknown | 43,54,56,57 |
| t(8;21) | *AML1-ETO* | 8-15 | *37-60** | 34,43,54,55,57 |
| 11q23 abnormalities | *MLL* | 8-28 | *22-33** | 54-58 |
| t(15;17) | *PML-RARα* | 2-20 | *20-57** | 34,54-57 |
| complex karyotypes | - | 6-11 | *25-66** | 54-56 |

Freq.=frequency, *4-year event-free survival

**Early response to treatment**

Early response is defined as the disappearance of leukemic blasts from the peripheral blood and bone marrow during induction therapy and is a reflection of the characteristics of the leukemic cell as well as the pharmacokinetic characteristics of the host. Several study groups have demonstrated that the persistence of blasts in peripheral blood or bone marrow after the first 7 or 14 days of treatment is highly predictive of clinical outcome (reviewed in ref.[59]). For instance, children with a reduction in peripheral blast count below 1000 blasts/µl after 1 week systemic monotherapy with prednisone and a single intrathecal dose of methotrexate, i.e. a good prednisone window response, have a significant better outcome than patients with a higher number of circulating blasts.[20] In addition, the persistence of circulating blasts after 1 week of multi-agent remission induction therapy was the most significant adverse feature of patients enrolled on St Jude Total Therapy Study XI.[60]

Most relapses occur in the largest group of children with no signs of residual blasts after induction therapy as detected by conventional morphological examination of bone marrow or peripheral blood aspirates. A more specific and sensitive technique to

assess *in vivo* response involves monitoring minimal residual disease (MRD) at consecutive time points during (induction) therapy. Various methods are used to detect MRD, including flow cytometric detection of leukemic clone-specific antigen patterns and real-time quantitative polymerase chain reaction (RQ-PCR) analysis of leukemic clone-specific fusion transcripts or immunoglobulin and/or T-cell receptor gene rearrangements.[20] A number of studies independently demonstrated the prognostic importance of the detection of MRD in the first 1-3 months of therapy.[61-63] Consequently, monitoring of MRD is now being incorporated in many clinical protocols.

***In vitro* drug resistance**

The *in vitro* response to chemotherapy can be studied by exposure of primary patient samples to cytostatic drugs in a cell kill assay such as the methyl-thiazol-tetrazolium (MTT) assay. The independent prognostic significance of *in vitro* resistance to single drugs or a combination of drugs, i.e. prednisolone, vincristine and L-asparaginase (PVA), was demonstrated for childhood ALL.[64-68] Furthermore, i*n vitro* resistance, especially towards prednisolone, is associated with numerous unfavorable risk factors such as a high white blood cell count, age at diagnosis of less than 1 or more than 10 years, pro-B-ALL or T-lineage ALL immunophenotype, the presence of *MLL* gene arrangements and t(9;22)/[*BCR-ABL*], poor prednisone window response and MRD (Table 3).[21,69-72] Compared to ALL, children with AML are more resistant to almost all drugs used, with the exception of cytarabine and thiopurines.[73] Unlike in ALL, *in vitro* resistance at initial diagnosis does not correlate with long-term clinical outcome in childhood AML.[74]

**Table 3: Correlation of *in vitro* drug response in childhood and prognostic factors acute leukemia**

| Prognostic factor | Resistant to: | Sensitive to: | Reference |
|---|---|---|---|
| **Age at diagnosis** | | | |
| <1.5 years | PRED, ASP, VM26 | ARA, CdA | 21,69 |
| >10 | PRED, DEX, ASP, MP, IDA | - | 21 |
| **Immunophenotype** | | | |
| pro-B-lineage ALL | PRED, ASP, DNR, TG, MP, DEX, DOX, IFOS | ARA, CdA | 21,69 |
| T-lineage ALL | PRED, VCR, ASP, DNR, DOX, IDA, DEX, IFOS, ARA | - | 21 |
| AML | PRED, VCR, ASP, DNR, TH, IDA, DEX, MIT, VP16, IFOS | - | 73,75 |
| **Genetic abnormalities** | | | |
| Hyperdiploid >50 | - | ASP, MP, TG, ARA | 76 |
| t(12;21) | - | ASP, DOX, VP16 | 77-79 |
| 11q23 rearrangements | PRED, DEX, ASP | ARA, CdA | 69,80 |
| t(9;22) | PRED, ASP, DNR, VBL, VP16, BLM, MEL, MIT | - | 71,81-83 |
| t(8;21) | IDA | - | 84 |
| t(9;11) | - | VCR, DNR, DOX, CdA, ARA, VP16, MIT, Amsa | 84 |
| 5/7 abnormalities | ARA | - | 84 |
| **Early response** | | | |
| high level of MRD | PRED | - | 85,86 |
| poor PRED window response | PRED | - | 72 |

*Abbreviations:* Amsa=amsacrine, ARA=cytarabine, ASP=L-asparaginase, CdA=2-chlorodeoxyadenosine, BLM=bleomycin, DNR= daunorubicin, DOX=doxorubicin, IDA=idarubicin, IFOS=4HOO-ifosfamide, MAF=mafosfamide, MEL=melphalan, MIT=mitoxantrone, MP=6-mercaptopurine, MTX=methotrexate, PRED=prednisolone, TG=6-thioguanine, TH=thiotepa, VCR=vincristine, VBL=vinblastine, VDS=vindesine, VP16=etoposide VM26=teniposide.

### Gene-expression profiling in acute leukemia

Micro-array technology research allows investigators to make a snapshot of the transcriptional status of the complete genome in a population of leukemic cells. This snapshot provides unique insights into the altered biology underlying the characteristics of these cells.

As described earlier in this chapter, acute leukemia is a heterogeneous disease entity, which consists of various subgroups that differ markedly in treatment outcome. Most leukemic subtypes, however, can hardly be distinguished on the basis of conventional assessment of morphological and histochemical characteristics. The use of gene-expression profiles as a classification tool in leukemia was first demonstrated by Golub *et al*., who accurately distinguished between AML and ALL using a set of genes as a class predictor.[87] Since then, micro-array technology has been successfully used to distinguish additional subgroups of acute leukemia. For instance, several independent groups demonstrated that ALL cells with *MLL* rearrangements have a unique gene-expression profile that clearly distinguishes them from *MLL* germline ALL or AML.[88-90] These results were confirmed and extended by others, who demonstrated distinct gene

expression signatures for each of the known prognostic subtypes in ALL[91-94] and AML.[95-97]

Micro-array technology has also contributed to the identification of previously unrecognized and prognostically significant subgroups of leukemia. For instance, Yeoh *et al* identified a previously unrecognized subset of pediatric ALL based solely on gene expression profiling.[92] In addition, Ferrando *et al* identified gene expression signatures in T-lineage ALL that reflected leukemic arrest at specific stages of normal thymocyte development, i.e. *LYL1* (pro-T), *HOX11* (early cortical thymocyte), and *TAL1* signatures (late cortical thymocyte).[47] Furthermore, they identified *HOX11L2* activation as a novel event involved in T cell leukemogenesis. *HOX11* expression was associated with a favorable prognosis, whereas activation of *TAL1* or *LYL1* was associated with a less favorable prognosis.

More recently, micro-array technology has been applied to gain insight in the determinants of treatment response. Analysis of gene-expression before and after treatment with methotrexate and 6-mercaptopurine, alone or in combination, showed that each of these 3 treatment regimens generated a unique *in vivo* response reflected by treatment-specific changes in gene-expression. However, different ALL subtypes responded with identical changes in gene-expression to the same treatment.[98]

### 1.4. The treatment of acute leukemia.

#### 1.4.1 The backbone of current chemotherapeutic protocols.

The purpose of treatment of acute leukemias is to induce remission and thereafter treat the residual cells to prevent relapse. Remission is conventionally defined by the presence of less than 5% blasts in the bone marrow and the regeneration of normal hematopoiesis. Relapse is defined as a reappearance of leukemic blasts in the bone marrow, peripheral blood or elsewhere in the body following complete remission. The backbone of current therapy for childhood ALL consists of several elements: induction, post-induction (consolidation and intensification), central nervous system (CNS)-directed therapy, reinduction (delayed intensification) and maintenance treatment.[20] In childhood AML, treatment regimens consist of: remission induction, central nervous system (CNS)-directed therapy, and consolidation/intensification. The clinical significance for maintenance therapy is questionable in childhood AML and most groups do not use maintenance therapy.[19,99] In case of the availability of a HLA

matched sibling donor, allogeneic bone marrow transplant is recommended for all patients with high-risk AML, i.e. unfavorable karyotype and a poor response to induction therapy.[10,12]

**Remission induction**

The aim of induction therapy is to achieve a complete remission and the restoration of normal hematopoiesis within approximately 4-6 weeks. In most ALL regimens this is achieved by systemic administration of a glucocorticoid, vincristine and L-asparaginase. Some protocols also include anthracyclines as a fourth drug.[20] One or two short courses of high-dose cytarabine and anthracyclines form the backbone of remission induction in most protocols for childhood AML. Some treatment regimens combine cytarabine and anthracyclines with either etoposide or 6-thioguanine.[12,19]

**Post-induction therapy**

Consolidation of the complete remission and eradication of residual (sub-detection) leukemic cells is the primary aim of consolidation and intensification therapy. Consolidation involves the repeated administration of drugs already used during induction therapy and intensification involves addition of drugs that were not previously administered to circumvent drug resistance. Intensification therapy for ALL includes high-dose methotrexate and 6-mercaptopurine in most study groups.[20] The most important component of intensification therapy for childhood AML are several courses of high-dose cytarabine, either alone or combined with etoposide, amsacrine, mitoxantrone or L-asparaginase.[12,19]

**CNS-directed therapy**

Leukemic cells in the meninges are beyond the reach of most chemotherapeutic drugs and repopulation of the bone marrow from the meninges was a frequent cause of therapy relapse in the past. Therefore, treatment of presymptomatic CNS relapses is an integral part of present therapeutic regimens for ALL and AML. Historically, the most effective CNS-directed therapy was cranial irradiation. However, due to its association with neurotoxicity, hormonal disturbances, development of secondary malignancies and long-term neurocognitive effects, most protocols have eliminated cranial irradiation and use multiple cycles of high dose systemic MTX and/or intrathecal chemotherapy instead (most often in combination with a glucocorticoid and cytarabine).[33] Cranial irradiation is only used in children with high-risk ALL or overt CNS disease.

**Reinduction therapy**

The benefit of reinduction or delayed intensification was first demonstrated in high-risk patients with ALL in the Berlin-Frankfurt-Münster (BFM) studies in the late 1970s and has become an integral component of most treatment regimens. Reinduction therapy is administered approximately 3 months after remission in most protocols and usually involves a repetition of the initial remission induction therapy.[33,100]

**Maintenance therapy**

In order to kill residual, slowly dividing blasts and to suppress emergence of a drug-resistant clone, children with ALL require long-term maintenance therapy. The general rule is to continue therapy for at least 2 years. The usual continuation regimen for children with ALL involves the combination of 6-mercaptopurine administered daily and methotrexate administered weekly. Some protocols also administer intermittent pulses of vincristine and a glucocorticoid.[20]

### 1.4.2 Risk-adapted therapy.

Because the childhood leukemias consist of many prognostically distinct subtypes, a uniform treatment would be inappropriate. Instead, risk stratification is used to assess the risk of relapse before the onset of treatment and tailor treatment intensity accordingly; patients at high risk of relapse will receive augmented treatment while patients at lower risk will receive less-intensive regimen to reduce treatment-related toxicities and long-term side effects. Risk-adapted therapy regimens are usually subdivided into three categories: low, standard (intermediate) and high risk. Most risk-classification schemes consider white blood cell count and age at diagnosis, immunophenotype, genetic abnormalities (especially t(9;22) and *MLL* rearrangements) and prednisone window response.[8] The German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL) study group uses in addition the *in vitro* cytotoxicity to prednisolone, vincristine and L-asparaginase as risk-stratification tool.[64,101]

### 1.4.3 Drugs commonly used in anti-leukemic therapy.

Various drug combinations are used in the current chemotherapeutic regimens for pediatric leukemia. Four chemotherapeutic agents, that are an integral part of all protocols, and which resistance mechanisms will be investigated in this thesis will be briefly discussed in this paragraph.

**Glucocorticoids**

Glucocorticoids such as prednisolone and dexamethasone have been the most important drugs used in the treatment for ALL for more than 50 years. Glucocorticoids exert their effects by binding to the glucocorticoid receptor (GR), which subsequently migrates to the nucleus to affect the transcription of various genes.[102] In near-physiological concentrations, glucocorticoids induce G1 cell cycle arrest and cell death or apoptosis, which will be discussed in more detail in chapter 2.

**Vinca alkaloids**

Vincristine is a vinca alkaloids found in the *Catharanthus roseus* (*Vinca rosea*). The vinca alkaloids are extensively being used in clinical treatment of ALL and other pediatric malignancies since the discovery of their anti-tumor properties in 1959. Vinca alkaloids interact with monomeric β-tubulin and hence inhibit tubulin polymerization into microtubules. This results in a disappearance of both interpolar and mitotic microtubules, leading to mitotic arrest at the $G_2$-M stage and apoptosis.[103]

**L-asparaginase**

L-asparaginase is an enzyme-derived drug purified from *Erwinia chrysanthemi* or *Escherichia coli* which hydrolyzes the amino acids asparagine and glutamine. L-asparaginase is a standard component of treatment protocols for pediatric acute leukemia and causes complete remission in 40-60% of ALL cases as a mono-agent.[104,105] Administration of L-asparaginase leads to rapid depletion of the amino acids asparagine and glutamine form the blood circulation.[106] The resulting asparagine deficiency leads to G1 cell cycle arrest and apoptosis of leukemic cells.[107,108]

**Anthracyclines**

Daunorubicin, doxorubicin, idarubicin and epirubicin are anthracycline antibiotics, originally isolated from the fungus *Streptomyces caeruleorubidus* or *S. peucetius*, which antileukemic activity in pediatric ALL was demonstrated in 1963.[109] Nowadays, anthracyclines are widely used in the treatment of childhood acute leukemia. Various mechanisms have been proposed to explain anthracycline-induced cytotoxicity, including induction of DNA damage by interaction with DNA topoisomerase and DNA helicase, induction of cellular DNA and membrane damage by the generation of free radicals. Anthracycline-inflicted cellular damage has been shown to induce apoptosis in leukemic cells.[110,111]

**1.5 Acute leukemia, apoptosis and mechanisms of cellular drug resistance**

Most, if not all, chemotherapeutic agents ultimately induce cell death by triggering apoptosis.[112] Apoptosis is characterized by a series of stereotypic morphological and biochemical alterations.[113] The morphological changes include cell shrinkage, plasma and nuclear membrane blebbing, organelle relocalization and chromatin condensation. Biochemical hallmarks of apoptosis include loss of sialic acid, translocation of phosphatidylserine to the outer leaflet of the plasma membrane and fragmentation of nuclear DNA into oligonucleosomal fragments. At the end of the apoptotic process, the cell disintegrates into membrane-enclosed vesicles, which are subsequently recognized and cleared by phagocytes.

A family of enzymes called caspases, which are activated upon exposure to chemotherapeutic agents, is responsible for triggering the typical morphological and biochemical features of an apoptotic cell. Once activated, caspases are capable of cleaving a wide array of structural and regulatory cellular proteins. There are two possible routes by which caspases can get activated after exposure to chemotherapeutic agents; the intrinsic or mitochondrial apoptosis pathway and the extrinsic or death receptor apoptosis pathway. Both routes are tightly regulated by various apoptosis-regulatory proteins in healthy cells in order to prevent unnecessary caspase activation. For instance, heat shock proteins and Bcl-2 family members are known regulators of the intrinsic pathway and decoy receptors and FLIP of the extrinsic pathway.[114-118] In addition, there are regulatory proteins, capable of regulating both the extrinsic and the intrinsic apoptosis pathway, i.e. IAP family members.[119-121] The exact

role of these apoptosis-regulatory proteins in controlling apoptosis is discussed in more detail in chapter 2.

The rationale behind combination therapy protocols is that the use of multiple drugs with distinct targets will decrease the probability that an individual malignant clone will be resistant against all used drugs and may survive treatment. However, one of the major causes of failure to contemporary combination therapy protocols still is cellular drug resistance. Defects in normal cell death mechanisms allow cells to survive and accumulate further transforming genetic alterations[122,123] and are thought of as one of the major mechanisms that govern the transformation of normal lymphoblasts into leukemia.[124,125] This and the fact that most chemotherapeutic agents induce apoptosis into their target cells suggests that defects in the apoptosis pathway may lead to cellular drug resistance. Indeed, functional blocks in apoptosis pathways have been found in AML and correlate with poor prognosis.[126] Although the investigation of defects in the apoptosis pathway is limited in children with acute leukemia, various aberrations have been identified. The nature of these defects as well as their relation to cellular drug resistance and clinical outcome is discussed in more detail in chapter 2.

### 1.6 Aims of this thesis

In the past 4 decades, event-free survival has increased to almost 80% for children with ALL and 60% for children with AML. Key clinical contributors to this progress have been better use of old drugs, central nervous system prophylaxis, and risk-adapted therapy. As described in §1.6, a large part of the failures of contemporary chemotherapeutic protocols are caused by cellular drug resistance, which may be caused by defects in the apoptosis pathway. However, little is known about the presence of defects in the apoptosis pathway and their relation to cellular drug resistance in childhood acute leukemia.

In **chapter 2** a literature overview is given of the current knowledge on apoptosis and defects in the execution phase of apoptosis contributing to cellular drug resistance and treatment outcome in childhood acute leukemia. The major aim of the studies described in this thesis is to evaluate which aberrations, either in or outside the execution phase of apoptosis, contribute to cellular drug resistance and treatment failure in childhood acute leukemia.

In **chapter 3** we investigated whether cellular drug resistance was associated with decreased functional apoptosis in newly diagnosed children with ALL. Functional

apoptosis was assessed at various levels of the effector apoptosis route, i.e. phosphatidylserine externalization, collapse of mitochondrial transmembrane potential, caspase-3 activation and PARP inactivation.

In **chapter 4** micro-array technology was applied to analyze the expression patterns of 70 key apoptotic genes in leukemic cells of children with newly diagnosed ALL. The expression was subsequently correlated to immunophenotype, genetic subtype, *in vitro* drug resistance and clinical outcome.

In **chapter 5** the protein expression of Apaf-1, procaspase-2, -3, -6, -7, -8, -10 and PARP were studied in children with newly diagnosed ALL and AML and the question was addressed whether the expression was related to cellular drug resistance in these patients.

In **chapter 6** data are presented of a study in which we applied micro-array technology to identify gene-expression patterns related to cellular drug resistance and outcome in leukemic cells of children with newly diagnosed ALL.

In **chapter 7** the prognostic significance of *OPAL1*, a newly discovered gene shown to be highly predictive of outcome in childhood ALL, was investigated in an independent cohort of children with newly diagnosed ALL.

In **chapter 8** we investigated whether an enhanced glycolytic rate was associated with prednisolone resistance in human leukemia cell lines. In addition, we addressed the question whether inhibition of the glycolytic rate augmented prednisolone-induced cytotoxicity in these cell lines.

The work presented in this thesis is summarized and conclusion and perspectives are given in **chapter 9** (in English) and **chapter 10** (in Dutch).

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Chapter 2

# Occurrence of defects in the apoptosis pathways and their relevance to cellular drug resistance in childhood acute leukemia. A review

Chapter 2

## 1. Introduction

Acute leukemia is the most common cancer diagnosed in children. Approximately 80% of acute leukemia diagnoses among children represent acute lymphoblastic leukemia (ALL) and 20% acute myeloid leukemia (AML). The treatment of pediatric acute leukemia has greatly improved over the past 4 decades, resulting in long-term disease-free survival of approximately 75-80% for ALL[1-3] and 60% for AML.[4] The improved outcome of childhood acute leukemia can largely be attributed to the introduction of combination chemotherapy. The rationale behind this form of treatment is that the use of different drugs with distinct intracellular targets will decrease the probability that a malignant clone will be treatment resistant. Defects in normal cell death mechanisms allow cells to survive which may lead to further accumulation of transforming genetic alterations.[5,6] This process is thought of as one of the major mechanisms that govern the transformation of normal lymphoblasts into leukemia.[7,8] This and the fact that most, if not all, chemotherapeutic agents ultimately induce cell death by triggering programmed cell death or apoptosis[9] suggests that aberrations in the apoptosis pathway may explain a large proportion of the acute leukemia cases resistant to contemporary chemotherapeutic protocols. Indeed, functional blocks in apoptosis pathways appear to be common in AML and correlate with poor response to induction chemotherapy and decreased overall survival.[10] A thorough understanding of the defects in the apoptosis route is critical for understanding the causes of treatment failure and for a rational approach to drug design and therapy.

Induction of apoptosis by chemotherapeutic agents can be subdivided into three general phases: insult generation, signal transduction and execution (Figure 1).[9] Defects in the first two phases, i.e. upregulation of drugs efflux pumps[11-13] or detoxifying enzymes[14-16], mutations in p53[17,18] and overexpression of Mdm2[19], and their relevance to cellular drug resistance in childhood acute leukemia have been described elsewhere[20] and are outside the scope of this chapter. This review summarizes the current knowledge of genes involved in the execution phase of apoptosis and discusses which defects may contribute to cellular drug resistance and treatment failure in childhood acute leukemia.

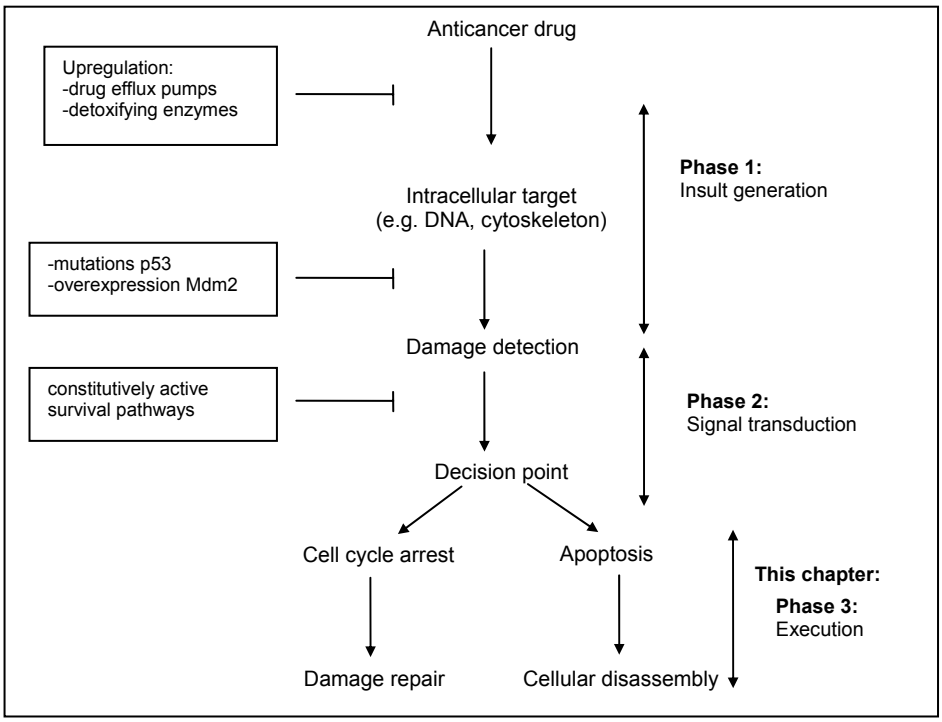


**Figure 1. The three phases of drug-induced apoptosis.** During the insult phase (phase 1), chemotherapeutic agents enter the cell and interact with and cause damage to their specific intracellular targets. Due to the large diversity of intracellular targets this phase is highly heterogeneous. During the downstream transduction of the apoptotic signal (phase 2), the severity of drug-induced damage is assessed and the cell determines if it arrests cell cycle progression and attempts to repair the damage or proceeds to the execution phase of apoptosis. The threshold for apoptosis is defined by the net balance of pro- and anti-apoptotic pathways activated in response to anticancer drugs. During the execution phase (phase 3), the morphological changes characteristic of apoptotic cell death occur. Aberrations in each of these phases, which have the potential to cause cellular drug resistance, are indicated in the boxes.

## 2. The executioners of apoptosis: caspases

During the execution phase of apoptosis the cell is disassembled by the activity of a family of cysteine-dependent aspartate-directed proteases called caspases. At present, the human caspase gene family contains 11 members, 7 of which function in apoptosis (caspase-2, -3, -6, -7, -8, -9, -10) and others mediate cytokine processing (caspase-1, -4, -5, -13).[21] Studies in knockout mice have shown that caspases have a highly cell-type specific expression pattern.[22,23] Activated caspases cleave a number of structural and regulatory cellular proteins which are responsible for the typical morphological and biochemical features of an apoptotic cell.

To prevent demolition of healthy cells, caspases are present in the cytoplasm as enzymatically inactive zymogens (procaspases). Only in cells that undergo apoptosis, procaspases are processed into the mature active enzymes.[24] Caspases have a unique substrate preference: they recognize a specific 4-amino acid motif and cleave this after the aspartic acid residue at the fourth position. The presence of an aspartic acid residue in caspases suggests that procaspases can be activated by active caspases their selves. Indeed, activation of a single caspase leads to a cascade of activated downstream caspases, also known as effector caspases. At least two different ways to activate the first or initiator caspase exist: the intrinsic and the extrinsic pathway.

**3. The intrinsic apoptosis pathway**

The intrinsic or mitochondrial apoptosis pathway is initiated by the release of the electron transport protein cytochrome *c* and other apoptogenic molecules, such as apoptosis-inducing factor (AIF), Smac/DIABLO and Omi/HtrA2 from the mitochondrial intermembrane space.[25] This release is accompanied by a dissipation of mitochondrial inner transmembrane potential ($\Delta\Psi_m$).[26,27] The subsequent binding of cytochrome *c* to the cytoplasmic protein Apaf-1 [apoptotic protease-activating factor-1] causes a dATP/ATP-dependent conformational change of Apaf-1. The more open conformation of Apaf-1 allows the formation of an oligomeric assembly, designated the apoptosome, which recruits and activates procaspase-9.[28] Activated procaspase-9 subsequently activates among others effector caspases-3, -6 and –7, which collectively work to disassemble the cell.[29,30] Given the lethal consequences of spontaneous caspase activation, it is not surprising that the intrinsic route is tightly controlled at multiple levels (Figure 2).

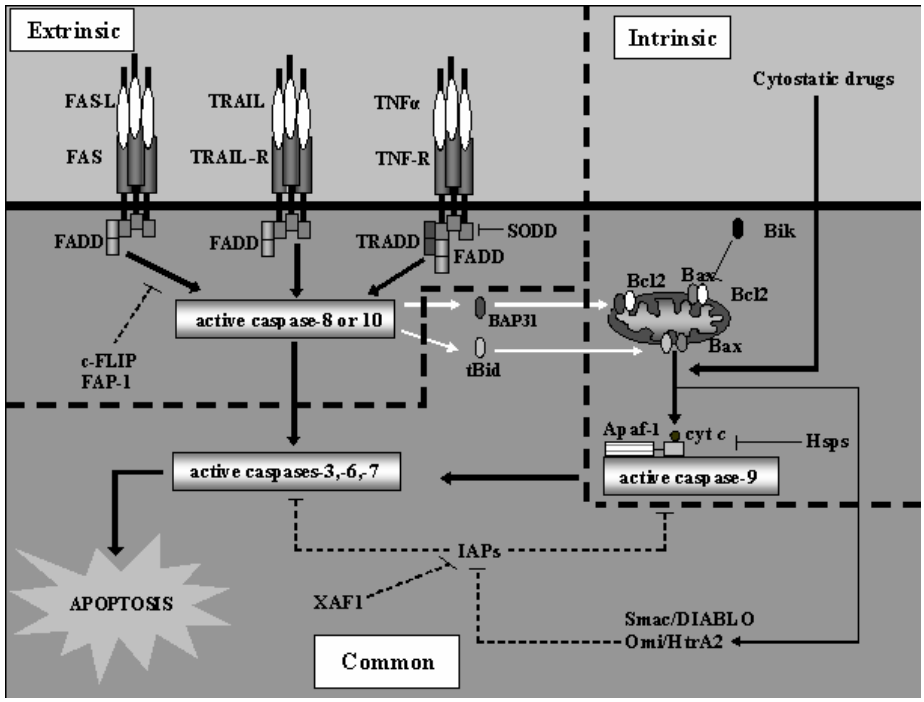


**Figure 2. The intrinsic, extrinsic and common apoptosis pathway.** Schematic representation of the main cellular routes of caspase activation. The core apoptotic route is indicated with bold arrows, dotted arrows indicate regulation mechanisms and white arrows indicate cross-talk between both pathways. Bold striped lines mark the boundary between the intrinsic, the extrinsic and the common apoptosis pathway. See main text for details of both pathways.

### 3.1 Regulation at the mitochondrial level: the Bcl-2 family

Bcl-2 family members are the central regulators of the intrinsic pathway, which sense intracellular damage, integrate pro- and anti-apoptotic signals and finally decide whether cytochrome *c* is released and apoptosis is engaged. The Bcl-2 family consists of more than 30 proteins and has pro- and anti-apoptotic members, which can form hetero- and homodimers.[31] Anti-apoptotic family members, such as Bcl-2, Bcl-$X_L$ and Mcl-1, localize primarily to the mitochondrial outer membrane where they can directly block the release of cytochrome *c*, preventing caspase activation.[32] The pro-apoptotic family members are subdivided according to the number of Bcl-2 homology (BH) domains into the multidomain and the BH3-only subfamily. Members of the multidomain subfamily, like Bax and Bak, are structurally very similar to the anti-apoptotic Bcl-2-like

subfamily but lack the fourth BH domain (BH4). During apoptosis, Bax and Bak both undergo conformation changes and form homo-oligomers within the mitochondrial outer membrane[33,34], which leads to mitochondrial permeabilization and release of cytochrome *c*.[35] The still growing BH3-only subfamily includes Bad, Bid and Bik and is characterized by the presence of only the third BH domain (BH3). BH3-only proteins are thought to induce mitochondrial permeabilization by either forming heterodimers with anti-apoptotic Bcl-2-like proteins or by directly activating the pro-apoptotic multidomain proteins.[31,36]

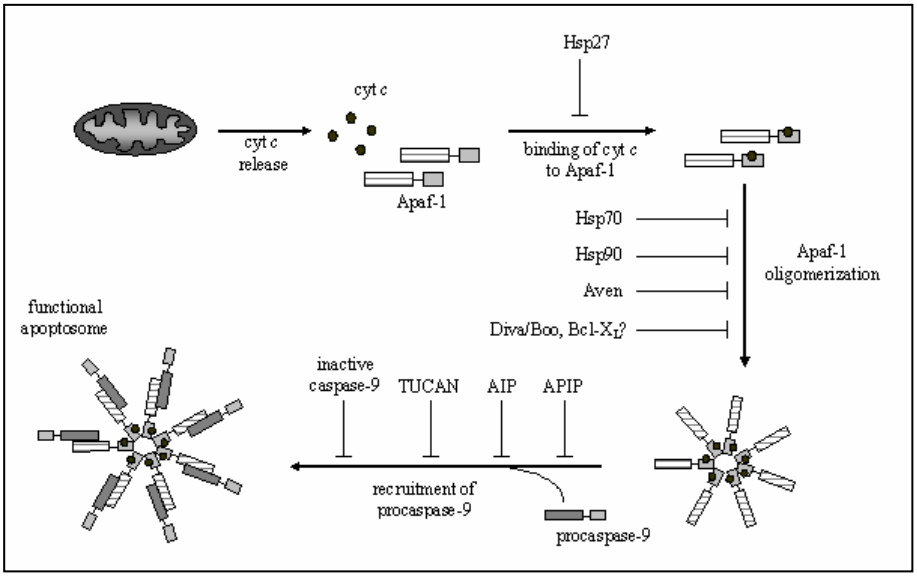


**Figure 3. Regulation of apoptosome formation.** The formation of the apoptosome occurs in various steps and finally leads to activation of the initiator caspase-9. The various steps of the formation of a functional apoptosome as well as the places where regulation occurs are indicated. See main text for details on these regulation mechanisms. Modified after Hajra et al.[37]

### 3.2 Regulation at the apoptosome level

The cell uses different strategies to prevent the formation of the apoptosome (Figure 3). One strategy, employed by heath shock protein 27 (Hsp27), is the binding to and sequestering of cytochrome *c*.[38,39] Another strategy, employed by two other Hsp family members, i.e. Hsp70 and Hsp90, is to prevent the formation of the apoptosome by binding Apaf-1.[40,41] It has been suggested that anti-apoptotic Bcl-2 members like Bcl-$X_L$ and Diva/Boo can interact with Apaf-1, thus preventing Apaf-1 oligomerization.[42,43]

However, the significance of this interaction has recently been questioned as various studies failed to confirm its existence.[44] Aven is a protein which was identified based in its ability to bind both Bcl-$X_L$ and Apaf-1 in a yeast 2-hybrid screen.[45] Aven was shown to interfere with the ability of Apaf-1 to self-associate, suggesting that Aven impairs Apaf-1-mediated caspase activation.[45] Alternatively, formation of the apoptosome can be prevented by competing with Apaf-1. In the last few years various CARD-containing proteins, such as TUCAN, AIP and APIP, have been identified that interfere with the binding of procaspase-9 to Apaf-1.[46-48] Finally, apoptosome formation can be prevented by inactivation of caspase-9, for instance by alternative splicing[49] or phosphorylation by the serine/threonine kinase Akt.[50]

**4. The extrinsic apoptosis pathway**

The activation of initiation caspase-8 and caspase-10 is mediated by the death receptor (DR) family of transmembrane receptors, which includes TNF-R1 [tumor necrosis factor (TNF) receptor 1], Fas (CD95), TRAMP [TNF receptor–related apoptosis-mediating protein] (DR3), TRAIL-R1 (DR4) [TNF-related apoptosis inducing ligand receptor 1], TRAIL-R2 (DR5) and DR6.[51,52] These receptors are characterized by the presence of a conserved cytoplasmic death domain. The best-studied death receptor signaling pathway is mediated by the Fas receptor (Fas). The natural ligand of Fas (Fas-L) is a type II transmembrane protein. Binding of Fas-L to the extracellular domain of Fas leads to receptor trimerization and recruitment of the FADD [Fas-associated death domain] to the cytoplasmic side of Fas. This recruitment involves homotypic interaction between the death domain present in Fas and FADD.[53,54] An additional homotypic interaction takes place between the death effector domains present in FADD and procaspase-8 or procaspase-10.[55,56] Fas, FADD and procaspase-8 or -10 together form the death-inducing signaling complex (DISC) where both procaspases are activated and released into the cytoplasm to activate the effector caspases. Other death receptors activate caspases in a similar way with the exception that TNF-R1 and DR6 first bind TRADD [TNR-R associated death domain], which in turn recruits FADD. The association of procaspase-2 with TNF-R1 via the adapters TRADD, RIP and RAIDD/CRADD suggests that this caspase is involved in TNF-R1-mediated signaling.[57] Like the intrinsic pathway, activation of the extrinsic pathway is regulated at various levels.

### 4.1 Regulation at the death receptor level

Some cells express so-called decoy receptors, i.e. proteins that bind death ligands with high affinity, but are incapable of recruiting cytoplasmic adapter molecules.[58] The *FAS* gene encodes two isoforms through alternative splicing: full-length Fas which has a cytoplasmic domain and a soluble form of Fas (DcR3).[59] DcR3 is secreted in the extracellular environment where it competes with Fas for Fas-L binding. Alternatively, a soluble form of Fas-L, generated through cleavage of the membrane form by metalloproteinases, has been described to compete with Fas-L for Fas binding.[60]
Two decoy receptors are known for TRAIL; TRAIL-R3 (DcR1/TRID), which lacks a cytoplasmic region, including the death domain[61] and TRAIL-R4 (DcR2/TRUNDD), which has a truncated cytoplasmic domain containing only one-third of the consensus death domain.[62] Both decoy receptors bind TRAIL with an affinity comparable to TRAIL-R1 and TRAIL-R2, but cannot transmit the death signal. Fas-associated phosphatase 1 (FAP-1) is a protein tyrosine phosphatase, which inhibits Fas-induced apoptosis by binding to the negative regulatory domain (C-terminal 15 amino acids) of the Fas receptor and reducing Fas cell surface expression[63,64]

### 4.2 Regulation at the death-inducing signaling complex (DISC) level

The assembly of a functional DISC can be blocked by the anti-apoptotic protein FLIP [FLICE-like inhibitory protein]. The first FLIPs that were identified were of viral origin (v-FLIPs). The v-FLIPs are characterized by the presence of two DED motifs and interfere with the recruitment of procaspase-8 to the DED of FADD.[65] Based on sequence homology, the mammalian homologue of v-FLIPs was identified and termed cellular FLIP (c-FLIP). Two forms of c-FLIP are encountered in the mammalian cell: a short form, which structurally resembles v-FLIP (c-$FLIP_S$) and a long form (c-$FLIP_L$), which resembles caspase-8 and 10 but is catalytically inactive. c-$FLIP_S$ and c-$FLIP_L$ interact with FADD and procaspase-8, and potently inhibit apoptosis induced by all known human death receptors.[66] In addition, the binding of silencer of death domains (SODD) to the DD of TNF-R1 is responsible for the negatively regulating downstream TNR-R1 signaling.[67]

## 5. The common downstream apoptotic pathway

Initially the intrinsic and the extrinsic pathways were thought of as two separate pathways that converge to utilize the same group of downstream effector caspases to execute apoptosis. More recent data suggest that this model is oversimplified and that both pathways are interconnected above the level of effector caspases. BAR, for instance, can bridge both pathways by forming a complex with procaspase-8 and the anti-apoptotic Bcl-2 members and Bcl-$X_L$.[68] Another type of cross-talk is observed for Bid and BAP31, which are both caspase-8 targets (Figure 2). [36,69,70]

### 5.1 Regulation of the common apoptotic pathway: the IAP family

The inhibitor of apoptosis proteins (IAPs) constitute a family of intracellular anti-apoptotic proteins that were first identified as homologues of genes present in baculoviruses. Thus far, eight human IAPs have been identified NAIP, c-IAP1, c-IAP2, XIAP, survivin, Apollon, Livin and ILP-2.[71] The anti-apoptotic activity of IAPs is dual: they bind and inhibit caspase-3, -7 and -9 directly [72-74] and, in addition, some IAPs can induce NF-κB signaling pathways that promote survival by induction of a variety of anti-apoptotic factors, including XIAP, c-IAP1 and c-IAP2.[75,76] A number of proteins have been identified that regulate the activity of IAPs. Smac/DIABLO and Omi/HtrA2 reside in the mitochondrial intermembrane space and translocate to the cytoplasm along with cytochrome *c* during apoptosis.[77,78] Both proteins promote apoptosis by binding to IAPs and preventing them from inhibiting caspases. XIAP-associated protein (XAF1) specifically binds and inhibits XIAP, probably by triggering the translocation of XIAP from the cytoplasm to the nucleus.[79]

## 6. Downstream caspase targets

Activated caspases cleave a number of structural and regulatory cellular proteins leading to apoptosis. For instance, cleavage of the inhibitory subunit of caspase-activated DNase (ICAD) leads to DNA fragmentation,[80] cleavage of cytoskeletal proteins actin, gelsolin and fodrin induces cell shrinkage and blebbing,[81] and cleavage of lamins present within the nuclear envelope is required for blebbing of the nuclear membrane.[82,83] Another protein cleaved by activated effector caspases is poly(ADP-

ribose) polymerase (PARP; also as PARP1 and ADPRT), which is involved in DNA repair and the maintenance of genomic integrity.[84]

**7. Apoptotic defects in childhood acute lymphoblastic leukemia**

*Caspases*

The role of caspases in cellular drug resistance in diagnostic childhood ALL samples is summarized in Table 2. The importance of caspase-2 in drug-induced apoptosis was demonstrated by the resistance of caspase-$2^{-/-}$ murine oocytes to cytostatic drugs.[22] The decreased procaspase-2 protein expression levels we observed in diagnostic *in vitro* drug resistant childhood ALL samples is in line with this finding.[85] The loss of caspase-3 expression in drug-resistant ALL and AML cell lines[86] and the loss of spontaneous caspase-3 activation observed at relapse in childhood ALL suggests that impaired caspase-3 activation is involved in therapy resistance.[87] Indeed, decreased drug-induced caspase-3 activation rather than decreased base-line procaspase-3 expression was associated with *in vitro* resistance to prednisolone and L-asparaginase in newly diagnosed childhood ALL.[27,85,88,89] No evidence exists for a contribution of caspase-6 and -7 to drug resistance in pediatric ALL.[85,88] Loss of caspase-8 expression though methylation of the promoter has been reported in various pediatric tumors including neuroblastoma, rhabdomyosarcoma, medulloblastoma and retinoblastoma.[90-92] Treatment with the demethylation agent 5-Aza-2'-deoxycytidine restored caspase-8 expression in various human cell lines and sensitized them to death receptor- and drug-induced apoptosis.[92] Until now, no evidence for procaspase-8 promoter methylation has been found in hematological malignancies.[93,94] Moreover, procaspase-8 expression did not correlate with cellular drug resistance in children with ALL.[85,88] The role of caspase-9 and caspase-10 in drug-induced apoptosis was demonstrated by the resistance of caspase-$9^{-/-}$ murine thymocytes[23] and leukemic cell lines with inactivated caspase-10[95] to drug-induced apoptosis. Pharmacological inhibition of caspase-9 and -10 activity caused partial resistance to glucocorticoid-induced apoptosis in human pre-B ALL cells.[96] These data suggest that caspases-9 and -10 may contribute to drug-induced apoptosis in leukemic cells, although evidence is lacking in clinical samples till date[88,97]

**Table 1. Caspases and cellular drug resistance in newly diagnosed childhood acute leukemia**

| Gene | Leukemia type | Examined | N | *In vitro* drug resistance | Refs |
|---|---|---|---|---|---|
| CASP2 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 43 | **Low expression: PVA, PRED resistance** | 85 |
| CASP3 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 60 | No relation with PRED resistance | 89 |
| | B-ALL and T-ALL | Activation | 50 | **Decreased drug-induced inactivation related to ASP, PRED resistance** | 27 |
| | B-ALL and T-ALL | protein | 43 | No relation with PVA resistance | 85 |
| CASP6 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 43 | No relation with PVA resistance | 85 |
| CASP7 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 43 | No relation with PVA resistance | 85 |
| CASP9 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| CASP10 | B-ALL and T-ALL | protein | 43 | No relation with PVA resistance | 85 |

N: number of patients, ASP: L-asparaginase, DNR: daunorubicine, PRED: prednisolone, VCR: vincristine, PVA: combined PRED, VCR, ASP resistance score, B-ALL: B-lineage ALL, T-ALL: T-lineage ALL

*Bcl-2 family members*

As was mentioned above, Bcl-2 and its family members play a central role in regulating the intrinsic apoptosis pathway. The role of Bcl-2 family members in cellular drug resistance in diagnostic childhood ALL samples is summarized in Table 2. Although increased Bcl-2 expression was observed in drug-resistant ALL and AML cell lines,[87] no relation has been established between Bcl-2 expression and *in vitro* response to a wide variety of chemotherapeutic agents in diagnostic childhood ALL samples.[88,89,98-100] The only study to date reporting a relation between high Bcl-2 expression and *in vitro* drug resistance in childhood acute leukemia was performed in relapsed ALL samples.[101] We recently observed increased Mcl-1 mRNA levels in *in vitro* prednisolone resistant pediatric ALL samples.[88] No such correlation was found for other drugs by us and other groups, suggesting that the role of Mcl-1 in resistance may be restricted to glucocorticoids such as prednisolone.[88,89,98,102] No association with drug resistance was found for other anti-apoptotic Bcl2-family members such as Bcl-$X_L$ in childhood acute leukemia.[88,89,98,102,103] Overexpression of pro-apoptotic Bax accelerates apoptosis[104] and inactivating *BAX* mutations have been documented in up to 20% of hematological malignancies.[105] The observation that Bax expression was decreased at relapse suggested that loss of Bax may be involved in the development of cellular drug resistance in childhood ALL.[87] In contrast, other studies in childhood acute leukemia found no significant association between Bax expression and cellular drug resistance.[88,89,98,100,103,106] In addition, no association was found between the expression of other pro-apoptotic family members such as Bad and Bak and drug resistance and outcome in childhood ALL.[88,98] We recently observed decreased mRNA expression of the pro-apoptotic Hrk and increased expression of the pro-apoptotic Bcl2-

like 13 (*Bcl2L13*) gene, also known as *Bcl-rambo*, in L-asparaginase resistant B-lineage ALL samples.[88] This gene was also linked to L-asparaginase resistance in TEL-AML1 positive ALL cases, and, moreover, was linked to an unfavorable prognosis in these patients.[107] Based on their capacity to form heterodimers, it has been proposed that the ratio of pro-apoptotic to anti-apoptotic Bcl-2 family members ultimately determines the cells' susceptibility to apoptosis.[104,108] The Bax:Bcl-2 ratio rather than Bcl-2 expression was linked to the response to dexamethasone-induced apoptosis in a panel of leukemia and lymphoma cell lines. However, this could not be confirmed in samples taken at initial diagnosis of ALL.[100]

**Table 2. Bcl-2 family members and cellular drug resistance in newly diagnosed childhood acute leukemia**

| Gene | Leukemia type | Examined | N | *In vitro* drug resistance | Refs |
|---|---|---|---|---|---|
| BCL2 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP and DNR | 88 |
| | B-ALL and T-ALL | protein | 77 | **High expression: ARA sensitive**<br>No relation with resistance to PRED and DEX | 101 |
| | B-ALL and T-ALL | protein | 52 | No relation with resistance to ARA, DEX, DNR, MTX, TG, VCR, VM26 | 99 |
| | B-ALL and T-ALL | protein | 78 | No relation with resistance to ASP, VCR, PRED | 98 |
| | B-ALL and T-ALL | protein | 60 | No relation with PRED resistance | 89 |
| | T-ALL | protein | 81 | No relation with resistance to DEX and DOX | 100 |
| MCL1 | B-ALL | mRNA | 190 | **High expression: PRED resistant**<br>No relation with resistance to VCR, ASP and DNR | 88 |
| | B-ALL and T-ALL | protein | 78 | No relation with resistance to ASP, VCR, PRED | 98 |
| | B-ALL and T-ALL | protein | 60 | No relation with PRED resistance | 89 |
| Bcl-XL | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP and DNR | 88 |
| | B-ALL and T-ALL | protein | 78 | No relation with resistance to ASP, VCR, PRED | 98 |
| | B-ALL and T-ALL | protein | 60 | No relation with PRED resistance | 89 |
| BAX | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP and DNR | 88 |
| | B-ALL and T-ALL | protein | 78 | No relation with resistance to ASP, VCR, PRED | 98 |
| | B-ALL and T-ALL | protein | 60 | No relation with PRED resistance | 89 |
| | T-ALL | protein | 81 | No relation with resistance to DEX and DOX | 100 |
| BAD | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP and DNR | 88 |
| | B-ALL and T-ALL | protein | 78 | No relation with resistance to ASP, VCR, PRED | 98 |
| BAK | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP and DNR | 88 |
| | B-ALL and T-ALL | protein | 78 | No relation with resistance to ASP, VCR, PRED | 98 |
| HRK | B-ALL | mRNA | 190 | **Low expression: ASP resistant**<br>No relation with PRED, VCR and DNR resistance | 88 |
| BCL2L13 | B-ALL | mRNA | 190 | **High expression: ASP resistant**<br>No relation with PRED, VCR and DNR resistance | 88 |
| BAX:BCL2 ratio | T-ALL | protein | 81 | No relation with resistance to DEX and DOX | 100 |

N: number of patients, ARA: cytarabine, ASP: L-asparaginase, DEX: dexamethasone, DNR: daunorubicine, DOX: doxorubicin, MTX: methotrexate, PRED: prednisolone, TG: 6-thioguanine, VCR: vincristine, VM26: teniposide , ASP resistance score, B-ALL: B-lineage ALL, T-ALL: T-lineage ALL

*The apoptosome*

The important role of Apaf-1 in drug-induced apoptosis was demonstrated by the resistance of Apaf-1$^{-/-}$ murine embryonic fibroblasts to cytostatic drugs.[109] In addition, methylation-induced loss of Apaf-1 expression leads to chemoresistance in human melanoma.[110] The finding that Apaf-1 overexpression promotes[111] and Apaf-1 deficiency inhibits[112] drug-induced apoptosis in leukemic cell lines suggests that

expression levels of Apaf-1 may play a role in resistance to apoptosis in human leukemia. However, no correlation was found between Apaf-1 protein expression and response to induction therapy in adult patients with acute leukemia.[97] Interestingly, a recent paper describes that the methylation frequency of *APAF1* promoter region was 35% in childhood ALL.[113] This may be clinically important since increasing Apaf-1 levels by demethylation treatment with 5-aza-2'-deoxycytidine sensitized a human leukemic cell line to UV light-induced apoptosis.[114]

**Table 3. The apoptosome and cellular drug resistance in newly diagnosed childhood acute leukemia**

| Gene | Leukemia type | Examined | N | *In vitro* drug resistance | Refs |
|---|---|---|---|---|---|
| APAF1 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 43 | No relation with PVA resistance | 85 |
| HSP27 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| HSP70 | B-ALL and T-ALL | mRNA | 20 | No relation with PRED resistance | 120 |
| HSP90 | B-ALL and T-ALL | mRNA | 20 | No relation with PRED resistance | 120 |

N: number of patients, ASP: L-asparaginase, DNR: daunorubicine, PRED: prednisolone, VCR: vincristine, PVA: combined PRED, VCR, ASP resistance score, B-ALL: B-lineage ALL, T-ALL: T-lineage ALL

The role of apoptosome members and regulators in cellular drug resistance in diagnostic childhood ALL samples is summarized in Table 3. We have recently shown that Apaf-1 expression does not correlate with *in vitro* resistance to prednisolone, vincristine, L-asparaginase and daunorubicine in diagnostic childhood ALL samples.[85,88] The expression of Apaf-1 splice variants has been linked to functional apoptosis in tumor cell lines.[115-117] The presence of an additional C-terminal WD-40 repeat encoded by exon 18 appears to be required for *in vitro* activation of procaspase-9 and -3.[116] We found no relation between the expression of any Apaf-1 splice variant and cellular drug resistance in B-linage ALL.[88] Aberrant expression of Hsp90 has been associated with glucocorticoid resistance in human leukemic cell lines.[118] However, Hsp90 expression failed to correlate to *in vitro* or *in vivo* prednisolone response in children with ALL.[119,120] In addition, Hsp70 nor Hsp27 mRNA levels correlated to *in vitro* drug resistance in childhood ALL.[88,120]

*The intrinsic apoptosis pathway*

Resistance to cytokine- and drug-induced apoptosis correlated with loss of Fas expression in human leukemia cell lines.[86] We and others showed that Fas expression is not related to *in vivo* and *in vitro* drug response in childhood acute leukemia.[88,100,121] Like Fas, mRNA levels of the TRAIL receptors and their ligand TRAIL were not related

to cellular drug resistance in childhood ALL.[88] At present the role of other death receptors in cellular drug resistance of acute leukemia samples is yet unanswered.

*The IAP family*

The role of IAP family members and regulators in cellular drug resistance in diagnostic childhood ALL samples is summarized in Table 4. Increased expression of IAPs has been shown to confer chemoresistance to several anticancer drugs in human leukemic cell lines.[122] Importantly, antisense-mediated downregulation of XIAP[123] and survivin[124] have been demonstrated to enhance chemosensitivity of human leukemic cells lines *in vitro.* We found no association between altered mRNA expression of any of the IAP family members and *in vitro* resistance to prednisolone, vincristine, L-asparaginase and daunorubicine in childhood ALL.[88] However, at the protein level, expression of XIAP and c-IAP2 but not of c-IAP1 correlated with *in vitro* prednisolone resistance in childhood ALL.[89]

**Table 4. The IAP family and cellular drug resistance in newly diagnosed childhood acute leukemia**

| Gene | Leukemia type | Examined | N | *In vitro* drug resistance | Refs |
|---|---|---|---|---|---|
| XIAP | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 60 | **High expression: PRED resistance** | 89 |
| cIAP1 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 60 | No relation with PRED resistance | 89 |
| cIAP2 | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |
| | B-ALL and T-ALL | protein | 60 | **High expression: PRED resistance** | 89 |
| SURVIVIN | B-ALL | mRNA | 190 | No relation with resistance to PRED, VCR, ASP or DNR | 88 |

N: number of patients, ASP: L-asparaginase, DNR: daunorubicine, PRED: prednisolone, VCR: vincristine, B-ALL: B-lineage ALL, T-ALL: T-lineage ALL

Transfection of the IAP inhibitor Smac/DIABLO increased the sensitivity of ALL and AML cell lines to UV light-induced apoptosis.[125] Although these data imply Smac/DIABLO in drug-induced apoptosis in acute leukemia, its role of Smac/DIABLO in drug-induced apoptosis yet remains to be confirmed in primary acute leukemia samples.

*Downstream targets*

Although downstream targets play a role at the final stage of apoptosis, defects in these downstream targets have shown to influence drug-induced apoptosis in cell lines. Lamin B1 has been shown to be upregulated in vincristine resistant ALL cell lines[126], and, moreover, an uncleavable mutant delayed the rate of apoptosis and fragmentation of DNA.[127] An other cell line study showed that caspase-3 activation may occur without any signs of nuclear apoptosis upon induction of apoptosis.[128] Pharmacological

inhibition of the caspase target PARP has been shown to confer chemoresistance to several anticancer drugs in human leukemic cell lines.[129,130] In contrast to these cell line studies, we observed that decreased PARP inactivation in resistant cells is likely to be caused by a defect upstream or at the level of mitochondrial function in pediatric ALL. Lack of PARP inactivation coincided with lack of mitochondrial membrane depolarization and phosphatidyl serine exposure at the outer membrane upon drug exposure in these cells.[27]

**8. Conclusions and perspectives**

An overview of the apoptotic defects in childhood acute leukemia that are discussed in relation to cellular drug resistance are provided in Table 1-4. The vast majority of studies failed to demonstrate an association between defects in apoptotic parameters and drug resistance. Moreover, the studies that did show an association were often contradictory. There are various potential explanations for these contradictory data.

Firstly, the discrepancies between various studies may be explained by the leukemia subtypes that were included. Acute leukemia is a heterogeneous disease composed of various subtypes defined by immunophenotype, chromosome number and the presence of chromosomal translocations, that differ markedly in their treatment response.[131] The expression of apoptotic proteins has been shown to vary between these subgroups. For instance, T-lineage phenotype and the presence of TEL-AML or E2A-PBX1 fusion proteins was associated with low expression levels of Bcl-2[98,132,133], whereas the presence of MLL-AF4 or Bcr-Abl fusion transcripts were associated with high levels of Bcl-2 expression in childhood ALL.[133] Likewise, the absence of expression of XIAP and Bcl-$X_L$ was highly correlated with the presence of favorable cytogenetics in childhood AML.[103] Moreover, we recently demonstrated that different ALL subtypes defined by immunophenotype and genotype have a unique expression pattern of apoptosis genes, indicating that the expression of apoptosis genes is cell-type specific.[88]

Secondly, the activity of apoptotic proteins is often determined by post-translational modifications, like phosphorylation[50,134,135] and cleavage.[136-138] For example, decreased caspase-3 activation rather than decreased base-line procaspase-3 expression has been associated with cellular drug resistance in childhood ALL.[27,87] Most studies described in this review quantified protein levels of the apoptotic machinery by flow cytometry, Western blotting or immunocytochemistry. The antibodies used in the vast

majority of these studies cannot distinguish between active and inactive forms of apoptotic proteins.

Thirdly, the discrepancies between various studies may be explained by the fact that the relation of an individual apoptotic protein with clinical outcome may depend on the treatment that was given.

Fourthly, treatment-induced changes in the expression level of various apoptotic proteins have been reported in leukemic cells.[139,140] Therefore, mRNA levels in untreated cells may not necessarily reflect the true cellular apoptotic potential.

In summary, aberrant expression of apoptosis proteins has been observed at various levels of the effector apoptosis route in childhood acute leukemia. However, the baseline expression of a single apoptosis protein as the cause of cellular drug resistance is highly unlikely since these apoptosis proteins are part of a complex signal transduction pathway in which many regulating proteins play a role. A genome-wide screening showed that defects in drug-specific pathways rather than the common apoptosis route may contribute to cellular drug resistance in childhood ALL.[141] Moreover, apoptosis linked genes were not found in samples that were cross-resistant to four unrelated classes of drugs.[142] Taken together, the current data suggest that resistance to multiple drugs is not caused by a single defect in the execution phase of apoptosis in leukemic cells. Future research therefore should focus on defects in drug-specific targets to elucidate causes of cellular drug resistance in pediatric ALL.

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Chapter 3

# Resistance to different classes of drugs is associated with impaired apoptosis in childhood acute lymphoblastic leukemia

Amy Holleman[1], Monique L. den Boer[1], Karin M. Kazemier[1], Gritta E. Janka-Schaub[2], and Rob Pieters[1]

*[1]Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Department of Pediatric Oncology and Hematology, Rotterdam, the Netherlands,*

*[2]COALL study group, University Children's Hospital, Hamburg, Germany.*

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**ABSTRACT**

Resistance of leukemic cells to chemotherapeutic agents is associated with an unfavorable outcome in pediatric acute lymphoblastic leukemia (ALL). To investigate the underlying mechanisms of cellular drug resistance, the activation of various apoptotic parameters in leukemic cells from 50 children with ALL was studied after *in vitro* exposure with 4 important drugs in ALL therapy (prednisolone, vincristine, L-asparaginase, and daunorubicin). Exposure to each drug resulted in early induction of phosphatidylserine (PS) externalization and mitochondrial transmembrane ($\Delta\Psi_m$) depolarization followed by caspase-3 activation and poly(ADPribose) polymerase (PARP) inactivation in the majority of patients. For all 4 drugs, a significant inverse correlation was found between cellular drug resistance and (1) the percentage of cells with PS externalization ($<0.001<P<0.008$) and (2) the percentage of cells with $\Delta\Psi_m$ depolarization ($0.002<P<0.02$). However, the percentage of cells with caspase-3 activation and the percentage of cells with PARP inactivation showed a significant inverse correlation with cellular resistance for prednisolone (P=0.001; P=0.001) and L-asparaginase (P=0.01; P=0.001) only. This suggests that caspase-3 activation and PARP inactivation are not essential for vincristine- and daunorubicin-induced apoptosis. In conclusion, resistance to 4 unrelated drugs is associated with defect(s) upstream or at the level of PS externalization and $\Delta\Psi_m$ depolarization. This leads to decreased activation of apoptotic parameters in resistant cases of pediatric ALL.

**INTRODUCTION**

Although combination chemotherapy has improved the prognosis of childhood acute lymphoblastic leukemia (ALL) over the last few decades, relapse still occurs in 20% to 30% of the cases.[1] Cellular drug resistance measured at initial diagnosis is associated with an increased relapse risk and unfavorable clinical outcome in childhood ALL.[2,3] In addition, the presence of adverse clinical prognostic factors such as older age (> 10 years) and pro-B and T-lineage immunophenotype have been shown to be associated with cellular resistance to drugs in children with ALL.[4] These findings indicate that cellular drug resistance (measured *in vitro*) can be used as a tool to identify patients at higher risk of treatment failure.

Chemotherapeutic agents have been described to induce apoptosis in malignant cells.[5] There are 2 major routes by which apoptosis can be induced: the

extrinsic or death receptor–associated route and the intrinsic or mitochondrial route. Although there is disagreement concerning the role of the extrinsic route in chemotherapy-induced apoptosis[6,7] there is a general agreement regarding the importance of the intrinsic route. The intrinsic route can be subdivided into 3 general phases:[8] (1) insult generation, (2) signal transduction, and (3) execution. During the insult generation phase, chemotherapeutic agents interact with and cause damage to their specific cellular targets. The signal transduction phase is the least-defined phase and is thought to involve integration of pro- and antiapoptotic signals. The relative abundances of pro- and antiapoptotic signals, that can be influenced by anticancer drugs,[9] ultimately determines if the execution phase is initiated.[10] The execution phase is initiated by release of cytochrome c and other polypeptides from the mitochondrial intermembrane space.[11] This release is accompanied by a dissipation of mitochondrial inner transmembrane potential ($\Delta\Psi_m$).[12] Once released in the cytoplasm, cytochrome c interacts with Apaf-1 (apoptotic protease-activating factor-1), ATP/dATP, and procaspase-9 to form a complex known as the apoptosome.[13] In the apoptosome, caspase-9 is activated which in turn activates effector caspases, like procaspase-3 and -7.[14] The effector caspases cleave a number of structural and regulatory cellular proteins (e.g., poly(ADP-ribose) polymerase [PARP, lamins) and are responsible for the typical morphologic and biochemical features of an apoptotic cell.[15,16] A simplified overview of the events taking place during chemotherapy-induced apoptosis is given in Figure 1.

The fact that a point of convergence in the cellular response to cytotoxic drugs appears to be apoptosis and that leukemic cells display cross-resistance to drugs with different mechanisms of action has led to the hypothesis that cellular drug resistance may be related to defects in the apoptotic route. Aberrations at various levels of the apoptotic route have been linked to a drug-resistant phenotype in cell lines: absence of cytochrome c release,[17,18] defective Apaf-1 activity,[19-21] and caspase deficiency.[22-24] However, the occurrence of apoptotic defects has not been studied in children with ALL. Therefore, the aim of this study was to determine whether cellular drug resistance is associated with defects in drug-induced apoptosis in pediatric ALL. To this aim, leukemic cells of 50 children with newly diagnosed ALL were exposed *in vitro* to 4 structurally unrelated drugs used in induction therapy of ALL, and activation of various apoptotic parameters was evaluated (Figure 1).

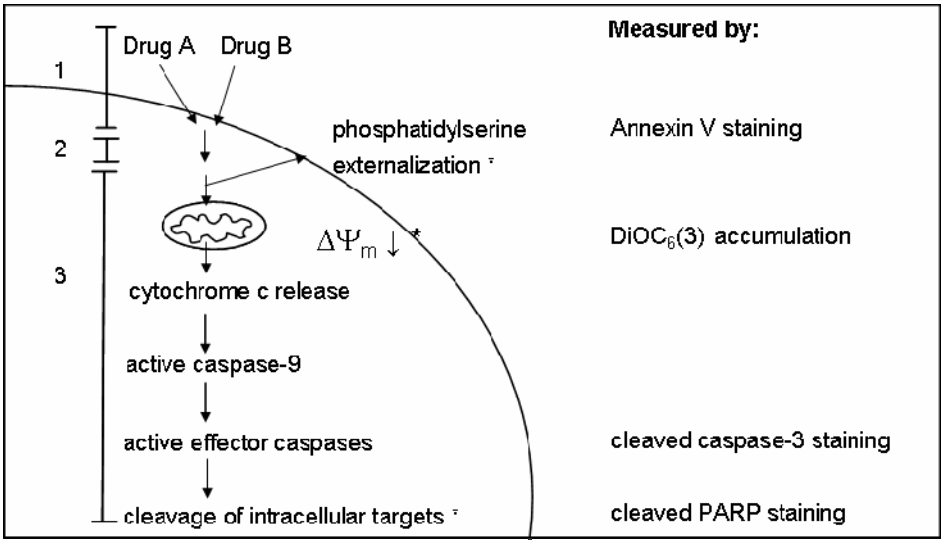


**Figure 1. Simplified overview of the events taking place during drug-induced activation of apoptotic parameters.** Drugs A and B represent two structurally unrelated drugs. Numbers 1, 2 and 3 refer to the three phases of the drug-induced apoptotic route as described in the Introduction section; 1: insult generation, 2: signal transduction and 3: execution. Parameters with an asterisk (*) are measured in this study.

## MATERIAL AND METHODS

### Patient samples

Bone marrow (BM) and/or peripheral blood (PB) were obtained from children with newly diagnosed ALL who entered the Sophia Children's Hospital or one of the hospitals participating in the German Cooperative Acute Lymphoblastic Leukemia (COALL) study. Within 24 hours after sampling, mononuclear cells were isolated by density gradient centrifugation with a Ficoll-Isopaque gradient (Lymphoprep 1.077 mg/mL; Nycomed Pharma, Oslo, Norway). Cells were resuspended in culture medium consisting of RPMI 1640 Dutch modification without L-glutamine (Gibco BRL, Breda, The Netherlands) supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 × $10^{3}$ µmol/L L-glutamine, 900 µmol/L gentamycin (Gibco BRL), 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.125 µg/mL fungizone (Gibco BRL), and 827 pmol/L insulin, 5 × $10^{-3}$ g/L transferrin, and 2.89 × $10^{-5}$ µmol/L sodium selenite (ITS media supplement; Sigma Aldrich, Zwijndrecht, The Netherlands). If necessary, the lymphoid cells were further purified to at least 90% leukemic blasts by removing nonmalignant cells with immunomagnetic beads (DynaBeads, Dynal, Oslo, Norway).

***In vitro* drug resistance assay**

*In vitro* drug resistance for daunorubicin (DNR; Cerubidine, Rhône-Poulenc Rorer, Amstelveen, The Netherlands), vincristine (VCR; TEVA Pharma, Mijdrecht, The Netherlands), L-asparaginase (ASP; Paronal, Christiaens, Breda, The Netherlands), and prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands) was determined using the 4-day MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay as described previously by Pieters et al.[25] Briefly, round-bottomed 96-well microculture plates were filled with 20 µL of different dilutions of a drug and stored at --20ºC. Six concentrations of each drug were tested in duplicate. The ranges of the final concentrations of these drugs were as follows: DNR: 0.002 µg/mL to 2.0 µg/mL; VCR: 0.05 µg/mL to 50 µg/mL; ASP: 0.003 IU/mL to 10 IU/mL; and PRED: 0.008 µg/mL to 250 µg/mL.

Aliquots of 80 µL cell suspension ($2 \times 10^6$ cells/mL) were added to each well. Four wells contained 100 µL culture medium without drugs or cells for blanking the plate reader and 8 wells contained 100 µL culture medium with cells and without drug for measuring control cell viability. After incubating plates for 4 days at 37°C in a humidified incubator in 5% $CO_2$, 10 µL MTT (5 mg/mL; Sigma) was added and the plates were incubated for an additional 6 hours. During these 6 hours, the living cells present in each well will reduce the yellow MTT tetrazolium salt to purple–blue formazan crystals. The formazan crystals were dissolved with 100 µL 0.04 N HCl-isopropanyl alcohol (acidified isopropanol). The optical density (OD) of the wells, which is linearly related to cell number,[26] was measured spectrophotometrically at 562 nm. Leukemic cell survival (LCS) was calculated by the equation: LCS=($OD_{day4}$ treated well/mean $OD_{day4}$ control wells) × 100%. The drug concentration lethal to 50% of the ALL cells, the $LC_{50}$ value, was used as a measure for cellular drug resistance. MTT-assay results were only used if the drug-free control wells contained at least 70% leukemic cells after 4 days of culture.

***In vitro* drug exposure for measuring apoptotic features**

Fresh leukemic cells ($2.0 \times 10^6$ cells/mL) with a purity of at least 90% leukemic blasts were cultured in the presence of drugs at 37°C in a humidified incubator in 5% $CO_2$. The ranges of the final drug concentrations were as follows: PRED: 0.061 µg/mL to 250 µg/mL; VCR: 0.195 µg/mL to 50 µg/mL; ASP: 0.016 IU/mL to 10 IU/mL; and DNR: 0.008 µg/mL to 2.0 µg/mL.

**Measurement of aberrant phosphatidylserine externalization on the outer cell membrane**

During the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner side of the plasma membrane to the outer leaflet of the cell membrane. Annexin V is a $Ca^{2+}$-dependent phospholipid-binding protein with high affinity for PS and can therefore be used to detect apoptotic cells.

Leukemic cells were resuspended in 200 µL Annexin V–Alexa 488 Reagent (Nexins Research BV, Kattendijke, The Netherlands) and incubated for 15 minutes at 4°C. A total of 5000 events was analyzed by flow cytometry (FACSCalibur, Becton Dickinson, Erembodegem, Belgium). Drug-induced apoptosis was calculated according to the following formula: percentage of apoptotic cells × 100% × (D-C)/(100-C), where D represents the percentage of Annexin V–positive cells in the presence of a drug and C is the percentage of Annexin V–positive cells in the absence of a drug (spontaneous apoptosis). The intra-assay coefficient of variation for measurements of PS externalization was 3.4%.

**Detection of apoptosis-associated alterations in $\Delta\Psi_m$**

Disruption of $\Delta\Psi_m$ was determined using 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$; Molecular Probes Inc., Eugene, OR), a lipophilic cationic dye that accumulates in the mitochondrial matrix driven by $\Delta\Psi_m$.[27] Loss of $\Delta\Psi_m$ was visualized as a reduction in the signal in the FL1 channel. Leukemic cells were incubated in 200 µl phosphate-buffered saline (PBS) containing 40 nM $DiOC_6(3)$ solution and incubated in a humidified incubator for 30 minutes at 37°C in 5% $CO_2$. A total of 5000 events was analyzed by flow cytometry. Percentage of cells with decreased mitochondrial transmembrane depolarization ($\Delta\Psi_m\downarrow$) was calculated with the following formula: 100% × (D-C)/(100-C), where D represents the percentage of cells with reduced $DiOC_6(3)$ accumulation in drug-treated samples and C represents the percentage of cells with reduced $DiOC_6(3)$ accumulation in untreated samples. The intra-assay coefficient of variation for measurements of disruption of $\Delta\Psi_m$ was 4.5%.

**Measurement of caspase-3 and PARP cleavage**

Leukemic cells were fixed using 2% (v/v) 37% formaldehyde solution in 100% acetone. Fixed cells were washed twice with PBS/0.1%BSA and incubated with an antibody directed against cleaved caspase-3 (Cell Signaling Technology, Beverly, MA, USA) or cleaved PARP (Cell Signaling Technology) at room temperature for 30 minutes. Both

antibodies recognize an epitope exposed only when both proteins are cleaved during apoptosis. Subsequently, cells were washed and incubated with fluorescein isothiocyanate (FITC) conjugated rabbit anti-rabbit $F(ab')_2$ (DAKO, Glostrup, Denmark) for caspase-3 and FITC-conjugated pork anti-mouse $F(ab')_2$ (DAKO) for PARP at room temperature for 30 minutes. A total of 5,000 events were measured by flow cytometry. Caspase-induced PARP cleavage leads to PARP inactivation,[16] hence we measure caspase-3 activation and PARP inactivation. The percentage of cells with caspase-3 activation or PARP inactivation was determined with the following formula: 100% × (D-C)/(100-C), where D represents the percentage of cells that stain positive for the antibody in drug-treated samples, and C in untreated samples. Intra-assay variation of caspase-3 and PARP cleavage measurements was 11.2 and 11.8% respectively.

**Measurement of caspase-3 and PARP cleavage**

Leukemic cells were fixed using 2% (vol/vol) 37% formaldehyde solution in 100% acetone. Fixed cells were washed twice with PBS/0.1% bovine serum albumin (BSA) and incubated with an antibody directed against cleaved caspase-3 (Cell Signalling Technology, Beverly, MA) or cleaved PARP (Cell Signalling Technology) at room temperature for 30 minutes. Both antibodies recognize an epitope exposed only when both proteins are cleaved during apoptosis. Subsequently, cells were washed and incubated with fluorescein isothiocyanate (FITC)–conjugated rabbit antirabbit F(ab')2 (DAKO, Glostrup, Denmark) for caspase-3 and FITC-conjugated pork antimouse $F(ab')_2$ (DAKO) for PARP at room temperature for 30 minutes. A total of 5000 events was measured by flow cytometry. Caspase-induced PARP cleavage leads to PARP inactivation,[15] hence we measured caspase-3 activation and PARP inactivation. The percentage of cells with caspase-3 activation or PARP inactivation was determined with the following formula: 100% × (D-C)/(100-C), where D represents the percentage of cells that stain positive for the antibody in drug-treated samples, and C is the percentage of cells that stain positive for the antibody in untreated samples. Intra-assay variation of caspase-3 and PARP cleavage measurements was 11.2% and 11.8%, respectively.

**Statistics**

Correlations between different apoptotic parameters as well as between the $LC_{50}$ values and apoptotic parameters were calculated using the Spearman rank ($r_s$) correlation test. Statistical tests were performed at a 2-tailed significance level of 0.05.

**RESULTS**

Time-dependent induction of apoptotic parameters was studied in 5 children with ALL in order to determine the most suitable time point for testing a larger group of children with ALL. *In vitro* exposure to each of the 4 drugs tested caused a time-dependent activation of apoptotic parameters in ALL cells as assessed by an increase of cells with PS externalization, $\Delta\Psi_m$ depolarization, caspase-3 activation, and PARP inactivation (Figure 2).

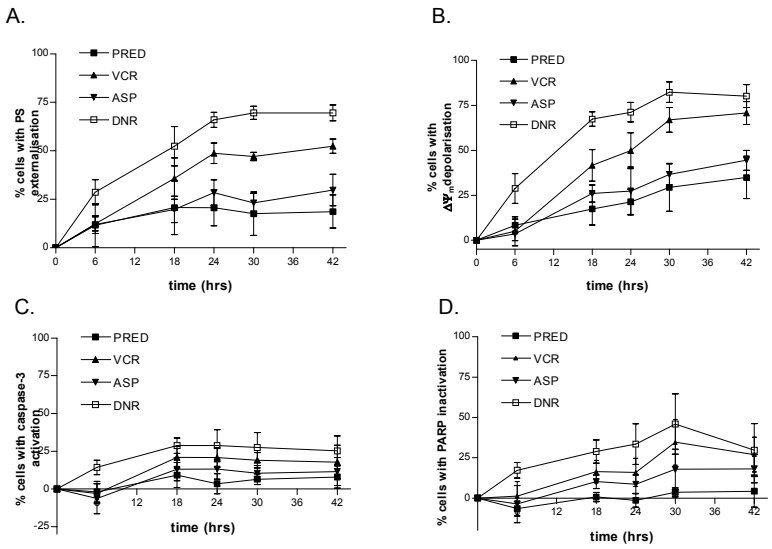


**Figure 2. Time-dependent drug-induced apoptosis in ALL.** Freshly isolated ALL cells were cultured in the presence of 2.0 μg/ml daunorubicin (DNR), 50 μg/ml vincristine (VCR), 10 IU/ml L-asparaginase (ASP) or 250 μg/ml prednisolone (PRED) for the indicated time points. Drug-induced PS externalization (A), mitochondrial transmembrane disruption (B), caspase-3 activation (C) and PARP inactivation (D) were determined by flow cytometry and calculated by the formula described in Materials and Methods. Results are expressed as mean ± SD of 5 patients with ALL.

In only one patient were sufficient cells available to perform an extensive concentration series. The data indicated a concentration-dependent increase in the activity of all apoptotic parameters (data not shown).

Although exposure to all 4 drugs resulted in activation of similar apoptotic parameters, a difference in apoptosis kinetics was observed. Whereas daunorubicin and vincristine trigger a relatively fast activation of apoptotic parameters, L-asparaginase and prednisolone consistently induced apoptosis more slowly (Figure 2). After 18 hours of daunorubicin or vincristine exposure the mean percentage of cells

with PS externalization in the 5 ALL samples is 52% ± 23% and 36% ± 21%, respectively. In contrast, the mean percentage of cells with PS externalization after 18 hours of L-asparaginase and prednisolone exposure was 20% ± 15% and 21% ± 31% compared with 30% ± 18% and 19% ± 19%, respectively, after 42 hours. The 2 types of kinetics could be confirmed in subsequent experiments; the mean percentage of cells with PS externalization in the 50 patients measured in this study after 18 hours of daunorubicin or vincristine treatment are 60% ± 24% and 42% ± 25%, respectively, compared with 31% ± 19% and 30% ± 30% after 42 hours L-asparaginase and prednisolone exposure, respectively. To be able to study the relationship between apoptosis and cellular drug resistance in a large group of patients, activation of apoptotic parameters was measured after 18 hours of incubation with daunorubicin and vincristine and after 42 hours of incubation with L-asparaginase and prednisolone in further experiments.

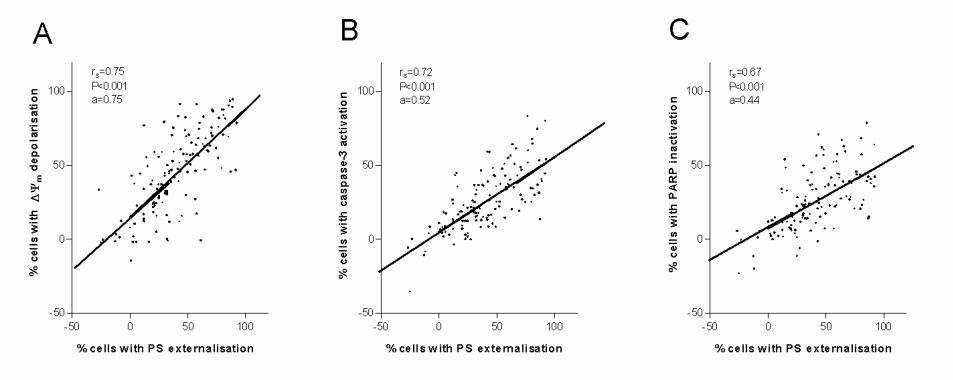


**Figure 3. Correlation between drug-induced apoptotic parameters in pediatric ALL.** Correlation between the percentage of cells with PS externalization and $\Delta\Psi_m$ depolarization (A), activated caspase-3 (B) or inactivated PARP (C) in leukemic cells *in vitro* incubated with prednisolone, vincristine, L-asparaginase or daunorubicin in 50 children with ALL. The dashed line represent the line x = y and the solid line represents the linear regression line.

Figure 3 shows that the percentage of cells with PS externalization is proportional to the percentage of cells with reduction in mitochondrial transmembrane potential ($r_s$=0.75, P<0.001), caspase-3 activation ($r_s$=0.72, P<0.001), and the percentage of cells with PARP inactivation ($r_s$=0.67, P<0.001). Significant correlations were also found when analyzing data from each of the 4 drugs separately (Table 1). The slopes of the regression lines in Figure 3A-C are a=0.75, a=0.52, and a=0.44,

respectively. The closer the slope of the regression line approaches a=1.0, the closer the event probably follows after PS externalization. This indicates that upon drug exposure, PS externalization and $\Delta\Psi_m$ depolarization are early events, whereas caspase-3 activation and PARP inactivation are occurring relatively later.

**Table 1. Correlation between PS externalization and the downstream apoptotic parameters upon drug exposure in pediatric ALL.** Freshly isolated ALL cells were cultured for 18 hours in the presence of 50 μg/ml vincristine or 2.0 μg/ml daunorubicin or 42 hrs in the presence of 250 μg/ml prednisolone or 10 IU/ml L-asparaginase. Drug-induced activation of apoptotic parameters was determined by flow cytometry. Correlation between apoptotic parameters was calculated using the Spearman's rank correlation test.

| Drug | | $\Delta\Psi_m$ depolarization | caspase-3 activation | PARP inactivation |
|---|---|---|---|---|
| **Prednisolone** | Correlation coefficient | 0.81 | 0.76 | 0.76 |
| | P-value | <0.001 | <0.001 | <0.001 |
| | N | 31 | 29 | 29 |
| **Vincristine** | Correlation coefficient | 0.80 | 0.56 | 0.64 |
| | P-value | <0.001 | <0.001 | <0.001 |
| | N | 30 | 32 | 32 |
| **L-asparaginase** | Correlation coefficient | 0.42 | 0.49 | 0.56 |
| | P-value | 0.017 | 0.006 | 0.002 |
| | N | 32 | 30 | 29 |
| **Daunorubicine** | Correlation coefficient | 0.63 | 0.50 | 0.41 |
| | P-value | <0.001 | 0.005 | 0.029 |
| | N | 29 | 31 | 29 |

Large interindividual variability in the extent of drug-induced activation of apoptotic parameters was observed between patients. For instance, prednisolone-induced PS externalization after 42 hours ranged between -26% and 86% (median: 27%). Figure 4 and Table 2 show for each individual drug highly significant inverse correlations between the $LC_{50}$ and (1) the percentage of cells with PS externalization and (2) the percentage of cells with $\Delta\Psi_m$ depolarization. However, caspase-3 activation and PARP inactivation showed a less-consistent inverse correlation pattern with cellular drug resistance. A significant inverse correlation between cellular drug resistance and the percentage of cells with caspase-3 activation was observed for prednisolone ($r_s$= -0.60, P<0.001) and L-asparaginase ($r_s$= -0.46, P=0.01) but not for vincristine and daunorubicin.

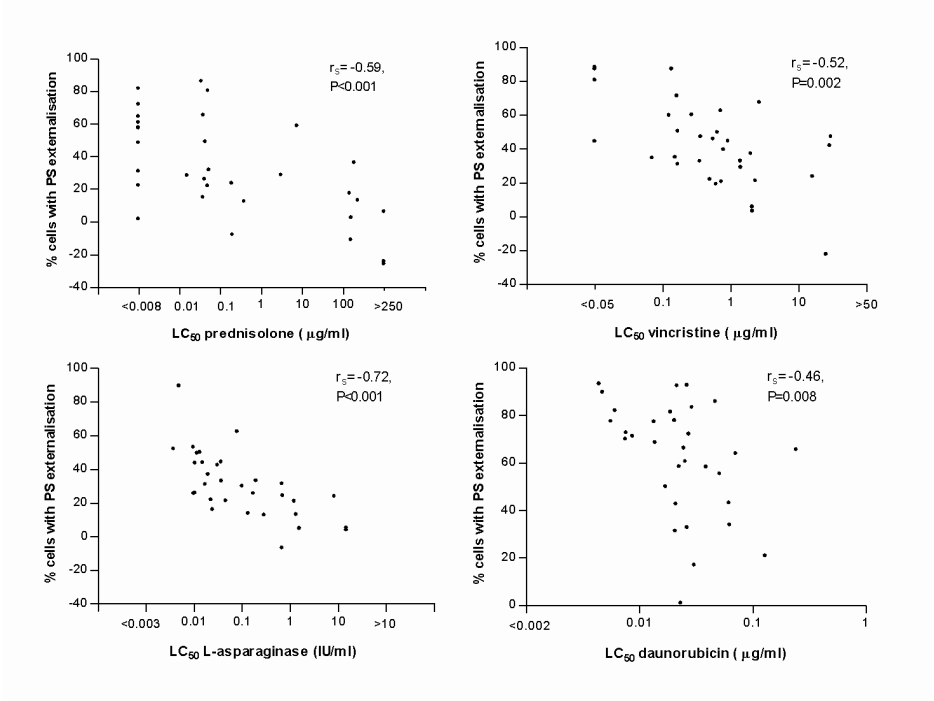


**Figure 4. Drug-induced apoptosis inversely correlates with cellular drug resistance in pediatric ALL.** Freshly isolated ALL cells were incubated in the presence of vincristine or daunorubicin for 18 hrs or prednisolone or L-asparaginase for 42 hrs at 37°C in a humidified incubator in 5% $CO_2$.

Likewise, PARP inactivation was inversely correlated to cellular drug resistance for prednisolone ($r_s$= -0.58, P<0.001) and L-asparaginase ($r_s$= -0.58, P<0.001) only (Table 2).

## DISCUSSION

Cellular drug resistance may reflect disruptions in the apoptotic route.[17-24] Low caspase-3 activity has been previously linked to a poor prognosis in adult chronic myelogenous leukemia (CML)[28] and high levels of caspase-3 with improved survival in adult acute myeloid leukemia (AML).[29] In addition, loss of spontaneous caspase-3 activation *in vivo* is associated with relapse in adults with ALL.[30] However, the presence and clinical significance of these disruptions in the apoptotic route have not been studied well in pediatric ALL. In the present study, we have analyzed drug-induced activation of apoptotic parameters in leukemic cells taken at initial diagnosis of ALL. PS externalization, $\Delta\Psi_m$ disruption, caspase-3 activation, and PARP inactivation were

measured after *in vitro* exposure to 4 cytotoxic drugs that form the backbone of ALL therapy: prednisolone, vincristine, L-asparaginase, and daunorubicin.

**Table 2. Inverse correlation between cellular drug resistance and the activation of parameters along the effector route of apoptosis in pediatric ALL.** See Table 1 for legends.

| Apoptotic parameter | $LC_{50}$ Prednisolone | $LC_{50}$ Vincristine | $LC_{50}$ L-asparaginase | $LC_{50}$ Daunorubicine |
|---|---|---|---|---|
| **PS externalization** | | | | |
| Correlation coefficient | -0.59 | -0.52 | -0.72 | -0.46 |
| P-value | <0.001 | 0.002 | <0.001 | 0.008 |
| N | 32 | 33 | 32 | 32 |
| **$\Delta\Psi_m$ depolarization** | | | | |
| Correlation coefficient | -0.43 | -0.45 | -0.45 | 0.54 |
| P-value | 0.016 | 0.014 | 0.010 | 0.002 |
| N | 31 | 30 | 32 | 29 |
| **caspase-3 activation** | | | | |
| Correlation coefficient | -0.60 | -0.28 | -0.46 | -0.27 |
| P-value | 0.001 | *N.S.* | 0.011 | *N.S.* |
| N | 29 | 32 | 30 | 31 |
| **PARP inactivation** | | | | |
| Correlation coefficient | -0.58 | -0.34 | -0.58 | -0.27 |
| P-value | 0.001 | *N.S.* | 0.001 | *N.S.* |
| N | 29 | 32 | 29 | 29 |

*N.S.* = non-significant, i.e. P-value≥0.05

Time series experiments showed a fast activation of apoptotic parameters for daunorubicin and vincristine and a slower activation for L-asparaginase and prednisolone (Figure 2). One may speculate that this reflects differences in primary cellular targets of the different drugs. Hypothetically, a cell is likely to respond quickly to the direct damaging effect of daunorubicin and vincristine treatment, that is, DNA damage and microtubule damage, respectively. In contrast, it may take a cell relatively longer to respond to the indirect effects of L-asparaginase and prednisolone treatment, that is, induction of gene expression or depletion of the intracellular stock of the amino acid asparagine.

Our data suggest that PS externalization and disruption of $\Delta\Psi_m$ are both early features of apoptosis induced by 4 structurally unrelated drugs in childhood ALL (Figures 2-3). The spread of data points around the line x = y in Figure 3 indicates that in half of the patients, disruption of $\Delta\Psi_m$ appears to precede PS externalization (dots above the line x=y). However, in the other half of the patients, disruption of $\Delta\Psi_m$ follows or coincides with PS externalization. No consensus is reached in literature concerning

the sequence of these 2 apoptotic events. Conflicting reports have been published showing that disruption of $\Delta\Psi_m$ either preceded or coincided with or followed PS externalization.[31-34] An explanation for this phenomenon is proposed by Denecker et al,[33] who suggest that both $\Delta\Psi_m$ disruption and PS externalization are not necessarily 2 dependent but rather parallel events initiated after an apoptotic stimulus. Consequently, the sequence of these 2 apoptotic events may be cell type-, stimulus-, and apparently also patient-specific.

The present data show that resistance of leukemic cells to each of 4 unrelated drugs is associated with decreased PS externalization and $\Delta\Psi_m$ depolarization compared with sensitive cells. Caspase-3 activation or PARP inactivation was linked to cellular resistance to prednisolone and L-asparaginase, but not with cellular resistance toward vincristine and daunorubicin (Table 2). A possible explanation for this observation is that caspase-3 and PARP cleavage may be an epiphenomenon, which is not essential for vincristine- and daunorubicin-induced apoptosis. Multiple caspases, which are redundant in function, are expressed in acute leukemic cells.[35] Possibly, in case of vincristine- and daunorubicin-induced apoptosis, a caspase other than caspase-3 may function as the main effector caspase in primary ALL cells.

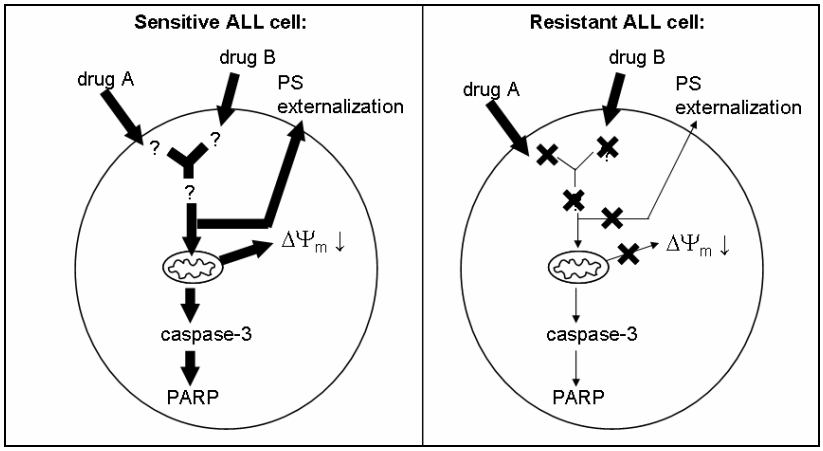


**Figure 5. Impaired apoptosis in resistant compared to sensitive ALL cells.** A defect localized upstream of the mitochondria may lead to decreased activation of downstream apoptotic parameters in resistant ALL patients. Potential sites of defects are indicated with a cross. Decreased activation of apoptotic parameters is illustrated by the decreased size of the arrows in resistant compared to sensitive patients.

We found that cellular drug resistance is associated with decreased PS externalization and $\Delta\Psi_m$ depolarization compared with sensitive cells. Decreased

activation of these apoptotic parameters is likely to result from a defect upstream or at the level of both PS externalization and disruption of $\Delta\Psi_m$ (Figure 5).

Aberrations in the expression of various molecules associated with cellular drug resistance in mainly adult leukemia and cell lines have been described in literature.[36-49] Treatment with chemotherapeutic drugs increases intracellular ceramide levels.[36] Significantly reduced ceramide levels have been linked to drug resistance in adult patients with ALL, CML, and AML.[37] Deficient up-regulation of CD95 ligand and down-regulation of CD95 receptor expression has been shown to confer drug resistance in leukemic cell lines.[6,38] Aberrant expression of both anti- and proapoptotic Bcl-2 family members is known to prevent mitochondrial permeability transition pore opening and release of apoptogenic proteins from mitochondria.[39] Data regarding the role of the expression levels of Bcl-2 family members and clinical outcome in ALL are contradictory.[40-43] Overexpression of the p53 regulator MDM2 has been associated with early relapse, adriamycin resistance, and failure to respond to reinduction therapy in childhood leukemia.[44] In addition, constitutive activation of antiapoptotic proteins such as both Akt/PKB[45] and c-Raf[46] as well as inactivation of the proapoptotic protein PTEN[47] have been linked to drug resistance in various types of cancers. Other proteins whose overexpression is associated with resistance to apoptosis in acute leukemia are members of the heat shock protein family, including Hsp27[48] and Hsp70.[49] To find out (1) which molecules play an actual role in cellular drug resistance in children with ALL and (2) whether resistance to different drugs is associated with drug-specific defects, we currently perform gene expression studies using high-density oligonucleotide microarrays.

In conclusion, the present study shows that decreased PS externalization and $\Delta\Psi_m$ depolarization are found in children with ALL who are *in vitro*-resistant to structurally unrelated drugs. These data suggest that cellular resistance to these drugs is caused by defects upstream or at the level of mitochondrial function. Caspase-3 activation and PARP inactivation are suggested to play a role in prednisolone- and L-asparaginase-induced apoptosis, but are not essential to vincristine- and daunorubicin-induced apoptosis. The nature of the defects upstream or at the level of PS externalization and $\Delta\Psi_m$ depolarization in resistant cells of children with ALL are not elucidated and will be the subjects of further research.

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Chapter 4

# The expression of 70 apoptosis genes in relation to lineage, genetic subtype, cellular drug resistance, and outcome in childhood acute lymphoblastic leukemia

[1]Amy Holleman, [1]Monique L. den Boer, [1,2]Renée X. de Menezes, [3]Meyling H. Cheok, [4]Cheng Cheng [1]Karin M. Kazemier, [5,6]Gritta E. Janka-Schaub, [5,7]Ulrich Göbel, [5,8]Ulrike B. Graubner, [3]William E. Evans, and [1]Rob Pieters

*[1]Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Department of Pediatric Oncology and Hematology, Rotterdam, the Netherlands, [2]Center for Human and Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands, [3]Department of Pharmaceutical Sciences and [4]Biostatistics, St. Jude Children's Research Hospital, Memphis, Tennessee, USA, [5]COALL study group, [6]University Children's Hospital, Hamburg, Germany, [7]Department of Pediatric Hematology and Oncology, University Medical Center, Heinrich-Heine-University, Düsseldorf, Germany, [8]Department of Pediatric Oncology, Dr. von Haunersches Children's Hospital, University of Munich, Munich, Germany.*

*Blood. (accepted)*

**ABSTRACT**

Childhood acute lymphoblastic leukemia (ALL) consists of various subtypes that respond differently to cytotoxic drugs and therefore have a markedly different clinical outcome. We used microarrays to investigate in 190 children with ALL at initial diagnosis whether 70 key apoptosis genes were differentially expressed between leukemic subgroups defined by lineage, genetic subtype, *in vitro* drug resistance and clinical outcome. The expression of 44 of 70 genes was significantly different in T- versus B-lineage ALL, 22 genes differed in hyperdiploid versus non-hyperdiploid, 16 in TEL-AML1 positive versus negative, and 13 in E2A-rearranged versus germline B-lineage ALL. Expression of MCL1 and DAPK1 was significantly associated with prednisolone sensitivity, whereas BCL2L13, HRK and TNF were related to L-asparaginase resistance. BCL2L13 overexpression was also associated with unfavorable clinical outcome (P<0.001). Multivariate analysis including known risk factors revealed that BCL2L13 expression was an independent prognostic factor (P=0.011). The same trend was observed in a validation group of 92 children with ALL treated on a different protocol at St. Jude (P=0.051). In conclusion, ALL subtypes have a unique expression pattern of apoptosis genes and our data suggest that selective genes are linked to cellular drug resistance and prognosis in childhood B-lineage ALL.

**INTRODUCTION**

The treatment of pediatric acute lymphoblastic leukemia (ALL) has greatly improved over the past three decades, resulting in long-term disease-free survival (DFS) of approximately 80%.[1] Despite this progress, therapy resistance still forms a major obstacle to successful treatment in a significant number of children. Childhood ALL is a heterogeneous disease consisting of various genetic subtypes such as t(9;22)/BCR-ABL, t(12;21)/TEL-AML1, hyperdiploid (>50 chromosomes), 11q23/MLL rearranged, t(1;19)/E2A-PBX1, and T-lineage ALL, which differ markedly in their treatment response.[2] The *in vitro* response to chemotherapy can be studied by exposure of primary patient samples to cytostatic drugs in a cell kill assay such as the methyl-thiazol-tetrazolium (MTT) assay. We and others have previously demonstrated that children with ALL whose leukemia cells exhibit *in vitro* resistance to single drugs or a combination of drugs, i.e. prednisolone, vincristine and L-asparaginase (PVA). have a significantly worse prognosis than patients with sensitive leukemic cells.[3-7] In addition, leukemia subtypes with a relatively unfavorable prognosis have been associated with *in vitro* drug resistance[8-10] and subtypes with a favorable prognosis with *in vitro* drug sensitivity.[11,12]

Apoptosis is the predominant form of cell death triggered *in vivo* and *in vitro* by drugs in hematological malignancies.[13] There are two major routes by which apoptosis can be induced: (1) the mitochondrial or intrinsic apoptosis pathway and (2) the death receptor-mediated or extrinsic apoptosis pathway. Both apoptotic pathways have been extensively reviewed elsewhere.[14-17] Briefly, the intrinsic route is initiated by mitochondrial damage that leads to release of apoptogenic factors, such as cytochrome *c*, Smac/Diablo, and apoptosis-inducing factor (AIF), from the mitochondrial intermembrane space.[17] The release of these factors is mediated by Bcl-2 family proteins, a group of key regulators of the intrinsic apoptosis pathway that consists of pro-apoptotic and anti-apoptotic members. Upon its release into the cytoplasm, cytochrome *c* forms a complex known as the apoptosome consisting of apoptotic protease-activating factor-1 (Apaf-1), ATP/dATP and procaspase-9.[18] Following its activation within the apoptosome, caspase-9 activates the downstream effector caspase cascade.[19] Initiation of the extrinsic apoptosis pathway involves ligand-induced aggregation of death receptors and activation of procaspase-8 or procaspase-10 within the death-inducing signaling complex (DISC).[20,21] Activated procaspase-8 or -10 is released into the cytoplasm where it induces activation of downstream effector caspases. The intrinsic and extrinsic apoptotic pathways converge at the level of caspase-3 activation.

Leukemia subtypes with a relatively unfavorable prognosis have been associated with *in vitro* drug resistance.[8-10] Moreover, cellular drug resistance is associated with decreased ability to induce apoptosis in pediatric ALL.[22] Therefore, one of the factors that may contribute to the different treatment response of genetic leukemia subtypes may be a differential propensity to undergo apoptosis. Apoptosis is controlled by various positive and negative regulators, responding to stimuli from inside and outside the cell.[23,24] Most papers to date addressing causes of cellular drug resistance, however, only focus on a limited number of apoptosis molecules. In the present study we analyzed the expression patterns of 70 key apoptosis genes in leukemic cells of 190 children at initial diagnosis of ALL. The expression of these genes was tested for association with (1) lineage and genetic subtype, (2) *in vitro* drug resistance to four widely used drugs in treatment of ALL, i.e. prednisolone, vincristine, L-asparaginase and daunorubicin, and (3) clinical outcome. Lastly, we analyzed the relation between the expression of active Apaf-1 isoforms and cellular drug resistance.

## MATERIAL AND METHODS

### Patient samples

Bone marrow (BM) and peripheral blood (PB) were obtained after informed consent from 190 children with newly diagnosed ALL who were enrolled on treatment protocols 92 and 97 at the hospitals participating in the German Cooperative Study Group for Acute Lymphoblastic Leukemia (COALL) study or the ALL-9 Dutch Childhood Oncology Group (DCOG) protocol at the Erasmus MC - Sophia Children's Hospital in Rotterdam (study cohort); and of 92 children enrolled as part of the Total Therapy protocols 13A[25] and 13B[26] of St. Jude Children's Research Hospital (SJCRH) in Memphis, Tennessee (validation cohort).[27] Approval was obtained from the Erasmus MC/Sophia Children's Hospital and SJCRH institutional review board for these studies. Clinical characteristics of these patients are provided in Table 1.

**Table 1: Clinical and biological characteristics of patients included in this study.**

| | COALL/DCOG cohort | St. Jude cohort |
|---|---|---|
| **Variable** | Number of patients | Number of patients |
| **ALL subtype** | | |
| B-other | 48 | 27 |
| *BCR-ABL* | 5 | 8 |
| *E2A** | 9 | 12 |
| Hyperdiploid[#] | 44 | 15 |
| *MLL** | 4 | 5 |
| *TEL-AML1* | 44 | 17 |
| T-lineage | 36 | 9 |
| **Folluw-up (yrs)** | | |
| median | 4.8 | 7.1 |
| P25-P75 | 3.8-5.9 | 4.9-9.0 |
| **Age** | | |
| median | 6.0 | 6.2 |
| P25-P75 | 3.5-10.2 | 3.3-11.8 |
| **WBC** | | |
| median | 34.4 | 36.9 |
| P25-P75 | 10.7-89.1 | 8.4-93.3 |
| **Clinical response** | | |
| CCR | 143[†] | 69 |
| relapse | 45 | 15 |

CCR: continuous complete remission
[#]: Cytogenetic analysis revealed more than 50 chromosomes
*: COALL: *MLL* and *E2A* rearranged, St. Jude: *MLL-AF4* and *E2A-PBX1*
[†]: 2 patients had a competing event; a secondary malignancy and myelodysplastic symdrome (MDS)

### Isolation of leukemia cells

Mononuclear cells were isolated by sucrose density gradient centrifugation (Lymphoprep, density 1.077 g/ml; Nycomed Pharma, Oslo, Norway), within 24 hours after sampling. Cells were resuspended in culture medium consisting of RPMI 1640 (Dutch modification without L-glutamine; Gibco BRL, Life Technologies, Breda, The Netherlands) supplemented with

20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 mM L-glutamine, 200 μg/ml gentamycin (Gibco BRL) 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone (Gibco BRL), and 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (ITS media supplement; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands). If necessary, leukemic samples were further enriched to more than 90% leukemic blasts by removing non-malignant cells with immunomagnetic beads (DynaBeads, Dynal Inc., Oslo, Norway).[28]

***In vitro* drug-resistance assay**

Responsiveness of leukemia cells to prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands), vincristine (VCR; TEVA Pharma, Mijdrecht, The Netherlands), L-asparaginase (ASP; Paronal, Christiaens, Breda, The Netherlands), and daunorubicin (DNR; Cerubidine, Rhône-Poulenc Rorer, Amstelveen, The Netherlands) was determined by the 4-day *in vitro* MTT drug resistance assay[3]. The concentration ranges tested for these drugs were: PRED: 0.008-250 μg/ml; VCR: 0.05-50 μg/ml; ASP: 0.003-10 IU/ml and DNR: 0.002-2.0 μg/ml. The drug concentration lethal to 50% of the ALL cells ($LC_{50}$ value) was used as the measure of cellular drug resistance. The cut-off $LC_{50}$ values used to assign cases as sensitive or resistant to each agent, were those previously shown to be associated with a good or poor treatment outcome in children with ALL[3,4].

**Gene expression profiling: purification, labeling and hybridization of RNA**

Total cellular RNA was extracted from leukemic cells of 190 patients with acute lymphoblastic leukemia and hybridized to the U133A GeneChip® oligonucleotide microarray containing 22,283 probe sets (~12,700 genes) according to manufacturer's protocols (Affymetrix, Santa Clara, CA). Gene-expression values were scaled to the target intensity of 2500, using Affymetrix Microarray Analysis Suite® (MAS) 5.0 software.[29,30] Probe sets expressed in fewer than 5 patients were omitted, leaving 14,550 probe sets in the filtered dataset for subsequent analyses. Gene expression analysis of 173 out of these 190 patients was published previously[29] in this paper we focus solely on genes involved in apoptosis. All analyses were carried out on $log_2$-transformed gene-expression values.

**Real time quantitative PCR**

Total cellular mRNA was extracted using Trizol reagent (Gibco BRL) and cDNA was synthesized using random hexamers and oligo dT. mRNA expression levels of total *Apaf-1*, "active" *Apaf-1* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference,

were quantified using real-time quantitative (RTQ) PCR analysis on a ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) as previously described.[31,32] The comparative cycle time ($C_t$) value of the target PCR was normalized by subtracting the $C_t$ value of GAPDH ($\Delta C_t$). The $\Delta C_t$ value was used to calculate the relative expression level to GAPDH for each target PCR using the following formula: relative mRNA expression = $2^{-\Delta Ct}$ x 100%.[33] Primer sequences used were: upper: 5'-ACCAGCCGCATAC TCTT-3, lower: 5'-CAGGGCCTACAAGTTCTG-3' (total *Apaf-1*), upper: 5'-GGACCCTCAA GAGGATATG-3', lower 5'-GTGGGGAGAAGTCACAGTAC-3' ("active" *Apaf-1*) and upper: 5'-GTCGGAGTCAACGGATT-3', lower: 5'-AAGCTTCCCGTTCTCAG-3' (*GAPDH*). Probe sequences were: '5-CACATGGCCAGTGCCAAGAT-3' (total *Apaf-1*), 5'-AAGTGTTGTTCG TGGTCTGCTGAT-3' ("active" *Apaf-1*) and 5'-TCAACTACATGGTTTACATGTTCCAA-3' (*GAPDH*).

**Statistical analysis**

A selection of genes with known involvement in apoptosis was made by a search at https://www.genmapp.org and in literature. Corresponding probe sets were retrieved using Affymetrix® NetAffx (https://www.affymetrix.com). From the total of 179 selected probe sets, 118 were present in the filtered dataset corresponding to 70 apoptosis genes (intrinsic pathway: 40, extrinsic pathway: 30) for subsequent analysis.

We applied the global test[34] to identify those probe sets that are simultaneously differentially expressed between different subgroups defined by: lineage, genetic subtype, *in vitro* drug resistance and clinical outcome. Briefly, the global test compares two or more groups taking into account the association between probe sets as well as their individual effects.[34] The advantage of the global test is that it is applied to the entire set of probe sets under study at the same time, yielding a single overall P-value, rather than on individual probe sets consecutively. Thus, there are no multiple testing issues associated with the global test. In addition, the global test can be applied to multiple probe sets encoding one gene, since this test investigates the influence of each single probe set on the discrimination between the two studied groups. One of the outputs of this test is a so-called gene plot, which displays the individual influences of the probe sets on the test result. The gene plot was used to select those probe sets that were most strongly explaining the difference between two subgroups.

In addition, we applied the Wilcoxon rank-sum test to each probe set to identify those probe sets that were individually associated with the subgroups. P-values were corrected for multiple testing using the false discovery rate (FDR) step-up procedure

proposed by Benjamini & Hochberg.[35] The global test has more power to detect differential expression when dealing with multiple probe sets with small effects, compared with tests applied probe set-wise, such as the Wilcoxon rank-sum test. The output of the global test and the FDR-corrected Wilcoxon rank-sum test were combined in Table 2, 3 and 4.

The duration of disease-free survival (DFS) was defined as the time from diagnosis until the date of leukemia relapse (event) or the last follow-up (censored). Univariate analysis using Cox proportional hazard regression models estimated the relative risk of an event. Significant probe sets from the univariate analysis were entered in a multivariate analysis using Cox's proportional hazards regression model, which included the known risk factors white blood cell count (WBC), age, lineage and genetic subtype. DFS curves were calculated by reversing the cumulative incidence curve[38]. Presence of competing events were accounted for in comparisons of DFS curves [36,37] and in multivariate analysis.[38]

The Wilcoxon rank-sum test was applied to compare Apaf-1 isoform mRNA expression in sensitive and resistant patients for each individual drug.

## RESULTS

### Apoptosis-related genes and immunophenotypic and genetic subtypes of pediatric ALL

The expression of 118 probe sets corresponding to 70 apoptosis-associated genes was compared between various leukemic subgroups, i.e. T-lineage and B-lineage ALL (lineage); hyperdiploid (i.e. more than 50 chromosomes present at cytogenetic analysis) and non-hyperdiploid B-lineage ALL patients (ploidy); TEL-AML1 positive and negative B-lineage ALL patients (TA) and E2A-rearranged and E2A-germline B-lineage ALL patients (E2A). The global test applied to all 118 probe sets generated P-values <0.001 for lineage, ploidy, TA and E2A. Gene plots that visualize influences for individual probe sets on the global test P-value are indicated in Figure 1S of the Supplementary Appendix (available at http://www2.eur.nl/fgg/kgk/onco/Supplementary_Appendix_Holleman_et_al.pdf or http://www.bloodjournal.org /cgi/content/full/2005-07-2930/DC1).

Probe sets were selected that had an influence on the global test P-value of larger than 2 standard deviations above the expected value under the null hypothesis of no association (Table 2). Table 2 also includes the probe sets that were selected by Wilcoxon rank-sum test after correction for multiple testing (FDR controlled at 5%).

**Table 2. Differential expression of apoptosis genes between ALL subtypes**

| Gene Name | Pro- or anti-apoptotic | Probe set ID | lineage | ploidy | TA | E2A |
|---|---|---|---|---|---|---|
| **Caspases** | | | | | | |
| CASP3 | pro | 202763_at | 1.23 | | | |
| CASP6 | pro | 211464_x_at | 1.32 | | | 0.61 |
| CASP6 | pro | 209790_s_at | 1.73 | | | |
| CASP7 | pro | 207181_s_at | 0.61 | 1.5 | | |
| CASP9 | pro | 203984_s_at | 1.25 | 0.78 | | |
| CASP9 | pro | 210775_x_at | 0.88 | | | |
| **Bcl2 family** | | | | | | |
| BAD | pro | 1861_at | 1.22 | 0.71 | 1.12 | |
| BAD | pro | 209364_at | 1.5 | 0.6 | 1.28 | 1.53 |
| BAK1 | pro | 203728_at | 1.25 | | | |
| BAX | pro | 211833_s_at | | | | |
| BAX | pro | 208478_s_at | | 0.92 | 1.24 | |
| BCL2 | anti | 207005_s_at | 0.57 | | | 0.49 |
| BCL2 | anti | 207004_at | 0.90 | 1.27 | 0.82 | |
| BCL2 | anti | 203685_at | 0.59 | | | 0.66 |
| BCL2L1 | anti | 215037_s_at | | | | 1.56 |
| BCL2L1 | anti | 206665_s_at | | 0.71 | | |
| BCL2L1 | anti | 212312_at | 1.39 | 0.66 | 1.38 | |
| BCL2L13 | pro | 217955_at | 1.45 | | 0.70 | |
| BID | pro | 204493_at | 1.24 | 0.77 | | |
| BIK | pro | 205780_at | | 0.64 | 0.78 | 14.40 |
| HRK | pro | 206865_at | 0.32 | 1.90 | | |
| HRK | pro | 206864_s_at | 0.62 | | | |
| MCL1 | anti | 200797_s_at | 0.66 | | | 0.62 |
| MCL1 | anti | 200798_x_at | 0.59 | | | 0.75 |
| MCL1 | anti | 214056_at | 0.73 | | 0.79 | |
| MCL1 | anti | 214057_at | | | | 0.61 |
| MCL1 | anti | 200796_s_at | 0.56 | | | |
| **Bcl2-interacting** | | | | | | |
| BAG1 | anti | 211475_s_at | 1.23 | 0.84 | 0.81 | 1.31 |
| BAG1 | anti | 202387_at | 1.43 | 0.80 | | |
| BAG3 | anti | 217911_s_at | 3.23 | 0.39 | 2.61 | |
| BAG4 | anti | 219624_at | | | | |
| BAG5 | anti | 202985_s_at | 0.70 | 1.58 | | |
| BAG5 | anti | 202984_s_at | 0.74 | 1.27 | | |
| BFAR | anti | 218056_at | | 1.07 | 0.91 | |
| PUMA | pro | 211692_s_at | | 0.81 | 1.34 | |
| **IAP family** | | | | | | |
| NAIP | anti | 204861_s_at | 1.87 | | | |
| NAIP | anti | 204860_s_at | | | | |
| cIAP1 | anti | 202076_at | 1.56 | | 0.80 | |
| cIAP2 | anti | 210538_s_at | 1.73 | | | |
| XIAP | anti | 206536_s_at | | | | |
| XIAP | anti | 206537_at | 0.64 | | | |
| SURVIVIN | anti | 202094_at | 1.65 | | | 1.82 |
| SURVIVIN | anti | 210334_x_at | 1.35 | | | |
| SURVIVIN | anti | 202095_s_at | 1.95 | | | |
| LIVIN | anti | 220451_s_at | 0.55 | 0.71 | 5.79 | |
| **CARD-containing** | | | | | | |
| APAF1 | pro | 211553_x_at | 0.80 | 1.26 | | |
| APAF1 | pro | 204859_s_at | 0.74 | 1.84 | 0.87 | |
| NOD1 | pro | 221073_s_at | 0.79 | | 0.92 | |
| TUCAN | anti | 204950_at | 0.7 | | | 0.59 |
| **Mitochondrial** | | | | | | |
| AIF | pro | 205512_s_at | 1.43 | 1.02 | | |
| Omi-HtrA2 | pro | 203089_s_at | | 0.89 | 1.15 | |
| Omi-HtrA2 | pro | 211152_s_at | | 0.84 | | |
| CYCS | pro | 208905_at | 1.41 | 0.83 | | |
| SMAC | pro | 219350_s_at | | | 1.13 | |
| **Miscellaneous** | | | | | | |
| DFFA | pro | 203277_at | 1.25 | 0.88 | 0.98 | |
| DFFB | pro | 206752_s_at | 2.84 | | 0.59 | |
| HSP27 | anti | 201841_s_at | 0.27 | | 2.00 | |
| PARP | pro | 208644_at | 0.71 | 0.83 | | 1.90 |
| PARPL1 | anti | 202239_at | | | | 0.86 |
| PARPL2 | pro | 204752_x_at | 1.21 | 1.19 | 0.86 | |
| PARPL2 | pro | 214086_s_at | 1.29 | 1.60 | | 0.38 |
| PARPL2 | pro | 215773_x_at | 1.13 | 1.26 | 0.84 | |
| PECAM-1 | anti | 208983_s_at | | 6.50 | 0.58 | |
| PECAM-1 | anti | 208982_at | | 1.83 | | 0.54 |
| PECAM-1 | anti | 208981_at | | 2.37 | | 0.43 |

| Gene Name | Pro- or anti-apoptotic | Probe set ID | lineage | ploidy | TA | E2A |
|---|---|---|---|---|---|---|
| **Ligands** | | | | | | |
| FAS-L | pro | 211333_s_at | | | | |
| FAS-L | pro | 210865_at | | | | |
| TNF | pro | 207113_s_at | 0.27 | 1.94 | 2.07 | |
| TRAIL | pro | 202687_s_at | | | | |
| TRAIL | pro | 202688_at | | | | |
| TRAIL | pro | 214329_x_at | | | | |
| **Death receptors** | | | | | | |
| FAS | pro | 215719_x_at | | | | |
| FAS | pro | 216252_x_at | | | | |
| FAS | pro | 204780_s_at | 1.45 | | 1.23 | |
| FAS | pro | 204781_s_at | 1.42 | | 1.06 | |
| DcR3 | anti | 206092_x_at | | | | |
| DcR3 | anti | 206467_x_at | | | 1.32 | |
| DcR3 | anti | 213829_x_at | | 0.90 | | |
| TNFR1 | pro | 207643_s_at | 0.55 | 1.79 | | 0.27 |
| TNFR2 | pro | 203508_at | 0.54 | 1.62 | | 0.59 |
| TRAIL-R2 | pro | 210405_x_at | 0.24 | 2.29 | | 0.26 |
| TRAIL-R2 | pro | 209295_at | 0.38 | 1.93 | | |
| TRAIL-R3 | anti | 206222_at | | | | |
| TRAIL-R4 | anti | 210654_at | 0.37 | 1.54 | | |
| TRAMP | pro | 219423_x_at | 1.39 | | | |
| TWEAK-R | pro | 218368_s_at | 0.58 | | | |
| **Caspases** | | | | | | |
| CASP1 | pro | 211368_s_at | | 1.68 | 0.38 | |
| CASP1 | pro | 209970_x_at | | | 0.75 | |
| CASP1 | pro | 211367_s_at | | 1.43 | 0.36 | |
| CASP1 | pro | 211366_x_at | | | 0.72 | |
| CASP2 | pro | 34449_at | | | | |
| CASP2 | pro | 209811_at | | | | |
| CASP8 | pro | 207686_s_at | 1.48 | | | |
| CASP8 | pro | 213373_s_at | 2.81 | 1.00 | | 0.37 |
| CASP10 | pro | 205467_at | 1.69 | | | 0.29 |
| **Adapters** | | | | | | |
| DAXX | pro | 201763_s_at | | | 1.37 | |
| FADD | pro | 202535_at | 1.99 | | | |
| FLASH | pro | 222201_s_at | | | | |
| FLIP | anti | 211862_x_at | 1.55 | 0.79 | | |
| FLIP | anti | 208485_x_at | 1.21 | | | |
| FLIP | anti | 211317_s_at | 1.29 | | | 1.32 |
| FLIP | anti | 209939_x_at | 1.69 | 0.81 | | |
| FLIP | anti | 214618_at | | | | |
| FLIP | anti | 209508_x_at | | 0.86 | | |
| FLIP | anti | 211316_x_at | 1.21 | | | |
| FLIP | anti | 210563_x_at | 1.54 | 0.79 | 1.03 | |
| RIPK1 | pro | 209941_at | | | | |
| TANK | pro | 207616_s_at | | | 0.89 | 1.20 |
| TANK | pro | 209451_at | 1.41 | | | |
| TANK | pro | 210458_s_at | | | | |
| TRADD | pro | 1729_at | 2.17 | | | |
| TRAF1 | anti | 205599_at | | | | |
| TRAF2 | anti | 204413_at | | | | |
| TRAF3 | pro | 208315_x_at | | | | |
| TRAF4 | pro | 211899_s_at | | | | 2.15 |
| TRAF6 | anti | 205558_at | 0.83 | | 1.26 | |
| **Miscellaneous** | | | | | | |
| CRADD | pro | 209833_at | 0.71 | | 0.91 | 0.67 |
| DAPK1 | pro | 203139_at | 1.45 | | | 0.29 |

The global test and Wilcoxon's rank-sum test were performed to identify which of the indicated 118 apoptosis probe sets were differentially expressed in various ALL subgroups defined by T- versus B-lineage ALL (lineage), or genetic subtype, i.e. TEL-AML1 status (TA), ploidy and E2A status (E2A). For each subgroup, probe sets selected only by the global test (P<0.001) are marked light gray, probe sets selected only by Wilcoxon's rank-sum test with false discovery rate (FDR) <5% are marked dark gray, probe sets selected by the global test (P<0.001) and Wilcoxon's rank-sum test with false discovery rate (FDR) are marked black and probe sets selected by none of these tests are

marked white. The numbers indicated in colored boxes are the ratio per significant gene between: T-lineage ALL and B-lineage ALL samples (column: lineage), hyperdiploid and non-hyperdiploid B-lineage ALL samples (column: ploidy), TEL-AML1 positive and TEL-AML1 negative B-lineage ALL samples (column: TA) and E2A-rearranged and E2A-germline B-lineage ALL samples (column: E2A).

Sixty-six probe sets (44 different genes) were differentially expressed in T-lineage versus B-lineage ALL and selected by both tests (indicated black in Table 2), 31 probe sets (22 different genes) genes in hyperdiploid versus non-hyperdiploid B-lineage ALL, 20 probe sets (16 different genes) in TEL-AML1 positive versus negative B-lineage ALL, and 15 probe sets (13 different genes) in E2A-rearranged versus E2A-germline B-lineage ALL. The probe-set identification, gene names and median expression of all probe sets are listed in Table 1S of the Supplementary Appendix.

**Apoptosis-related genes and cellular drug resistance in pediatric B-lineage ALL**

Due to the large difference in the expression of apoptosis genes between T- and B-lineage ALL ($P<0.001$, global test) and the limited number of T-lineage ALL patients, differences in expression of apoptosis genes between drug sensitive and resistant patients was only addressed in the B-lineage ALL group. The global test generated significant P-values for prednisolone (16 probe sets corresponding to 14 different genes, $P=0.007$), vincristine (14 probe sets corresponding to 13 different genes, $P=0.002$) and L-asparaginase (20 probe sets corresponding to 15 different genes, $P<0.001$), but not for daunorubicin (Table 3). The probe sets most strongly associated with resistance to individual drugs in the global test and the Wilcoxon rank-sum test (FDR controlled at 5%) are indicated in black in Table 3. While no probe sets were associated with resistance to vincristine or daunorubicin, 4 probe sets (corresponding to 2 genes, i.e. MCL1 and DAPK1) and 3 probe sets (i.e. BCL2L13, HRK and TNF) were significantly associated with resistance in both tests towards prednisolone or L-asparaginase, respectively. Gene plots for each drug are shown in Figure 2S and probe-set identification, gene names and median expression are shown for each drug in Table 2S of the Supplementary Appendix.

**Table 3. Apoptosis genes associated with resistance to four individual chemotherapeutic agents in B-lineage ALL**

| Gene Name | Pro- or anti-apoptotic | Probe set ID | PRED | VCR | ASP | DNR |
|---|---|---|---|---|---|---|
| **Caspases** | | | | | | |
| CASP3 | pro | 202763_at | | 1.19 | | |
| CASP6 | pro | 211464_x_at | | | | |
| CASP6 | pro | 209790_s_at | | | | |
| CASP7 | pro | 207181_s_at | | | | 0.73 |
| CASP9 | pro | 203984_s_at | | | | |
| CASP9 | pro | 210775_x_at | | | | |
| **Bcl2 family** | | | | | | |
| BAD | pro | 1861_at | | | | |
| BAD | pro | 209364_at | | | | |
| BAK1 | pro | 203728_at | | | | |
| BAX | pro | 211833_s_at | | | | |
| BAX | pro | 208478_s_at | | | | |
| BCL2 | anti | 207005_s_at | | | | |
| BCL2 | anti | 207004_at | 1.12 | | | |
| BCL2 | anti | 203685_at | | 1.43 | | |
| BCL2L1 | anti | 215037_s_at | | | | |
| BCL2L1 | anti | 206665_s_at | | | | |
| BCL2L1 | anti | 212312_at | | | | |
| BCL2L13 | pro | 217955_at | 1.25 | | 1.35 | 1.24 |
| BID | pro | 204493_at | | | | |
| BIK | pro | 205780_at | | | 3.25 | |
| HRK | pro | 206865_at | 0.3 | | 0.34 | |
| HRK | pro | 206864_s_at | | | 0.63 | |
| MCL1 | anti | 200797_s_at | 1.42 | | | 1.28 |
| MCL1 | anti | 200798_x_at | | | | |
| MCL1 | anti | 214056_at | 1.44 | | | |
| MCL1 | anti | 214057_at | 1.6 | | | 1.12 |
| MCL1 | anti | 200796_s_at | | | | |
| **Bcl2-interacting** | | | | | | |
| BAG1 | anti | 211475_s_at | | 0.78 | 1.2 | |
| BAG1 | anti | 202387_at | | | 1.11 | |
| BAG3 | anti | 217911_s_at | | 1.71 | | |
| BAG4 | anti | 219624_at | | | | |
| BAG5 | anti | 202985_s_at | | | 0.83 | |
| BAG5 | anti | 202984_s_at | | | | |
| BFAR | anti | 218056_at | | | | |
| PUMA | pro | 211692_s_at | | | | |
| **IAP family** | | | | | | |
| NAIP | anti | 204861_s_at | | | | |
| NAIP | anti | 204860_s_at | | | | |
| cIAP1 | anti | 202076_at | 1.19 | | | |
| cIAP2 | anti | 210538_s_at | | | | |
| XIAP | anti | 206536_s_at | | | | |
| XIAP | anti | 206537_at | | | | |
| SURVIVIN | anti | 202094_at | | | | |
| SURVIVIN | anti | 210334_x_at | | | | |
| SURVIVIN | anti | 202095_s_at | 0.66 | | | |
| LIVIN | anti | 220451_s_at | | 2.05 | | |
| **CARD-containing** | | | | | | |
| APAF1 | pro | 211553_x_at | | | | |
| APAF1 | pro | 204859_s_at | | | 0.76 | |
| NOD1 | pro | 221073_s_at | 1.4 | | | |
| TUCAN | anti | 204950_at | | 1.04 | | |
| **Mitochondrial** | | | | | | |
| AIF | pro | 205512_s_at | | | 0.93 | |
| Omi-HtrA2 | pro | 203089_s_at | | | | |
| Omi-HtrA2 | pro | 211152_s_at | | | | |
| CYCS | pro | 208905_at | | | | |
| SMAC | pro | 219350_s_at | | | | |
| **Miscellaneous** | | | | | | |
| DFFA | pro | 203277_at | | | 1.01 | |
| DFFB | pro | 206752_s_at | | | | 2.24 |
| HSP27 | anti | 201841_s_at | | | | |
| PARP | pro | 208644_at | 0.81 | | | |
| PARPL1 | anti | 202239_at | | | | 1.28 |
| PARPL2 | pro | 204752_x_at | | 0.98 | | |
| PARPL2 | pro | 214086_s_at | | | 0.84 | |
| PARPL2 | pro | 215773_x_at | | | | |
| PECAM-1 | anti | 208983_s_at | | | 0.43 | |
| PECAM-1 | anti | 208982_at | | | 0.83 | |
| PECAM-1 | anti | 208981_at | | 1.2 | 0.73 | |

| Gene Name | Pro- or anti-apoptotic | Probe set ID | PRED | VCR | ASP | DNR |
|---|---|---|---|---|---|---|
| **Ligands** | | | | | | |
| FAS-L | pro | 211333_s_at | | | | |
| FAS-L | pro | 210865_at | | | | |
| TNF | pro | 207113_s_at | | | 0.63 | |
| TRAIL | pro | 202687_s_at | 1.43 | | | |
| TRAIL | pro | 202688_at | | | | |
| TRAIL | pro | 214329_x_at | | | | |
| **Death receptors** | | | | | | |
| FAS | pro | 215719_x_at | | | | |
| FAS | pro | 216252_x_at | | | | |
| FAS | pro | 204780_s_at | | | | |
| FAS | pro | 204781_s_at | | | | |
| DcR3 | anti | 206092_x_at | | | | |
| DcR3 | anti | 206467_x_at | | | | |
| DcR3 | anti | 213829_x_at | | | | |
| TNFR1 | pro | 207643_s_at | | 1.54 | | |
| TNFR2 | pro | 203508_at | | | | |
| TRAIL-R2 | pro | 210405_x_at | | | 0.74 | |
| TRAIL-R2 | pro | 209295_at | | | 0.83 | |
| TRAIL-R3 | anti | 206222_at | | | | |
| TRAIL-R4 | anti | 210654_at | | | 0.76 | |
| TRAMP | pro | 219423_x_at | | | | |
| TWEAK-R | pro | 218368_s_at | | | | |
| **Caspases** | | | | | | |
| CASP1 | pro | 211368_s_at | | | | |
| CASP1 | pro | 209970_x_at | 1.16 | | | |
| CASP1 | pro | 211367_s_at | | 0.86 | | |
| CASP1 | pro | 211366_x_at | | 0.86 | | |
| CASP2 | pro | 34449_at | | | | |
| CASP2 | pro | 209811_at | | | | |
| CASP8 | pro | 207686_s_at | | | | |
| CASP8 | pro | 213373_s_at | | 0.98 | 0.69 | |
| CASP10 | pro | 205467_at | | | | |
| **Adapters** | | | | | | |
| DAXX | pro | 201763_s_at | | | | |
| FADD | pro | 202535_at | | | | |
| FLASH | pro | 222201_s_at | | | | |
| FLIP | anti | 211862_x_at | | | | |
| FLIP | anti | 208485_x_at | | | | |
| FLIP | anti | 211317_s_at | | | | |
| FLIP | anti | 209939_x_at | | | | |
| FLIP | anti | 214618_at | | | | |
| FLIP | anti | 209508_x_at | | | | |
| FLIP | anti | 211316_x_at | | | | |
| FLIP | anti | 210563_x_at | | | | |
| RIPK1 | pro | 209941_at | | 1.16 | | |
| TANK | pro | 207616_s_at | | | 1.12 | |
| TANK | pro | 209451_at | | | | |
| TANK | pro | 210458_s_at | | | | |
| TRADD | pro | 1729_at | | 0.59 | | |
| TRAF1 | anti | 205599_at | | | | 1.54 |
| TRAF2 | anti | 204413_at | | | | |
| TRAF3 | pro | 208315_x_at | | | | |
| TRAF4 | pro | 211899_s_at | 0.81 | | | |
| TRAF6 | anti | 205558_at | 0.93 | | | 0.95 |
| **Miscellaneous** | | | | | | |
| CRADD | pro | 209833_at | 1.3 | | | |
| DAPK1 | pro | 203139_at | 1.71 | | | 1.77 |

The global test and Wilcoxon's rank-sum test were performed to identify which of the indicated 118 apoptosis probe sets were differentially expressed in B-lineage ALL cells sensitive and resistant to prednisolone (PRED), vincristine (VCR), L-asparaginase (ASP) and daunorubicin (DNR). For each drug, probe sets selected only by the global test (P<0.001) are marked gray, probe sets selected by the global test (P<0.001) and Wilcoxon's rank-sum test with false

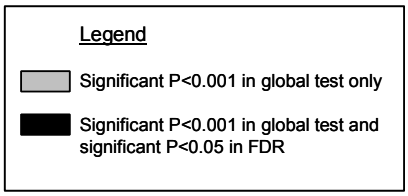
discovery rate (FDR) <5% are marked black and probe sets not selected by none of the tests are marked white. The numbers indicated in colored boxes are the ratio per significant gene between: PRED resistant and PRED sensitive ALL samples (column: PRED), VCR resistant and VCR sensitive ALL samples (column: VCR), ASP resistant and ASP sensitive ALL samples (column: ASP) and DNR resistant and DNR sensitive ALL samples (column: DNR).

**The expression of apoptosis-related genes and clinical outcome in pediatric ALL**

From the 190 patients included in this study (median follow-up at risk of event: 4.8 years, range: 0.3-10.5 years), 45 had disease-related events and 2 had a competing event, which was censored at the time of occurrence. Apoptosis gene expression profiles measured at initial diagnosis were compared between patients who entered and remained in continuous complete remission (CCR) and those who relapsed during follow-up.

**Table 4: Apoptosis genes associated with disease-free survival in pediatric ALL.**

| Gene Name | Probe set ID | outcome |
|---|---|---|
| BAG1 | 211475_s_at | 0.91 |
| BAG1 | 202387_at | 0.81 |
| BAG5 | 202985_s_at | 1.26 |
| BAG5 | 202984_s_at | 1.20 |
| BCL2L13 | 217955_at | 0.72 |
| BID | 204493_at | 0.87 |
| BIK | 205780_at | 0.25 |
| XIAP | 206537_at | 1.30 |
| CASP9 | 203984_s_at | 0.79 |
| CYCS | 208905_at | 0.83 |
| PECAM-1 | 208982_at | 1.33 |
| PECAM-1 | 208981_at | 1.66 |
| CASP1 | 211367_s_at | 0.77 |
| FAS | 216252_x_at | 0.99 |
| TRAIL-R2 | 210405_x_at | 1.73 |
| TRAIL-R2 | 209295_at | 1.36 |
| TRAIL-R4 | 210654_at | 1.53 |




The global test and Wilcoxon's rank-sum test with false discovery rate (FDR) controlled at 5% were performed to identify which of the 118 apoptosis probe sets under study were differentially expressed in leukemic cells taken at initial diagnosis of ALL from patients who achieved and remained in continuous complete remission (CCR) and patients who achieved a complete remission but relapsed during or after completion of chemotherapy.

Gene plots for each drug are shown in Figure 3S and probe-set identification, gene names and median expression are shown for each drug in Table 3S of the Supplementary Appendix. 17 Probe sets (13 genes) influenced the global test P-value by more than 2 standard deviations. Out of these 17 probe sets, 4 probe sets (3 genes) were also selected by the univariate Cox regression analysis with FDR controlled at 5% (marked black in Table 4).

Subsequently, each of these 4 significant probe sets was analyzed in a multivariate Cox regression analysis with inclusion of conventional risk criteria, i.e. age, white blood cell count, lineage and genetic subtype (Table 5). BCL2L13 was the only gene that was independently and significantly associated with treatment outcome (P=0.011; Table 5A). BCL2L13 expression was significantly associated with treatment outcome when used as continuous variable (P<0.0001) and when divided into two equally sized groups (P=0.002; Figure 1). The 5-year probability of disease-free survival (pDFS) ± SE was 85% ± 5.2% for patients with low (i.e. below median) and 66% ± 7.3% for patients with high (i.e. above median) expression of BCL2L13.

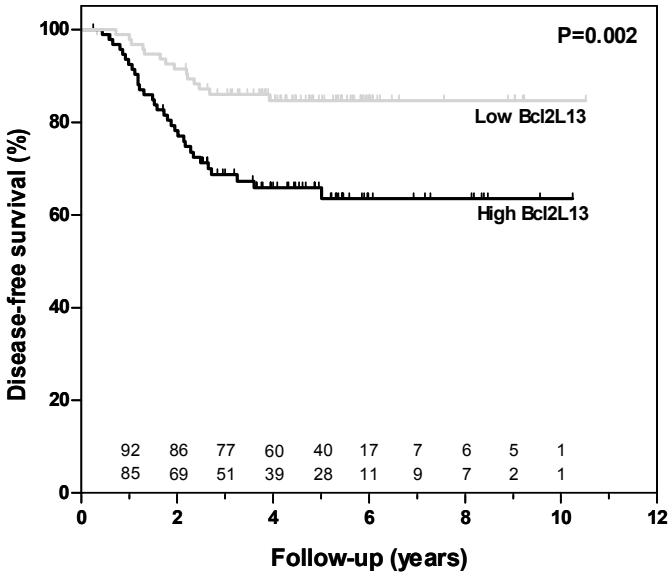



**Figure 1. Disease-free survival according to BCL2L13 expression in pediatric ALL.** The disease-free survival (DFS) of patients was estimated according to a Kaplan-Meier among 190 patients of the COALL/DCOG study cohort. Patients were grouped according to on their expression of BCL2L13, i.e. expression higher than (black line) or lower than (gray line) the median.

For 92 patients enrolled at St. Jude the median follow-up was 6.2 years. Of these patients, 15 had disease-related events and 8 had a competing event, which was censored at the time of occurrence. In this independent cohort treated with the same chemotherapeutic agents but on a different protocol at the St. Jude Children's Research Hospital the association between BCL2L13 expression and outcome was significant in a univariate analysis when treated as a continuous variable (P=0.025), but not significant when patients below and above the median were compared (P=0.28). In a multivariate Cox analysis

including the above mentioned known risk factors BCL2L13 expression showed the same trend for an association with outcome in patients treated according to St. Jude protocols (P=0.051; Table 5B).

**Table 5. Multivariate proportional-hazards analysis of the risk of relapse.** Multivariate Cox regression analysis among 190 patients of the original COALL/DCOG cohort (A) and 92 patients in the validation St. Jude cohort (B) was performed to quantify the independent contribution of BCL2L13 to disease-free survival. Age, white blood cells count (WBC) and genetic subtype were considered as discrete and BCL2L13 expression as a continuous variable in the analysis. A hazard ratio >1 indicates increased probability of relapse. CI denotes confidence interval.

| | A. COALL/DCOG cohort | | | | B. St. Jude cohort | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **N** | **HR** | **95% CI** | **P-value** | **N** | **HR** | **95% CI** | **P-value** |
| **Age** | | | | | | | | |
| <10 years | 138 | 1.0* | | | 63 | 1.0* | | |
| >10 years | 52 | 1.47 | 0.77-5.13 | 0.24 | 29 | 7.88 | 1.45-42.90 | *0.017* |
| **White-cell count** | | | | | | | | |
| <10/nL | 44 | 1.0* | | | 26 | 1.0* | | |
| 10-49/nL | 71 | 0.56 | 0.21-1.48 | 0.24 | 26 | 1.06 | 0.21-5.46 | 0.95 |
| 50-100/nL | 31 | 1.12 | 0.40-3.17 | 0.83 | 18 | 6.48 | 1.25-33.62 | *0.026* |
| >100/nL | 44 | 1.49 | 0.54-4.06 | 0.44 | 22 | 2.57 | 0.52-12.64 | 0.24 |
| **ALL subtype** | | | | | | | | |
| B-other | 48 | 1.0* | | | 24 | 1.0* | | |
| *BCR-ABL* | 5 | 1.33 | 0.34-5.13 | 0.68 | 8 | 5.49 | 1.49-20.23 | *0.011* |
| *E2A*[£] | 9 | 1.10 | 0.31-3.86 | 0.89 | 12 | 0.70 | 0.07-6.62 | 0.75 |
| Hyperdiploid[#] | 44 | 0.28 | 0.08-0.97 | *0.045* | 15 | 0.48 | 0.05-4.44 | *0.51* |
| *MLL*[£] | 4 | 9.45 | 2.42-36.88 | *0.001* | 8 | 1.01 | 0.15-6.99 | *0.99* |
| *TEL-AML1* | 44 | 0.23 | 0.07-0.80 | *0.021* | 16 | 1.40 | 0.14-14.30 | *0.78* |
| T-lineage | 36 | 0.66 | 0.28-1.60 | 0.36 | 9 | 4.85 | 0.64-36.60 | 0.13 |
| ***BCL2L13*** | | | | | | | | |
| expression | 190 | 1.94 | 1.17-3.24 | *0.011* | 92 | 4.09 | 0.99-16.78 | *0.051* |

N: number of patients, HR: hazard ratio, CI: confidence interval, *: this group served as the reference group to calculate the ratio, [#]: Cytogenetic analysis revealed more than 50 chromosomes, [£]: COALL: *MLL* and *E2A* rearranged, St. Jude: *MLL-AF4* and *E2A-PBX1*

**Expression of Apaf-1 isoforms and cellular drug resistance in pediatric ALL**

The expression of Apaf-1 splice variants has been linked to functional apoptosis in tumor cell lines.[39-41] The presence of an additional C-terminal WD-40 repeat encoded by exon 18 appears to be required for *in vitro* activation of procaspase-9 and -3. The Affymetrix probe sets are unable to distinguish between the individual isoforms of Apaf-1 (Figure 2). To investigate whether the relative expression of pro-apoptotic (active) Apaf-1 isoforms, i.e. the isoforms containing exon 18, is linked to sensitivity to antileukemic agents, real-time quantitative PCR was carried out in 36 children with ALL at initial diagnosis. Two primer pairs were used: one pair recognizes both pro- and anti-apoptotic Apaf-1 isoforms and one pair hybridizes to exon 18 and is thus specific for the pro-apoptotic isoform of Apaf-1 (Figure 2).


**Figure 2.** Structure of the Apaf-1 gene and two Apaf-1 transcript variants. Indicated is the location of the Affymetrix probe sets and the Taqman primer pairs (1 and 2).

The relative expression of pro-apoptotic Apaf-1 isoform ranged between 2%-69% of total Apaf-1 and did not differ significantly in patients sensitive and resistant to prednisolone (P=0.74), vincristine (P=0.33), L-asparaginase (P=0.79) or daunorubicin (P=0.95). In addition, the absolute expression of the pro-apoptotic isoform did not differ significantly in patients sensitive and resistant to prednisolone (P=0.96), vincristine (P=0.20), L-asparaginase (P=0.25) or daunorubicin (P=0.67).

## DISCUSSION

Leukemic subtypes with an unfavorable prognosis may have a decreased tendency to undergo apoptosis compared to subtypes with a favorable prognosis. Gene expression signatures discriminative for lineage,[27,42] genetic subtype,[27,42] *in vitro*,[29] and *in vivo*[29,42] drug response were previously reported. Among the discriminative genes identified in these studies were virtually no apoptosis genes. This does not rule out a role for apoptosis genes in these leukemic subtypes per se, because these genes may be significant at a lower level than the cut-off P-values used for the construction of these signature models. Therefore, we analyzed the expression patterns of 70 selected key apoptotic genes in leukemic cells of 190 children at initial diagnosis of ALL and correlated the expression of these genes to lineage, genetic subtype, *in vitro* drug resistance and clinical outcome.

Children with T-lineage ALL have an increased risk of treatment failures compared to children with B-lineage ALL[43], which can be attributed to the presence of numerous adverse presenting features, such as older age, high white blood cell count and *in vitro* resistance to a variety of drugs.[8,44] However, intensification of treatment regimens has resulted in remarkably improved outcomes for children with T-ALL.[45] Although T-ALL has been associated with aberrant expression of some apoptosis genes,[46,47] the underlying causes of *in vitro* drug resistance have not yet been fully determined. Global test analysis

indicated that the expression of apoptosis genes differs between T-lineage and B-lineage ALL ($P<0.001$). A large number of apoptosis genes, i.e. 44 out of 70 examined genes, were most discriminative between T-lineage and B-lineage ALL (as defined by $\geq$2 sd influence on the global test P-value, see explanation in Materials and Methods).

The death-receptor Fas has been linked to apoptosis and NF-kB-related inflammatory response pathways.[48] Activation of NF-kB inhibits drug-induced apoptosis in various cell line studies and was shown to be linked to drug resistance in childhood ALL.[49-51] Interestingly, we observed simultaneous upregulation of Fas and its downstream effectors, i.e. FADD, caspase-8 and caspase-10 in T- compared to B-lineage ALL and upregulation of several NF-κB target genes, i.e. cIAP1, cIAP2, survivin and FLIP. The relative high expression of NF-kB associated genes may point to enhanced NF-kB activity in T-lineage compared to B-lineage ALL.

Children with hyperdiploid and TEL-AML1 positive ALL have a favorable prognosis, which is associated with a relatively high *in vitro* sensitivity to various drugs, including L-asparaginase[11,12] Interestingly, the TNF receptor ligand (TNF) is expressed higher in hyperdiploid and TEL-AML1 positive B-lineage ALL patients (Table 2). Moreover, TNF is 0.6-fold lower expressed in L-asparaginase sensitive cases (Table 3). Since both hyperdiploid and TEL-AML1 positive B-lineage ALL are *in vitro* sensitive to L-asparaginase,[11,12] these data point to novel insights in the apoptotic changes underlying L-asparaginase cytotoxicity. Another notable feature of hyperdiploid B-lineage ALL cells is the simultaneous overexpression of TNF-R1, TRAIL-R2 and TRAIL-R4. The overexpression of these cytokine receptors was not previously observed in hyperdiploid B-lineage ALL but may contribute to their marked apoptotic propensity in allogeneic bone marrow-derived stromal layers that contain the micro-environment to trigger these receptors.[52]

The relation between the expression of apoptosis genes and *in vitro*,[53-55] or *in vivo* response[56-59] has been extensively studied in ALL. However, these studies each focused on the expression of only a few genes out of the large family of apoptosis-related genes. In this study, analysis of 70 key apoptotic genes revealed that only 2 and 3 genes were significantly associated with resistance towards prednisolone and L-asparaginase respectively. Bcl2-family members are thought of as the central regulators of apoptosis by regulating cytochrome *c* release upstream of the mitochondria.[60] We observed increased expression of the anti-apoptotic Bcl-2 family member MCL1 in prednisolone resistant B-lineage ALL cells and decreased expression of the pro-apoptotic Bcl2-family member HRK in L-asparaginase resistant B-lineage ALL cells. The differential expression of these Bcl2-

family members may contribute to the apoptotic blockage we previously observed upstream of the mitochondria in prednisolone and L-asparaginase resistant ALL cells.[22] The fact that we observed decreased apoptosis in vincristine and daunorubicin resistant ALL cells in the former study[22] and no apoptosis gene was associated with vincristine and daunorubicin resistance in the present study suggests that resistance to these drugs is caused by mechanisms that do not appear transcriptionally. Alternatively, VCR and DNR resistance in childhood ALL may be caused by a defect further upstream of the mitochondria. Aberrant expression and function of cytoskeleton-associated genes[29,61] and lack of ceramide generation[62] are examples of upstream defects that were previously observed in leukemic samples resistant to VCR and DNR respectively.

BCL2L13 (Bcl-rambo) is a recently discovered member of the Bcl-2 family with pro-apoptotic activity.[63,64] Remarkably, however, in this study we observed that a high mRNA expression of BCL2L13 was associated with *in vitro* L-asparaginase resistance and an unfavorable long-term clinical outcome in children with ALL. This finding suggests BCL2L13 may have a different apoptotic role in primary leukemic cells of children compared to the cell lines used to describe its apoptotic role. Alternative splicing is known to generate both anti- and pro-apoptotic variants of a single apoptosis gene (e.g., Apaf-1, Figure 2).[40,65,66] Therefore, an alternative explanation for our finding may be the existence of a previously unrecognized anti-apoptotic splice variant. Probe sets designed by Affymetrix are (in general) not suitable to recognize differential expression of splice variants of a single gene. Most importantly, high expression of the BCL2L13 probe set was associated with resistance towards L-asparaginase and independently linked to an unfavorable prognosis compared to other know risk factors. Since BCL2L13 expression was also associated with an inferior outcome in a second (differently treated) validation cohort, this gene may represent a new risk factor in childhood ALL. The fact that only 1 out of the 70 apoptosis genes was independently associated with treatment outcome in this study suggests that treatment outcome in childhood ALL is largely dependent on genes involved in other pathways than the apoptosis pathway. This notion is supported by the absence of apoptosis genes amongst the genes previously associated with treatment response in several studies in diagnostic childhood ALL samples.[42,67,68]

In conclusion, this study is the first to describe an association between the differential expression of key apoptosis genes and lineage, genetic subtype and *in vitro* drug resistance in children with ALL. In addition, we identified a single gene, i.e. BCL2L13, which is related to both L-asparaginase resistance and treatment outcome independent from known prognostic factors in two independent cohorts of children with B-lineage ALL.

To establish BCL2L13 expression as a true prognostic factor in childhood ALL, prospective validation is required. Also, the currently identified genes warrant further studies on expression and function at the protein level to further increase our insight in the causes of drug resistance and therapy failure in pediatric ALL. It was recently demonstrated that inhibition of Mcl-1 by the cyclin-dependent kinase (CDK) inhibitor Seliciclib induced significant cytotoxicity in multiple myeloma cells sensitive and resistant to conventional therapy.[69] In addition, depletion of Mcl-1 levels by antisense Mcl-1 oligonucleotides sensitized lung cancer cells to apoptosis induced by cytotoxic agents as well as by ionizing radiation.[70] Likewise, it can be hypothesized that downregulation of Bcl2l13 by antisense oligonucleotides or specific inhibitors may sensitize ALL cells to L-asparaginase and eventually other drugs.

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Chapter 5

# Decreased PARP and procaspase-2 protein levels are associated with cellular drug resistance in childhood acute lymphoblastic leukemia

Amy Holleman[1], Monique L. den Boer[1], Karin M. Kazemier[1], H. Berna Beverloo[1,2], Anne R. M. von Bergh[2], Gritta E. Janka-Schaub[3], and Rob Pieters[1]

*[1]Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Department of Pediatric Oncology and Hematology, Rotterdam, the Netherlands,*

*[2]Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Department of Clinical Genetics, Rotterdam, the Netherlands*

*[3]COALL study group, University Children's Hospital, Hamburg, Germany.*

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**ABSTRACT**

Drug resistance in childhood acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) is associated with impaired ability to induce apoptosis. To elucidate causes of apoptotic defects, we studied the protein expression of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and poly(ADPribose) polymerase (PARP) in cells from children with acute lymphoblastic leukemia (ALL; N=43) and acute myeloid leukemia (AML; N=10). PARP expression was present in all B-lineage samples, but absent in 4 of 15 T-lineage ALL and 3 of 10 AML cases, which was not caused by genomic deletions. PARP expression was a median 7-fold lower in T-lineage ALL (P<0.001) and 10-fold lower in AML (P<0.001) compared with B-lineage ALL. PARP expression was 4-fold lower in prednisolone, vincristine and L-asparaginase (PVA)–resistant compared with PVA-sensitive ALL patients (P<0.001). Procaspase-2 expression was 3-fold lower in T-lineage ALL (P=0.022) and AML (P=0.014) compared with B-lineage ALL. In addition, procaspase2 expression was 2-fold lower in PVA-resistant compared to PVA-sensitive ALL patients (P=0.042). No relation between apoptotic protease-activating factor 1 (Apaf-1), procaspases-3, -6, -7, -8, -10, and drug resistance was found. In conclusion, low baseline expression of PARP and procaspase-2 is related to cellular drug resistance in childhood acute lymphoblastic leukemia.

**INTRODUCTION**

The treatment of pediatric acute leukemia has greatly improved in the past 4 decades, resulting in long-term disease-free survival of approximately 80%[1] for acute lymphoblastic leukemia (ALL) and 60% for acute myeloid leukemia (AML).[2] Despite this progress, therapy resistance in a significant number of children still forms a major obstacle to successful treatment. We and others have previously shown that ALL patients whose leukemia cells exhibit

*in vitro* resistance to antileukemic agents have a significantly worse prognosis than patients whose leukemic cells are sensitive.[3-6] Compared with ALL, leukemic cells of children with AML have a less favorable prognosis and are in vitro more resistant to several antileukemic agents.[7] Apoptosis is the predominant form of cell death triggered *in vivo* and *in vitro* by chemotherapeutic agents in hematological malignancies.[8] There are 2 major routes by which apoptosis can be induced: the intrinsic and the extrinsic apoptosis pathways. The intrinsic pathway is initiated by mitochondrial damage that leads to release of cytochrome *c* from the mitochondrial intermembrane space.[9] Upon

entry in the cytoplasm, cytochrome *c* interacts with apoptotic protease-activating factor 1 (Apaf-1), deoxyadenosine triphosphate (dATP), and procaspase-9 to form a complex known as the apoptosome.[10] In the apoptosome, caspase-9 is activated, which in turn induces processing and activation of downstream effector caspases such as caspases-2, -3, -6, and -7.[11,12] Activation of the effector caspases results in the cleavage of a number of structural and regulatory cellular proteins (e.g., poly(ADPribose) polymerase [PARP; also known or PARP1 and ADPRT]) and lamins.[13,14] Initiation of the extrinsic apoptosis pathway involves ligand-induced aggregation of membrane receptors of the tumor necrosis factor receptor superfamily and subsequent cytoplasmic recruitment of Fas-associated protein with death domain (FADD), and procaspase-8 or procaspase-10 to form the death-inducing signaling complex (DISC).[15,16] Within the DISC procaspase-8 or procaspase-10 is activated and released back into the cytoplasm, where it induces activation of downstream effector caspases. Both pathways converge at the level of caspase-3 activation and therefore have several downstream effector caspases and substrates (e.g., PARP) in common.

Decreased apoptosis may be an important event in the acquisition of cellular drug resistance in pediatric acute leukemia.[17] Studies evaluating the expression levels of apoptotic proteins in clinical samples are limited in leukemia and mainly restricted to adult acute leukemia.[18-22] In the present study, we examined the expression of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and PARP in diagnostic samples containing at least 85% leukemic blasts of 43 children with ALL and 10 children with AML samples using quantitative Western blotting. The protein expression was compared between B-lineage ALL, T-lineage ALL, and AML, and between acute leukemia cases that are in vitro–sensitive and resistant to prednisolone (PRED), vincristine (VCR), L-asparaginase (ASP), and daunorubicine (DNR).

## MATERIAL AND METHODS

### Leukemia samples

Bone marrow and peripheral blood samples were obtained at initial diagnosis after informed consent from children with newly diagnosed ALL who were enrolled on protocol DCOG ALL-9 at the Erasmus MC/Sophia Children's Hospital or on COALL-97 treatment protocol at one of the hospitals participating in the German COALL study group. Approval was obtained from the Erasmus MC/Sophia Children's Hospital institutional review board for these studies. Mononuclear cells were isolated by sucrose

density gradient centrifugation (Lymphoprep, density 1.077 g/mL; Nycomed Pharma, Oslo, Norway), within 24 hours after sampling at the research laboratory of Pediatric Oncology, Erasmus MC/Sophia Children's Hospital, Rotterdam, the Netherlands. Cells were resuspended in culture medium consisting of RPMI 1640 (Dutch modification without L-glutamine; Gibco BRL, Life Technologies, Breda, the Netherlands) supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, the Netherlands), 2 mM L-glutamine, 200 µg/mL gentamycin (Gibco BRL) 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.125 µg/mL fungizone (Gibco BRL), and 5 µg/mL insulin, 5 µg/mL transferring, and 5 ng/mL sodium selenite (ITS media supplement; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands). If necessary, leukemic samples were further enriched to at least 85% leukemic blasts by removing nonmalignant cells with immunomagnetic beads (DynaBeads, Dynal Inc, Norway).[23] Cell pellets were immediately stored at -80°C until use.

**Antibodies**

Monoclonal mouse antibodies to human procaspase-2, PARP were purchased from PharMingen (San Diego, CA, USA). Polyclonal rabbit antibodies to human procaspase-6, procaspase-10, and a monoclonal mouse antibody to human procaspase-8 were purchased from Cell Signaling Technology (Beverly, MA). In addition, we used monoclonal mouse antibodies to human procaspase-3 (Transduction Laboratories, Lexington, KY, USA) and procaspase-7 (StressGen, Victoria, BC, Canada). Monoclonal antibodies tested for human procaspase-9 were purchased at Oncogene Research Products (Cambridge, MA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling Technology.

***In vitro drug* resistance assay**

Responsiveness of leukemia cells to PRED, VCR, ASP, and DNR was determined by the 4-day in vitro methyl thiazolyl tetrazolium (MTT) drug resistance assay as described previously.[3,4] The concentration ranges tested for these drugs were as follows: PRED, 0.008-250 µg/mL; VCR, 0.05-50 µg/mL; ASP, 0.003-10 IU/mL; and DNR, 0.002-2.0 µg/mL. The drug concentration lethal to 50% of the ALL cells ($LC_{50}$ value) was used as the measure of cellular drug resistance. The PVA score was calculated as previously described (ie, each patient was given a score according to the $LC_{50}$ value measured in the MTT assay).[4] Patients were given a score of 1 if sensitive, 2 if intermediate, and 3 if

resistant per agent. (The cut-off $LC_{50}$ values used to assign these scores were those previously associated with treatment outcome in ALL4,[24] and are provided in Table 1).

**Table 1. Cut-off LC50 values used to assign a patient as *in vitro* sensitive, intermediate, or resistant**

| Drug | *In vitro* sensitive | *In vitro* resistant |
|---|---|---|
| PRED (µg/ml) | ≤ 0.100 | ≥ 150 |
| VCR (µg/ml) | ≤ 0.391 | ≥ 1.758 |
| ASP (IU/ml) | ≤ 0.033 | ≥ 0.912 |
| DNR (µg/ml) | ≤ 0.075 | ≥ 0.114 |

The sum of the individual scores for PRED, VCR, and ASP resulted in the PVA score. Patients with a combined PVA score of 3 or 4 are relatively sensitive to PRED, VCR, and ASP, and have a favorable treatment outcome compared with patients with a combined PVA score of 7, 8, or 9, which are relatively resistant to these 3 drugs.

**Western blotting**

Leukemic cells were lysed in 50 mM Tris (pH 7.6) containing 150 mM NaCl, 10 mM EDTA, and 1% Triton X-100. Protease inhibitors (200 µg/mL pepstatin A, 200 µg/mL leupeptin and 2 mM PMSF were freshly added prior to use. Protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL, USA). All samples were kept on ice during the protein isolation and lysates were either used directly or stored immediately at -80°C until use. Twenty micrograms of protein in Laemmli buffer (4% (wt/vol) SDS, 100 mM Tris-HCl [pH 6.8], and 20% glycerol) was heated to 100°C for 3 minutes and loaded onto a 5%-15% gradient polyacrylamide gel. After electrophoresis, samples were transferred to nitrocellulose blots, blocked with skim milk, and incubated for 1 hour with primary antibodies. Blots were washed and incubated for 1 hour with peroxidase-conjugated secondary antibody (DAKO, Carpentaria, CA, USA). Presence of proteins was detected by enhanced chemiluminescent staining using the SuperSignal West Femto kit (Pierce). Signals were directly scanned by the ChemiGenius imaging system (SynGene, Cambridge, United Kingdom) and signal intensity was quantified using GeneTools 3.1 image analysis software (SynGene).

**Quantification of apoptotic proteins**

A dilution series of 30, 25, 20, 15, and 10 µg protein lysate of the human B-cell leukemia cell line Reh (N CRL-8286; ATCC, Rockville, MD, USA) was included in each

gel. Expression levels of protein were expressed in arbitrary units (AU) and were based on the relative quantity present in the patient's sample compared to the Reh cell line dilution series (Figure 1).

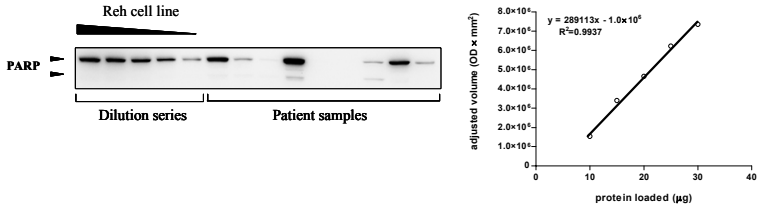


**Figure 1. Quantification of apoptotic proteins in newly diagnosed childhood acute leukemia.** A representative blot for cells of 9 acute leukemia samples incubated with monoclonal anti-PARP antibody is depicted in the top panel. The dilution series consists of 30, 25, 20, 15, and 10 μg Reh protein lysate. The presence of PARP was detected by enhanced chemiluminescent staining and the signal intensity was quantified by densitometry and plotted (bottom). This graph was used to estimate the relative quantity present in the patient's sample compared with the Reh cell line dilution series by linear regression. The 9 samples shown here are part of the group of 53 patients and include 3 B-lineage ALL samples, 3 T-lineage ALL samples, and 3 AML samples. OD indicates optical density.

Reproducibility of our method was assessed by subjecting 27 samples to duplicate Western blot analysis and is illustrated in Figure 2 for 16 representative samples.

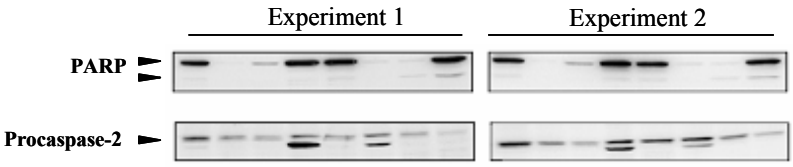


**Figure 2. Reproducibility of PARP and procaspase-2 protein detection in childhood acute leukemia samples.** Representative Western blots from 2 independent experiments incubated with monoclonal anti-PARP or anti–procaspase-2 antibodies are depicted.

### Real time quantitative PCR

Total cellular mRNA was extracted using Trizol reagent (Gibco BRL) and cDNA was synthesized using random hexamers and oligo dT primers. mRNA expression levels of *PARP* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a reference, were quantified using real-time polymerase chain reaction (PCR) analysis on a ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) as previously described.[25] PCR reactions were performed and optimized to an

amplification efficiency of more than 95%. The comparative cycle time (Ct) value of the target PCR was normalized by subtracting the Ct value of *GAPDH* (ΔCt). The ΔCt value was used to calculate the relative expression level to *GAPDH* for each target PCR using the following formula: relative mRNA expression = $2^{-\Delta Ct} \times 100\%$.[25] PARP primer sequences used were: sense, 5'-AGGCTGCTTTGTCAAGAA-3' and antisense: 5'-CTT GCTGCTTGTTGAAGAT-3' and the probe sequence: 5'-ATGAGGTGGATGGAGTGGA TGA-3'. *GAPDH* primers and probe sequences have been described elsewhere.[26]

**Fluorescence in situ hybridization analysis**

To investigate the PARP gene copy number, dual colored fluorescence in situ hybridization (FISH) was applied using 2 combinations of probes: (1) Two 1q42-specific bacterial artificial chromosome (BAC) clones (Roswell Park Cancer Institute, Buffalo, NY, USA), RP11-118H4 labeled with biotin-16-dUTP, and RP11-125A15 labeled with digoxigenin-12-dUTP, with a 50-kilobase (kb) overlap in the PARP gene; and (2) a combination of the PARP-specific BAC clone RP11-118H4 (labeled with biotin-16-dUTP) and 2 PAC clones 203H23 and 213H16 (RCPI-6 Human PAC Library, Roswell Park Cancer Institute) labeled with digoxigenin-12-dUTP specific for the AF1q gene on 1q21. Cytospins of leukemic samples stored at -20°C were used for FISH analysis. The FISH protocol was based on that described previously.[27] Briefly, slides were pretreated with RNase and pepsin and subsequently fixed with formaldehyde. Hybridization of 100 ng of each probe to the slides was performed overnight at 37°C. Biotinylated probes were detected via subsequent incubation with fluoroscein isothiocyanate (FITC)–labeled avidin-d, biotinylated goat-anti-avidin and avidin-d FITC (Vector, Burlingame, CA, USA), whereas digoxigenin-labeled probes were detected via sheep-anti-TRITC-labeled antibodies (Boehringer Mannheim, Mannheim, Germany), followed by Texas-red-conjugated donkey antisheep antibodies (Jackson ImmunoResearch, Westgrove, PA). Slides were counterstained with DAPI and embedded in Vectashield/DABCO. Hybridization results were examined using a Zeiss Axioplan 2 fluorescence microscope (Zeiss, Oberkochen, Germany).

**Statistics**

Protein expression in different leukemic subgroups (B-lineage ALL, T-lineage ALL, and AML) was compared using the Mann-Whitney *U* test. Spearman rank ($r_s$) correlation test was used to correlate protein expression to several variables on the study.

Statistical tests were performed at a 2-tailed significance level of 0.05. Whenever applicable, a Bonferroni correction was applied to correct for multiple comparisons.

## RESULTS

### Expression of various apoptotic proteins in ALL

The expression of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and PARP was first determined in a test group of 20 children with ALL. These 20 patients were selected by having a PVA score of 3 or 4 (i.e., relatively *in vitro* sensitive to PRED, VCR, and ASP; N=10) or 7, 8, or 9 (i.e., relatively in vitro–resistant to PRED, VCR, and ASP; N=10). The expression of procaspase-9 could not be analyzed in these samples since all procaspase-9 antibodies tested revealed abundant nonspecific staining, whereas staining of procaspase-9 itself was low. Most other apoptotic proteins were ubiquitously expressed in the ALL samples and varied markedly between patients (Figure 3).

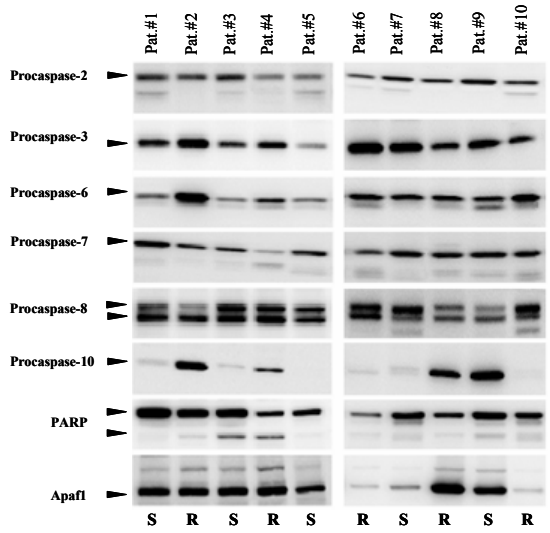


**Figure 3. Variation in expression of 8 apoptotic proteins in PRED, VCR, and ASP (PVA)–sensitive and –resistant childhood ALL.** Protein levels of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and PARP of leukemic cells from 10 patients compared by Western blot analysis. Arrows indicate the positions of full-length procaspase-2 (48 kDa), procaspase-3 (32 kDa), procaspase-6 (33 kDa), procaspase-7 (35 kDa), procaspase-8 (50/55 kDa), procaspase-10 (58 kDa), full-length and cleaved PARP (116/89 kDa), and Apaf-1 (140 kDa). The in vitro drug responsiveness of each patient is indicated below each lane; R indicates in vitro resistant and S indicates in vitro sensitive toward PRED, VCR, and ASP (PVA).

Exceptions were procaspase-10 and PARP, which were not expressed in 4 (2 B-lineage ALL, 2 T-lineage ALL) and 1 (T-lineage ALL) out of the tested 20 samples, respectively. Expression values in arbitrary units (A.U.) for each apoptotic protein are provided in Table 2.

**Table 2: Apoptosis-associated protein expression in PRED, VCR and ASP (PVA) sensitive and resistant childhood ALL patients.**

| Apoptotic protein[#] | PVA sensitive patients (N=10) median (range)[*] | PVA resistant patients (N=10) median (range)[*] | P-value |
|---|---|---|---|
| Apaf-1 | 21.6 (12.7-124.4) | 17.4 (4.0-175.3) | 0.529 |
| Procaspase-2 | 13.4 (1.1-27.2) | 6.2 (1.2-10.1) | 0.019 |
| Procaspase-3 | 18.5 (9.9-41.0) | 20.8 (8.0-35.7) | 0.912 |
| Procaspase-6 | 17.5 (11.0-78.8) | 25.2 (16.3-49.0) | 0.280 |
| Procaspase-7 | 10.8 (6.1-32.4) | 8.6 (1.0-25.2) | 0.247 |
| Procaspase-8 | 32.4 (21.5-75.2) | 42.3 (17.0-110.1) | 0.393 |
| Procaspase-10 | 5.4 (0.0-40.8) | 6.4 (0.0-47.0) | 0.912 |
| PARP | 35.8 (6.5-48.9) | 15.1 (0.0-38.5) | 0.042 |

[#]: the expression of the predominantly expressed isoform was quantitated for all proteins
[*]: In arbitrary units (expression of an apoptotic protein in the patient sample related to the expression of this protein in a dilution series of the Reh leukemic cell line), see materials and methods

The variation in expression ranged between 5-fold for procaspase-3 and 44-fold for Apaf-1. *In vitro* cross-resistance to PRED, VCR, and ASP (PVA) is an important independent predictor of treatment failure and long-term outcome in childhood ALL.[4,5,28] Therefore, the expression of the 8 apoptotic proteins was subsequently correlated to *in vitro* PVA resistance. Only procaspase-2 and PARP protein expression were significantly associated with *in vitro* PVA resistance in ALL. Procaspase-2 expression was 2.2-fold lower in cells of ALL patients resistant to PRED, VCR, and ASP, hereafter called PVA resistant (median: 6.2 A.U.) compared with cells of ALL patients sensitive to these 3 drugs, hereafter called PVA sensitive (median, 13.4 A.U.; P=0.019). PARP expression was 2.4-fold lower in PVA-resistant (median, 15.1 A.U.) compared with PVA-sensitive children with ALL (median, 35.8 A.U.; P=0.042). Based on these data, we examined the expression of PARP and procaspase-2 in a larger group of children with ALL (N=43) and AML (N=10).

**Expression of PARP in acute leukemia**

Twenty-eight patients with B-lineage ALL, 15 patients with T-lineage ALL, and 10 patients with AML were studied. PARP expression varied between 0 and 59.8 A.U. in these patients with the relatively highest expression level found in B-lineage ALL (Figure 4A).

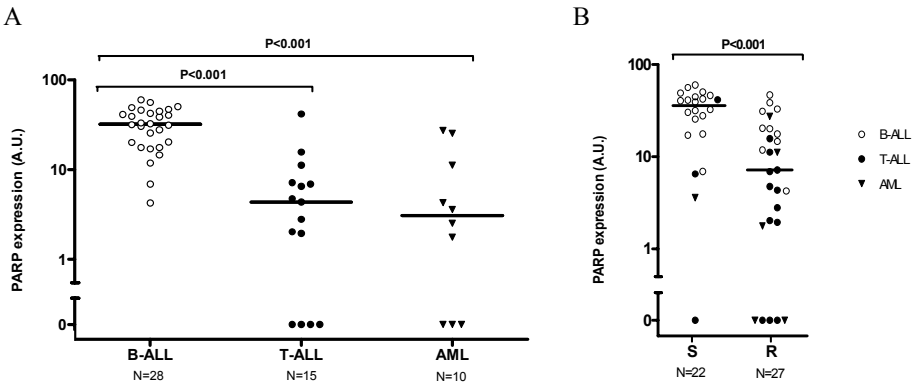


**Figure 4.** (A) PARP expression in different types of childhood acute leukemia. Each dot represents an individual patient and the horizontal bar represents the median protein level. Protein expression level of PARP was quantitated in 28 B-lineage ALL samples (○), 15 T-lineage ALL samples (●) and 10 AML samples (▼). (B) PARP expression versus in vitro PRED, VCR, and ASP (PVA) resistance in childhood acute lymphoblastic leukemia. The protein expression level of PARP quantitated in 21 PVA sensitive (S) and 22 PVA resistant (R) patients with B-lineage ALL (○) or T-lineage ALL (●). *Statistically significant after Bonferroni correction for multiple comparisons. A.U. indicates arbitrary units.

The expression of PARP was significantly lower in T-lineage ALL (median, 4.3 A.U.; P<0.001) and in AML (median, 3.1 A.U.; P<0.001) compared to B-lineage ALL (median, 32.0 A.U.). PARP expression did not differ significantly between AML and T-lineage ALL. Whereas all patients with B-lineage ALL showed PARP expression, we identified 4 T-ALL and 3 AML patients without detectable PARP expression (see below). PARP mRNA levels correlated with PARP protein levels ($r_s$=0.58, P=0.018). FISH analysis of the 4 patients with T-lineage ALL and 3 patients with AML with absent PARP protein expression revealed that the lack of PARP protein and mRNA expression was not associated with genomic deletions in the PARP gene region of these patients (data not shown). Epigenetic changes such as DNA methylation of promoter regions may result in decreased mRNA levels; however, no CpG islands have been reported within the promoter region of PARP, nor could we identify these using the CpGPlot program (http://www.ebi.ac.uk/emboss/cpgplot/).[29]

Next, we compared PARP expression in sensitive and resistant patients with acute leukemia to PRED, VCR, ASP, or DNR. After correction for multiple comparisons (Bonferroni model), we observed a significantly decreased PARP expression in samples resistant to PRED, VCR, and ASP (Table 3). PVA resistance data were available for 8 AML samples, 15 T-lineage ALL samples, and 28 B-lineage ALL samples; of these, 5 AML samples (63%) and 12 T-lineage ALL samples (80%) were PVA resistant, whereas only 10 B-lineage ALL samples (36%) were PVA resistant. This is in line with our previous findings that children with AML and T-lineage ALL are relatively *in vitro* resistant to various drugs including PRED, VCR, and ASP.[7,30]

**Table 3. PARP and procaspase-2 expression in childhood acute leukemia patients sensitive and resistant to PRED, VCR, ASP, and DNR.**

| | | PARP | | | Procaspase-2 | | |
|---|---|---|---|---|---|---|---|
| **Drug** | | **N** | **median (A.U.)** | **P-value** | **N** | **median (A.U.)** | **P-value** |
| **PRED** | sensitive | 20 | 39.8 | <0.001 | 15 | 24.7 | 0.012 |
| | resistant | 21 | 6.9 | | 19 | 8.8 | |
| **VCR** | sensitive | 24 | 31.0 | 0.002 | 19 | 22.0 | 0.142 |
| | resistant | 21 | 4.7 | | 18 | 9.3 | |
| **ASP** | sensitive | 21 | 31.7 | 0.004 | 18 | 23.3 | 0.070 |
| | resistant | 22 | 7.0 | | 19 | 9.5 | |
| **DNR** | sensitive | 30 | 31.0 | 0.017 | 24 | 12.8 | 0.188 |
| | resistant | 9 | 4.7 | | 8 | 7.3 | |

Since PRED, VCR, and ASP are an integral part of ALL therapy, PARP expression was compared between PVA-sensitive and PVA-resistant ALL samples. Within the total group of ALL cases, PVA-resistant cases (median, 39.2 A.U.) had a 4-fold lower expression of PARP compared with sensitive cases (median, 9.2 A.U., P<0.001). Our observation that PARP expression is lower in T-ALL and that this immunophenotype is associated with cellular drug resistance partly explains our finding that low PARP expression is associated with PVA resistance in pediatric ALL (Figure 4B). Within the B-lineage group, PVA resistant patients (N=10; median, 20.2 A.U.) also had a 2-fold lower expression of PARP compared with sensitive patients (N=18, median, 39.8, P=0.040; Figure 4B, open symbols). No difference was found in PARP expression between ALL patients with an M1 bone marrow response at day 15 and those having an M2 or M3 bone marrow response (P=0.11). Due to limited follow-up of patients no relevant comparisons with long-term outcome could be made.

**Expression of procaspase-2 in acute leukemia**

A slightly smaller group of 34 ALL cases (besides 10 AML) were analyzed for procaspase-2 expression due to limitations in availability of material. Procaspase-2 expression was detectable in all leukemic samples. As shown in Figure 5A, procaspase-2 expression varied between 2.1 and 65.4 A.U. in these patients, with the relatively highest expression level found in B-lineage ALL. The expression of procaspase-2 was 3-fold lower in T-lineage ALL (median, 7.0 A.U.; P=0.014) and in AML (median, 6.80 A.U.; P=0.022) compared with B-lineage ALL (median, 22.0 A.U.). Procaspase-2 expression did not differ significantly between AML and T-lineage ALL (P=0.976).

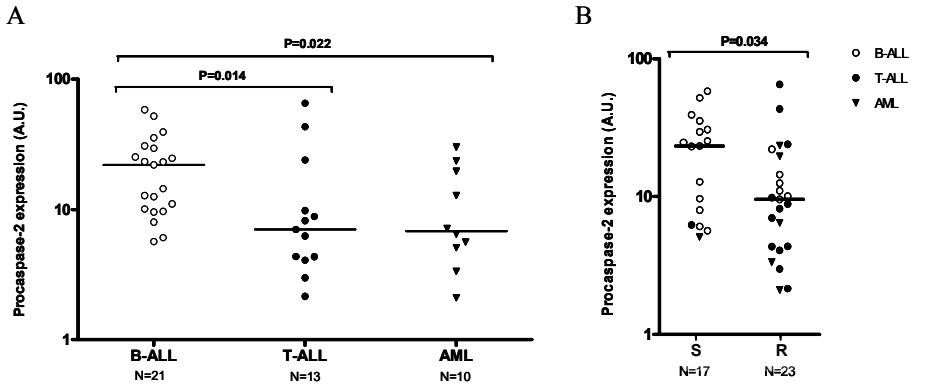


**Figure 5.** (A) Procaspase-2 expression in different types of childhood acute leukemia. Each dot represents an individual patient and the horizontal bar represents the median protein level. Protein expression level of procaspase-2 quantitated in 21 B-lineage ALL samples (○), 13 T-lineage ALL samples (●) and 10 AML samples (▼). (B) Procaspase-2 expression versus in vitro PRED, VCR, and ASP (PVA) resistance in childhood acute lymphoblastic leukemia. The protein expression level of PARP quantitated in 16 PVA sensitive (S) and 18 PVA resistant (R) patients with B-lineage ALL (○) or T-lineage ALL (●). *Statistically significant after Bonferroni correction for multiple comparisons.

Procaspase-2 expression was compared between *in vitro* sensitive and resistant patients for PRED, VCR, ASP, or DNR as single drugs. After correction for multiple comparisons, we observed that decreased procaspase-2 expression was related to *in vitro* PRED resistance only, although a trend with resistance to the other drugs was found (Table 3). Within the total group of ALL patients, PVA resistant cases (median, 9.7 A.U.) had a 2-fold lower expression of procaspase-2 compared with sensitive cases (median, 24.0 A.U., P=0.042; Figure 5B). Within the B-lineage group, PVA resistant patients (N=6; median, 11.8 A.U.) had a 2.1-fold lower expression of procaspase-2 compared with sensitive patients, although this difference was not

statistically significant (N=15, median, 24.7 A.U., P=0.132; Figure 5B, open symbols). Since the expression of procaspase-2 and PARP showed similar expression patterns, we examined the possibility whether the expression of both proteins was correlated. As shown in Figure 6 there is a strong positive correlation between the expression of PARP and procaspase-2 ($r_S$=0.58, P<0.001). Procaspase-2 expression did not differ between ALL patients with an M1 bone marrow response at day 15 and those having an M2 or M3 bone marrow response (P=0.30). Due to limited follow-up of patients no relevant comparisons with long-term outcome could be made.

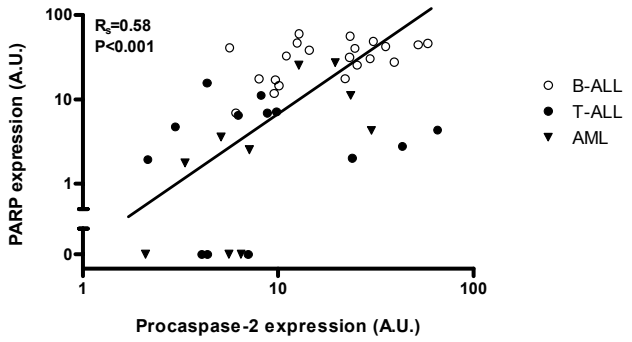


**Figure 6: Correlation between PARP and procaspase-2 protein levels in newly diagnosed childhood acute leukemia.** Comparison between PARP and procaspase-2 protein expression levels in 21 B-lineage ALL samples (○), 13 T-lineage ALL samples (●) and 10 AML samples (▼).

## DISCUSSION

The prognosis of childhood acute lymphoblastic leukemia (ALL) has improved remarkably over the past 4 decades due to the introduction of effective combination risk-adapted therapies. Conventional factors used to stratify patients are clinical and biological parameters such as age and white blood cell (WBC) count at diagnosis, immunophenotype, the presence of specific genetic abnormalities,[1] and initial response to PRED treatment.[31] Newer approaches are measurement of minimal residual disease after induction of initial remission[32] and *in vitro* drug resistance profiles.[4] We and others showed that children whose leukemic cells exhibit *in vitro* resistance to antileukemic agents, especially to PRED, VCR, and ASP, have a significantly worse prognosis than patients whose ALL cells are sensitive.[3-6] Children with T-lineage ALL and AML are, *in vitro,* more resistant to a variety of drugs.[7,30] The use of more intensive risk-adapted treatment regiments, however, has resulted in a prognosis for children with T-lineage

ALL similar to that of children with B-lineage ALL.[33] In the present study, the expression of Apaf-1, procaspases-2, -3, -6, -7, -8, -10, and PARP was studied in childhood ALL and AML and was related to immunophenotype and *in vitro* resistance to PRED, VCR, and ASP (PVA) as well as to resistance to PRED, VCR, and ASP and DNR individually.

We observed no relation between the expression of Apaf-1, procaspases-3, -6, -7, -8, and -10, and PVA resistance in childhood ALL in the present study. The absence of a relation with drug resistance may explain why no prognostic value was found for these proteins in adult ALL and AML,[22] although data in children and adults are not necessarily interchangeable. In a previous study we demonstrated that the degree of caspase-3 activation (ie, cleavage of procaspase-3 into caspase-3) correlates with the degree of resistance to PRED and ASP in childhood ALL.[17] In our present study no correlation was found between the baseline expression levels of procaspase-3 and resistance to these chemotherapeutic drugs. Taken together, these studies suggest that resistance to these 2 drugs may not be caused by decreased availability of the active enzyme due to decreased expression of procaspase-3, but rather may be caused by defects upstream of caspase-3 that inhibit caspase-3 activation upon PRED or ASP exposure.

PARP (EC 2.4.2.30) is an abundantly expressed nuclear enzyme, which binds to single- or double-stranded DNA breaks in response to DNA damage. At the breakage site, PARP catalyzes the transfer of the ADP-ribose polymers from the respiratory coenzyme $NAD^+$ to nuclear acceptor proteins involved in chromatin structure, DNA repair, and DNA metabolism.[34] Its poly(ADP ribosylation) activity as well as its association with components of base-excision repair[35] contribute to the role of PARP in DNA repair and the maintenance of genomic integrity. In the present study, we observed that *in vitro* drug resistant childhood leukemia subtypes (ie, T-lineage ALL and AML), have a decreased and occasionally even absent expression of PARP. Complete absence of PARP expression was observed in 4 out of 15 patients with T-lineage ALL and 3 out of 10 patients with AML, but in none of the patients with B-lineage ALL. The variation in PARP expression between leukemia subtypes suggests that caution should be taken when interpreting studies that examining protein expression in a mixed group of B-lineage ALL, T-lineage ALL, and AML samples without correcting for leukemia subtypes. The absence of PARP was not caused by deletions of the PARP gene. Given the absence of CpG islands within the PARP promoter, it seems unlikely that the absence of PARP is caused by methylation-induced PARP silencing in these patients. The presence of putative binding sites for several

transcription factors, including simian virus 40 protein 1 (Sp1),[36,37] activator protein-2 (AP-2),[37] v-ets erythroblastosis virus E26 oncogene homolog 1 ( Ets-1),[38] and yin-yang 1 (YY1)[39] within the PARP promoter region, suggests that PARP expression is regulated at the transcriptional level. In addition, we observed that decreased or absent expression of intact PARP is related to *in vitro* resistance to PRED, VCR, and ASP in childhood ALL. Other studies also reported that depletion of PARP, either by gene disruption,[40,41] antisense RNA,[40,42] or pharmacologic inhibitors,[40,43-45] resulted in decreased drug-induced apoptosis. Various explanations have been proposed for the requirement of PARP during apoptosis, including depletion of cellular $NAD^+$ and ATP pools,[46] modification of proteins involved in apoptosis like p53,[47,48] facilitation of oligonucleosomal DNA fragmentation,[49] and up-regulation of P-glycoprotein in PARP-depleted cells.[41] We previously observed that, in resistance to PRED and ASP, but not to VCR and DNR, is linked to decreased drug-induced PARP cleavage (i.e. inactivation of PARP).[17] Our current observation that resistant cells express lower baseline levels of this protein suggests that the suitability to use cleaved PARP as read-out for functional apoptosis is drug/stimuli dependent.

Caspase-2 activity is implicated in the initiation[50,51] as well as the effector phase[52,53] of apoptosis induced by various stimuli. The present study shows a decreased procaspase-2 expression in *in vitro* drug resistant childhood leukemia subtypes (i.e., T-ALL and AML). In addition, we observed a relation between decreased procaspase-2 expression and in vitro PVA resistance in childhood ALL. This suggests that sufficient intracellular amounts of procaspase- 2 are required to respond to these drugs, and that relative deficits in procaspase-2 expression levels may contribute to cellular PVA resistance in childhood acute leukemia. In adult acute leukemia, high expression of procaspase-2 was not linked to prognosis.[19-22] The lack of prognostic value for the response to combination chemotherapy in these studies does not rule out a role for procaspase-2 in cellular responses to PRED and/or other drugs in adult leukemias. Since this relationship has not been addressed in adults so far, no comparison between childhood and adult studies can be made. In cell lines the requirement of caspase-2 for drug-induced apoptosis was shown to be highly cell specific.[51,54,55]

Decreased PARP and procaspase-2 expression did correlate with *in vitro* PVA resistance, whereas these expression levels did not correlate with an early bone marrow response at day 15. One may argue that this is exactly the time period when the leukemia is treated with PRED, VCR, and ASP in most protocols. However, in the

COALL-97 protocol, patients are first treated with a DNR window (up-front combination chemotherapy) for 7 days, followed by PRED, VCR, and DNR for 4 weeks. After this period of 5 weeks, ASP is given for the first time. Since PARP and procaspase-2 expression are not linked to DNR resistance, and this drug is used up-front of combination chemotherapy as well as in the first 4 weeks, this may explain the lack of correlation between both proteins and day-15 bone marrow response of COALL-97 patients.

In our previous study we observed that resistance to each of the 4 studied drugs (PRED, VCR, ASP, and DNR) was associated with a decreased ability to expose phosphatidyl serine on the outer membrane and decreased collapse of the mitochondrial membrane potential.[17] Caspase-3 activation and PARP inactivation correlated to PRED and ASP resistance, but not to VCR and DNR resistance. This finding suggested that cleaved caspase-3 and PARP are not important determinants of the VCR- and DNR-triggered apoptotic route. Both phosphatidyl serine exposure and the collapse of the mitochondrial membrane potential occurred earlier than caspase-3 activation and PARP inactivation, implying that resistance to these 4 drugs is caused by a blockade at the level of or upstream of mitochondrial function. The identity of these upstream causes of resistance are part of extensive research in the past few years by different groups (reviewed by Pieters and Den Boer).[56]

Recently, new insights were obtained by comparing gene expression profiles of drug-resistant and -sensitive cells of children with ALL.[24] In this latter study, drug-specific genes rather than more general apoptosis-associated genes (such as caspases, Bcl-2 family members, and inhibitors of apoptosis [IAP] factors) were among the most discriminative genes that were associated with resistance to PRED, VCR, ASP, and DNR in ALL cells. These findings do not rule out a role for altered expression and/or activity f apoptosis genes, but suggest that, at the mRNA level, genes upstream of the apoptosis-execution pathway are more important or resistance to these drugs. At the protein level, we here show that the basal expression levels of Apaf-1 and procaspase-3, -6, -7, -8, and -10 are not differentially expressed between drug-resistant and -sensitive patients with acute leukemia. Expression of procaspase-2 and PARP were decreased in children with drug-resistant ALL. The causal aspect of this association should be further determined with functional studies showing the sensitizing effect of ectopic expression of PARP and procaspase-2 on drug resistance in leukemic cells of children with ALL.

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Chapter 6

# Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment.

Amy Holleman[1], Meyling H. Cheok[2], Monique L. den Boer[1], Wenjian Yang[2,5], Anjo J.P. Veerman[7], Karin M. Kazemier[1], Deqing Pei[4], Cheng Cheng[4], Ching-Hon Pui[3,5,6], Mary V. Relling[2,5,6], Gritta E. Janka-Schaub[8,9], Rob Pieters[1], William E. Evans.[2,5,6]

*[1] Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Department of Pediatric Oncology and Hematology, Rotterdam, the Netherlands, Departments of [2]Pharmaceutical Sciences, [3]Hematology-Oncology and [4]Biostatistics, St. Jude Children's Research Hospital, Memphis, TN, USA, [5]The Pharmacogenetics of Anticancer Agents Research Group in the Pharmacogenetics Research Network, Memphis, Tennessee, USA, [6]University of Tennessee Colleges of Pharmacy and Medicine, Memphis, Tennessee, USA, [7]VU Medical Center, Department of Pediatric Hematology Oncology, Amsterdam, the Netherlands, [8]University Children's Hospital, Hamburg, Germany. [9]COALL study group*

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**ABSTRACT**

Childhood acute lymphoblastic leukemia (ALL) is curable with chemotherapy in approximately 80 percent of patients. However, the cause of treatment failure in the remaining 20 percent of patients is largely unknown. We tested leukemia cells from 173 children for sensitivity *in vitro* to prednisolone, vincristine, asparaginase, and daunorubicin. The cells were then subjected to an assessment of gene expression with the use of 14,500 probe sets to identify differentially expressed genes in drug-sensitive and drug-resistant ALL. Gene-expression patterns that differed according to sensitivity or resistance to the four drugs were compared with treatment outcome in the original 173 patients and an independent cohort of 98 children treated with the same drugs at another institution. We identified sets of differentially expressed genes in B-lineage ALL that were sensitive or resistant to prednisolone (33 genes), vincristine (40 genes), asparaginase (35 genes), or daunorubicin (20 genes). A combined gene-expression score of resistance to the four drugs, as compared with sensitivity to the four, was significantly and independently related to treatment outcome in a multivariate analysis (hazard ratio for relapse, 3.0; P=0.027). Results were confirmed in an independent population of patients treated with the same medications (hazard ratio for relapse, 11.85; P=0.019). Of the 124 genes identified, 121 have not previously been associated with resistance to the four drugs we tested. In conclusion, differential expression of a relatively small number of genes is associated with drug resistance and treatment outcome in childhood ALL.

**INTRODUCTION**

Improvements in the treatment of childhood acute lymphoblastic leukemia (ALL) over the past four decades have resulted in rates of long-term disease-free survival of approximately 80 percent.[1,2] We have shown that children whose ALL cells exhibit *in vitro* resistance to antileukemic agents have a substantially worse prognosis than children whose ALL cells are drug-sensitive.[3-5] However, little is known about the genetic basis of resistance to chemotherapy. Multidrug-resistance genes[6] and genes involved in cell-cycle progression,[7,8] DNA repair,[9] drug metabolism,[9-11] and apoptosis[12] have been associated with the prognosis of ALL, but their role in determining the sensitivity of ALL cells to individual antileukemic agents is not known. Gene products arising from rearrangements of the *TEL-AML1,*[13] *BCR-ABL,*[14] and *MLL*[15] genes are also associated with prognosis and drug resistance, but for unknown reasons, many patients with a favorable genetic subtype (e.g., *TEL-AML1*) are not cured, whereas

many with an unfavorable subtype (e.g., certain *MLL* rearrangements) are cured. Although it is likely that multiple pathways and genes contribute to the sensitivity of ALL cells to specific agents,[16-18] all studies to date have focused on a small number of candidate genes instead of taking advantage of the genomic survey that is possible with the use of gene-expression profiling. Such profiles have been used successfully to investigate drug resistance in cancer cell lines[19,20] and human tumor xenografts,[21] but not in primary cancer cells.

Gene-expression profiles can differentiate lineage (T cell or B cell) and molecular subtypes of ALL[22-25] and identify treatment-specific changes in gene expression in ALL cells.[23] However, it is not known whether gene-expression profiles of leukemia cells are associated with resistance to individual drugs. The present study was undertaken to identify genes that are differentially expressed in primary ALL cells exhibiting resistance or sensitivity to prednisolone, vincristine, asparaginase, or daunorubicin and to determine whether the expression of such genes influences the response to treatment.

## MATERIAL AND METHODS

### Patients

The study population consisted of 271 children with newly diagnosed ALL: 173 were enrolled as part of the 9th ALL Dutch Childhood Oncology Group protocol at Erasmus Medical Center, Sophia Children's Hospital, in Rotterdam or treatment protocols 92 and 97 of the German Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia in Hamburg, and 98 were enrolled as part of the Total Therapy protocols XIIIA and XIIIB of St. Jude Children's Research Hospital in Memphis, Tennessee, USA[26,27] Patients were enrolled in the German protocol from 1992 to 2003, in the Dutch protocol from 1997 to 2004, and in the St. Jude protocols from 1991 to 1998. The original gene-profiling population consisted of the 173 children in the Dutch and German protocols, and the independent-validation population consisted of the 98 patients in the St. Jude protocols. The parents or guardians of the patients provided written informed consent, and the patients provided assent.

**Isolation of leukemia cells**

Bone marrow and peripheral blood were obtained before treatment, and mononuclear cells were isolated by means of sucrose density-gradient centrifugation (density, 1.077 g per milliliter; Lymphoprep, Nycomed Pharma) within 24 hours. Cells were resuspended in RPMI 1640 medium (GIBCO BRL) supplemented with 20 percent fetal-calf serum (Integro), 2 mM L-glutamine, 200 µg of gentamycin per milliliter (GIBCO BRL), 100 IU of penicillin per milliliter, 100 µg of streptomycin per milliliter, 0.125 µg of fungizone per milliliter (GIBCO BRL), 5 µg of insulin per milliliter, 5 µg of transferrin per milliliter, and 5 ng of sodium selenite per milliliter (ITS media supplement, Sigma-Aldrich Chemie). If necessary, ALL samples were further enriched to achieve more than 90 percent blasts by removing nonmalignant cells with the use of immunomagnetic beads (DynaBeads).

**Drug-resistance assay**

The sensitivity of leukemia cells to prednisolone (Bufa Pharmaceutical Products), vincristine (TEVA Pharma), asparaginase (Paronal, Christiaens), and daunorubicin (Cerubidine, Rhône-Poulenc Rorer) was determined with the use of the four-day *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT) drug-resistance assay.[3] The following concentrations of each drug were tested: 0.008 to 250 µg of prednisolone per milliliter, 0.05 to 50 µg of vincristine per milliliter, 0.003 to 10 IU of asparaginase per milliliter, and 0.002 to 2.0 µg of daunorubicin per milliliter. The drug concentration lethal to 50 percent of the leukemia cells (the $LC_{50}$ value) was used as the measure of drug resistance. The $LC_{50}$ values used to define cells as sensitive or resistant to each agent were those previously associated with a good or bad treatment outcome in patients with ALL (see Table 1 in the Supplementary Appendix, available with the full text of this article at www.nejm.org). [3-5]

**Purification, labeling, and hybridization of RNA**

Total cellular RNA was extracted from a minimum of $5 \times 10^6$ leukemia cells with the use of Trizol reagent (GIBCO BRL), RNA was additionally purified with phenol–chloroform–isoamylalcohol (25:24:1), and RNA integrity was assessed as previously described.[23,24] RNA processing and hybridization to the U133A GeneChip oligonucleotide microarray (Affymetrix) were performed according to the manufacturer's protocol.

**Statistical analysis**

Gene-expression values were calculated with the use of Affymetrix Microarray Suite version 5.0.2.[3,24] Expression signals were scaled to the target intensity of 2500 and log-transformed. Arrays were omitted if the scaling factor exceeded 3 SD of the mean or if the ratio of 3' to 5' messenger RNA for β-actin or glyceraldehyde-3-phosphate dehydrogenase was greater than 3. From the total of 22,283 probe sets, those expressed in fewer than five patients were omitted, leaving 14,550 probe sets for subsequent analyses.

For each antileukemic agent, we identified genes that were most discriminative for resistance and sensitivity using the Wilcoxon rank-sum test and t-test for each probe set and estimated the false discovery rate using the q value according to Storey and Tibshirani.[28,29] At the selected P value (alpha) for ranked discriminating genes (e.g., $P<0.001$), the overall significance of the estimated false discovery rate was computed as the probability of observing equal or lower false discovery rates on the basis of 1000 random permutations.

To assess the predictive accuracy using the top 30, 50, and 100 discriminating genes for drug sensitivity as compared with drug resistance, for each drug, we randomly divided the patients with drug-sensitive leukemic cells and the patients with drug-resistant leukemic cells into two groups, using two thirds to build the model and one third to assess the accuracy of the model. This process was repeated 1000 times; in each case we reselected a fixed number of probe sets to build a prediction model using support vector machines. Predictive accuracies of the various gene-expression profiles with respect to the sensitivity of each antileukemic agent and their confidence intervals were computed with the use of data from the 173 Dutch and German patients.

In the outcome analysis, we computed drug-resistance gene-expression scores for the 173 Dutch and German patients in the original population and the 98 St. Jude patients[25] in the validation population on the basis of the 172 gene-probe sets that discriminated between leukemic cells that were sensitive and those that were resistant to each of the four drugs. The scores were computed with the use of bagging algorithms.[30] For each of the four drugs, we assigned each patient a score of 1 if the cells were predicted to be sensitive and 2 if the cells were predicted to be resistant. After 1000 iterations, the average scores for each of the four drugs for each patient were combined as the final drug-resistance gene-expression score and used in the outcome analysis. For the analysis of disease-free survival, any type of leukemia relapse was considered. The duration of disease-free survival was defined as the time

from diagnosis until the date of treatment failure. Data were censored at the time of the last follow-up visit in the absence of treatment failure. Cox proportional-hazards regression analysis was used to assess the association between the combined gene-expression score and treatment outcome. Leukemia-free survival was analyzed with the use of Fine and Gray's estimator accounting for competing events.[30]

We used Fisher's exact test to determine the degree of overrepresentation or underrepresentation of discriminating genes in specific functional groups as compared with the genes on the U133A GeneChip, using the Gene Ontology database (http://www.geneontology.org/). Probe sets with the same gene symbol were counted as one. Primary data are available through the GeneExpression Omnibus of the National Center for Biotechnology Information at http://www.ncbi.nlm.nih.gov/geo/ (Platform, GPL91; Sample, GSM9653 to 9934; Series, GSE635 to 660). Additional information concerning the methods used is available at http://www.stjuderesearch.org/data/ALL4/index.html at http://www2.eur.nl/fgg/kgk/, and in the Supplementary Appendix.

## RESULTS

Gene expression was determined in ALL cells from 173 patients with newly diagnosed disease whose leukemia cells were either sensitive or resistant to prednisolone, vincristine, asparaginase, or daunorubicin, as assessed by the *in vitro* MTT assay. The distribution of $LC_{50}$ values (the drug concentration lethal to half the cultured lymphoblasts) in our study population did not differ significantly from that of the entire population of approximately 700 patients for whom we had previously determined the sensitivity status to each of these antileukemic agents (Figure 1). Likewise, the proportion of patients classified as having sensitive or resistant leukemia cells, according to previously defined $LC_{50}$ values (Table 1 in the Supplementary Appendix)[3-5] did not differ significantly between the study group and the entire population (Figure 1). The leukocyte counts, age at diagnosis, proportions of girls and boys, and immunophenotypes in the drug-sensitive and drug-resistant groups for each antileukemic agent are summarized in Table 2 in the Supplementary Appendix.

### Prediction of sensitivity and resistance with the use of differentially expressed genes

Unsupervised hierarchical clustering, which groups patients according to the predominant similarities in gene expression, did not cluster patients according to their resistance to any of the four antileukemic agents. Rather, patients were clustered predominantly according to immunophenotype or ALL genetic subtype (Figure 1 in the Supplementary Appendix).[24] Because cases of T-lineage ALL have a strong gene-expression signature, subsequent analyses were performed with the use of all samples or only the samples of B-lineage ALL (Table 2 in the Supplementary Appendix). At 28, the number of cases of T-lineage ALL was too small for a separate analysis. The false discovery rate was higher for daunorubicin than for the other three drugs. For all drugs, the false discovery rates were lower in the B-lineage ALL group than in the total group and highest for daunorubicin (Table 3 in the Supplementary Appendix). Using the top 30, 50, and 100 discriminating genes for each drug yielded predictive accuracies of 67 to 73 percent. For B-lineage ALL, the estimated predictive accuracies were higher, ranging from 71 to 76 percent (Table 5 in the Supplementary Appendix).

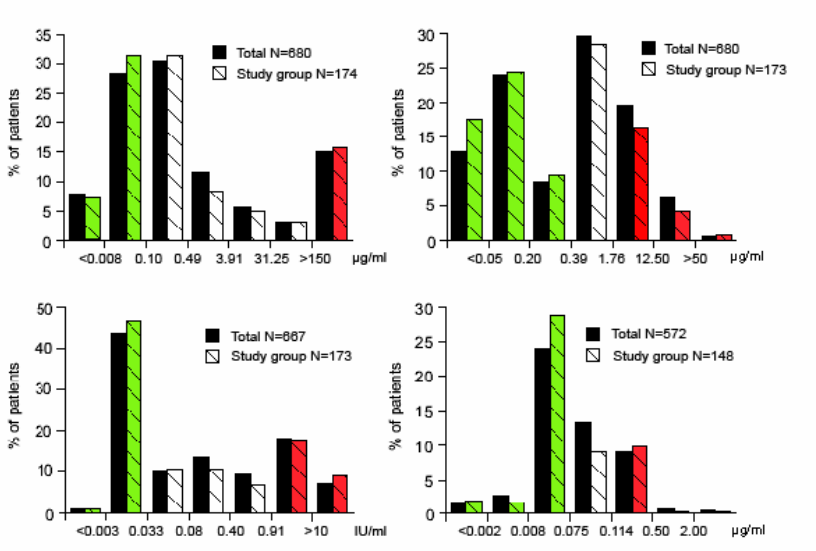


**Figure 1. Distribution of the Drug Concentrations Lethal to 50 Percent of Primary Leukemia Cells ($LC_{50}$) in the Study Group and in the Larger Population of Children with ALL.** The study group comprised 173 patients whose leukemia-cell samples were selected for gene-expression analysis from the total group of approximately 700 patients whose ALL blasts had been assessed at diagnosis for sensitivity to a panel of four antileukemic agents. The distribution of $LC_{50}$ values between the study group and the corresponding total group did not differ significantly for any of the drugs: P=0.89 for prednisolone, P=0.63 for vincristine, P=0.89 for asparaginase, and P=0.22 for daunorubicin (by the chi-square test).

### Supervised clustering and principal-component analysis

The number of genes used to build drug-resistance models for each antileukemic agent was based on the false discovery rate and predictive accuracy (Tables 3, 4, and 5 in the Supplementary Appendix). This determination resulted in 172 probe sets corresponding to 124 unique genes and 28 complementary DNA clones (some genes are represented on the array by multiple probe sets) that were differentially expressed in sensitive and resistant B-lineage ALL. This included 42 gene-probe sets for prednisolone, 59 for vincristine, 54 for asparaginase, and 22 for daunorubicin. Hierarchical clustering with the use of these probe sets correctly assigned the drug-sensitivity status (as sensitive or resistant) of 66 of 74 cases with respect to prednisolone, 84 of 104 with respect to vincristine, 83 of 106 with respect to asparaginase, and 86 of 105 with respect to daunorubicin (Figure 2) (Table 4 in the Supplementary Appendix). Similarly, principal-component analyses correctly grouped samples from most patients into the resistant or sensitive cluster for each of the four antileukemic agents (Figure 2). Hierarchical clustering and principal-component

analyses involving all 173 patients gave similar results (Figure 3 and 4 in the Supplementary Appendix). The probe-set identification, gene names, annotations, and the gene-expression ratio in resistant as compared with sensitive leukemia cells for discriminating genes are shown for each drug in Figures 5, 6, 7, and 8 (B-lineage ALL) and 9, 10, 11, and 12 (B-lineage and T-lineage ALL combined) in the Supplementary Appendix.

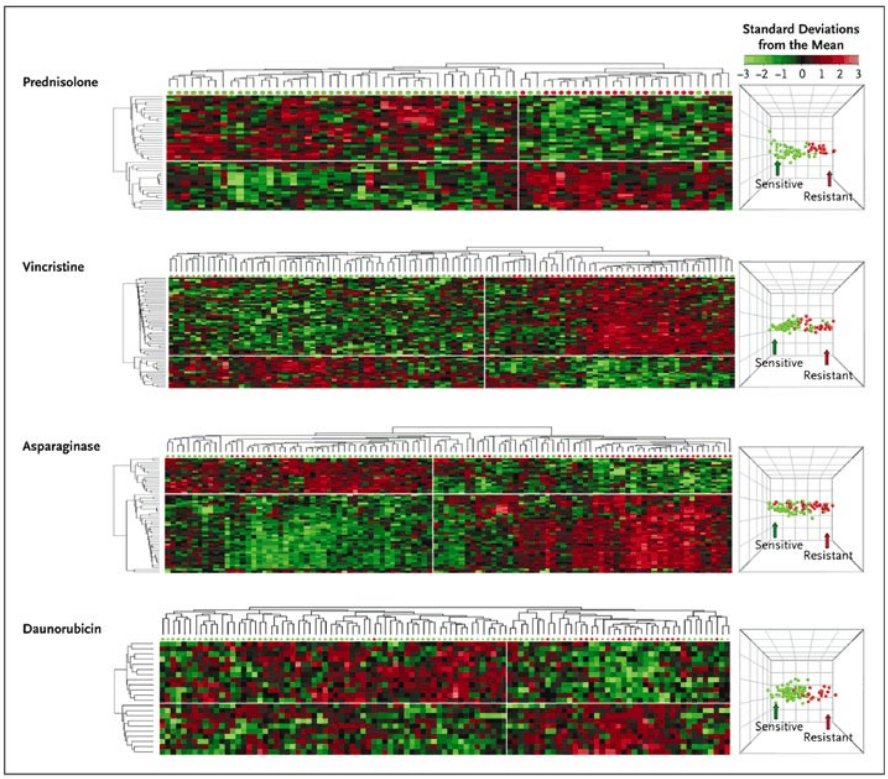


**Figure 2. Results of Supervised Hierarchical-Clustering and Principal-Component Analyses with the Use of Genes That Discriminate between Drug-Resistant and Drug-Sensitive B-Lineage ALL with Respect to Prednisolone, Vincristine, Asparaginase, and Daunorubicin.** The Wilcoxon rank-sum test and t-test were used to identify genes that were differentially expressed in sensitive and resistant ALL ($P<0.001$). Each column represents an ALL sample, labeled according to whether it was sensitive (green) or resistant (red) to a given drug, and each row represents a probe set. The "heat" maps on the left side of the figure indicate a high (red) or a low (green) level of expression relative to the number of standard deviations from the mean. The three-dimensional plots on the right show three principal components based on the significant discriminating genes for each drug. Each circle represents a patient with leukemia; red circles indicate those with drug-resistant ALL, and green circles those with drug-sensitive ALL.

**Resistance genes, combined gene-expression scores, and treatment outcome**

For the 173 patients treated according to the Dutch and German protocols, the median follow-up was 4.2 years; 132 patients remained in continuous complete remission, 40 patients relapsed, and 1 patient had a second cancer, at which time data on this patient were censored. A high combined gene-expression score indicative of resistance to the four drugs was associated with a significantly increased risk of relapse (P=0.001) (Figure 3A). The combined drug-resistance gene-expression score also predicted the outcome of treatment in a multivariate analysis that included the patient's age, ALL genetic subtype, ALL lineage, and leukocyte count at diagnosis (hazard ratio for relapse with a high score as compared with a low score, 3.0; P=0.027) (Table 1).

**Table 1. Multivariate proportional-hazards analysis of the risk of relapse.**

| | Study cohort (N=173) | | | | Validation cohort (N=98) | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **N** | **HR** | **95% CI** | **P-value** | **N** | **HR** | **95% CI** | **P-value** |
| **Age** | | | | | | | | |
| 1-10 years | 126 | 1.0* | | | 62 | 1.0* | | |
| <10 years | 0 | | | | 7 | 6.48 | 0.60-69.79 | 0.12 |
| >10 years | 47 | 1.37 | 0.68-2.75 | 0.37 | 29 | 7.61 | 1.23-46.95 | *0.029* |
| **White-cell count** | | | | | | | | |
| <10/nL | 36 | 1.0* | | | 25 | 1.0* | | |
| 10-49/nL | 68 | 0.84 | 0.30-2.34 | 0.74 | 27 | 1.93 | 0.24-15.15 | 0.53 |
| 50-100/nL | 28 | 0.97 | 0.28-3.35 | 0.96 | 20 | 12.02 | 1.30-110.79 | *0.028* |
| >100/nL | 39 | 2.74 | 0.92-8.12 | 0.07 | 26 | 8.35 | 0.84-83.11 | 0.07 |
| **ALL subtype** | | | | | | | | |
| B-other | 46 | 1.0* | | | 71 | 1.0* | | |
| *BCR-ABL* | 5 | 2.08 | 0.56-7.63 | 0.27 | 9 | 8.99 | 2.33-34.7 | *0.002* |
| *E2A-PBX1* | 8 | 1.75 | 0.47-6.52 | 0.40 | 14 | 0.53 | 0.07-4.17 | 0.69 |
| Hyperdiploid[#] | 3 | 13.63 | 2.98-62.27 | *0.001* | 51 | 0.75 | 0.26-2.18 | 0.81 |
| *MLL-AF4* | 43 | 0.13 | 0.03-0.58 | *0.007* | 5 | 3.92 | 0.59-26.2 | 0.81 |
| *TEL-AML1* | 40 | 0.34 | 0.09-1.24 | 0.10 | 71 | 0.83 | 0.20-3.35 | 0.94 |
| T-lineage | 28 | 0.63 | 0.24-1.66 | 0.35 | 36 | 3.40 | 1.25-9.28 | 0.15 |
| **Combined drug-resistance gene-expression score** | | | | | | | | |
| Sensitive (<4.7) | 60 | 1.0* | | | 29 | 1.0* | | |
| Interm.(4.7-5.6) | 56 | 2.58 | 0.97-6.87 | 0.07 | 48 | 4.00 | 0.58-27.73 | 0.16 |
| Resistant (>5.6) | 57 | 3.00 | 1.13-7.96 | *0.027* | 21 | 11.85 | 1.51-93.12 | *0.019* |

N: number of patients, HR: hazard ratio, CI: confidence interval, *: this group served as the reference group to calculate the ratio, [#]: Cytogenetic analysis revealed more than 50 chromosomes, [£]: COALL: *MLL* and *E2A* rearranged, St. Jude: *MLL-AF4* and *E2A-PBX1*

The combined gene-expression score was tested in an independent cohort of 98 U.S. patients who had been treated with these four drugs, but according to a different protocol. The median follow-up of these patients was 7.0 years; 17 patients relapsed, 9 had competing events (7 had second cancers, and remission failed in 2), and 72 remained in continuous complete remission. As in the training set, a high combined drug-resistance gene-expression score was associated with a significantly increased risk of relapse (P=0.003) (Figure 3B). When the patient's age, genetic subtype of ALL, ALL lineage, and leukocyte count at diagnosis were included in a multivariate analysis,

a high combined drug-resistance gene-expression score was independently associated with a higher probability of relapse than was a low score (hazard ratio, 11.85; P=0.019) (Table 1).

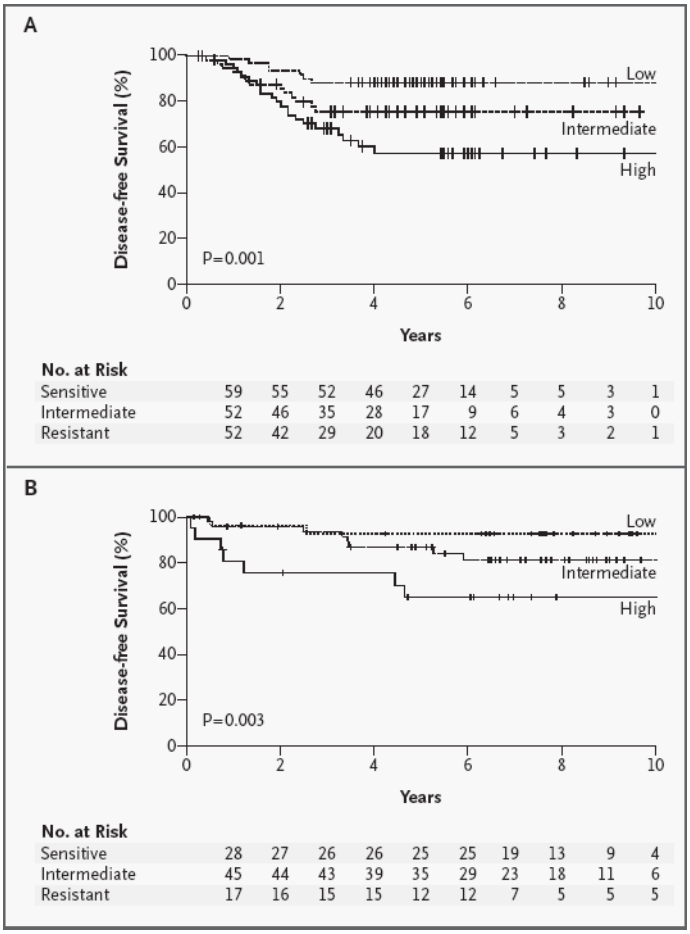


**Figure 3. Kaplan–Meier Estimates of Disease-free Survival among 173 Patients in the Original Study Group (Panel A) and 98 Patients in the Validation Cohort (Panel B), According to Whether the Pattern of Gene Expression Indicated Cellular Resistance or Sensitivity to the Four Antileukemic Agents.** In each panel, patients are grouped according to their combined drug-resistance gene-expression scores for 172 probe sets for prednisolone, vincristine, L-asparaginase, and daunorubicin. The 33 percent with the lowest score (indicating sensitivity), the 33 percent with an intermediate score (indicating an intermediate level of resistance), and the 33 percent with the highest score (indicating resistance) are shown.

**Ontology classification of discriminating genes**

Genes that could be used to identify B-lineage ALL that was resistant to each antileukemic agent were grouped into functional categories according to the Gene Ontology database (Figure 4).

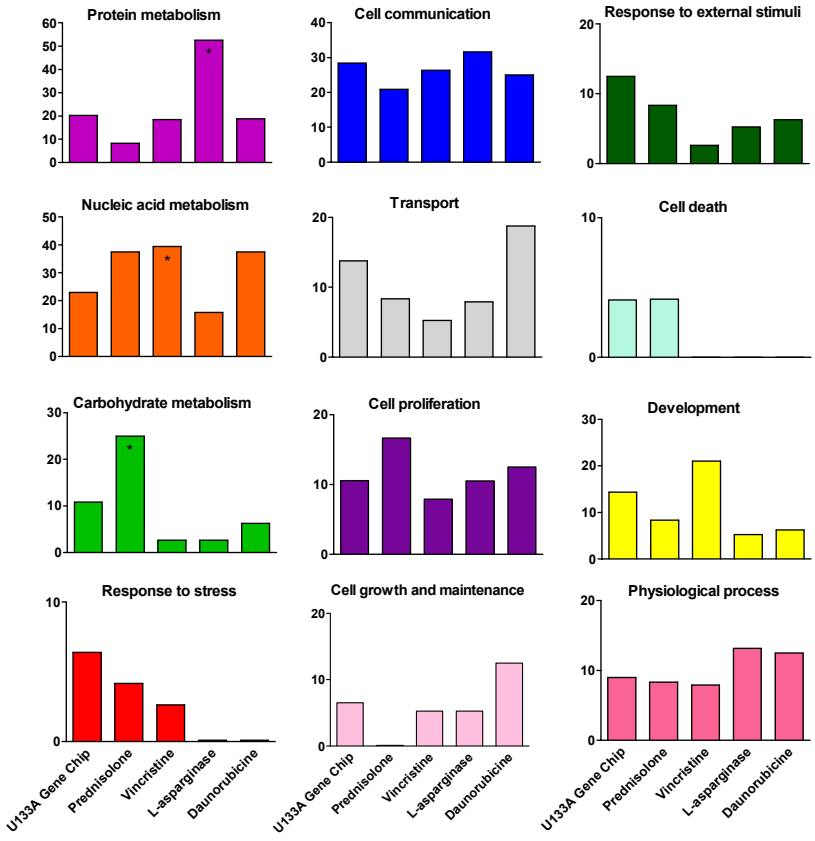


**Figure 4. Gene ontology (GO) functional classification of genes that discriminated between drug-sensitive and drug-resistant B-lineage ALL.** The functional GO classification of genes identified as discriminating B-lineage ALL cells that are resistant to each of the antileukemic agents, as compared with the entire genome as represented by all probe sets on the U133A GeneChip (222,283 probe sets, 12,893 with GO annotation). Functional categories that are overrepresented in the probe sets, as compared with the entire genome, are indicated by an asterisk (P<0.05 by Fisher's exact test).

As compared with the entire array, the 42 gene-probe sets related to prednisolone sensitivity had a higher percentage of genes involved in carbohydrate metabolism (25 percent vs. 11 percent, P=0.039). As compared with the entire array, the gene-probe

sets related to vincristine sensitivity had a higher percentage of genes involved in nucleic acid metabolism (39 percent vs. 23 percent, P=0.021), and the gene-probe sets related to L-asparaginase sensitivity had a higher percentage of protein metabolism genes (53 percent vs. 20 percent, P<0.001).

**Genes previously linked with drug resistance or prognosis in ALL**

Of the 124 differentially expressed genes, to our knowledge 121 have not previously been linked to resistance to the four agents investigated. Only three genes for which results were significant in our analyses (*RPL6, ARHA,* and *SLC2A14*) have previously been associated with resistance to doxorubicin (*RPL6*[31] and *ARHA*[32]) or vincristine (*SLC2A14*[33]). Other genes previously associated with drug resistance or prognosis were not associated with sufficient statistical significance (i.e., P<0.001) for inclusion in our models (Tables 4 and 7 in the Supplementary Appendix).

**DISCUSSION**

We have identified genes that are differentially expressed in ALL cells with resistance to four antileukemic drugs and have shown that the pattern of expression of these genes is related to the outcome of treatment. The expression of 172 gene-probe sets (representing 124 unique known genes and 28 complementary DNA clones) in primary B-lineage leukemia cells was associated with resistance to prednisolone (42 probe sets), vincristine (59 probe sets), L-asparaginase (54 probe sets), and daunorubicin (22 probe sets). Of these 124 genes, to our knowledge 121 have not previously been associated with resistance to the four agents. Twelve other genes that have previously been associated with drug resistance or prognosis in ALL were differentially expressed in sensitive and resistant ALL but not at the level required for inclusion in our models (P<0.001). No universal cross-resistance gene was identified, since no single gene was associated with resistance to all four drugs. Discriminating genes belong to numerous functional groups, and specific functional categories were significantly overrepresented for some antileukemic agents (Figure 4). These findings document that resistance to mechanistically distinct antileukemic agents is associated with the expression of different functional groups of genes and support the use of combination chemotherapy for cancer treatment.

Our findings point to previously unrecognized potential targets for new agents to augment the efficacy of current chemotherapy for ALL. For example, in prednisolone-resistant ALL there was overexpression of the anti-apoptosis gene *MCL1* and

underexpression of several transcription-associated genes (e.g., *SMARCB1, PRPF18,* and *CTCF*), in L-asparaginase-resistant ALL there was overexpression of several ribosomal protein genes (e.g., *RPL3, RPL4, RPL5, RPL6,* and *RPL11*), and in vincristine-resistant ALL there was altered expression of cytoskeleton and extracellular-matrix genes (e.g., *TMSB10, PDLIM1*, and *DSC3*). It will be important to determine whether modulation of the proteins encoded by these genes will enhance treatment efficacy in patients with drug-resistant ALL.

It is noteworthy that the gene-expression signatures associated with resistance to individual antileukemic agents were also related to the response to treatment. The robustness of these signatures was validated in an independent population of patients who were treated with these same drugs, but in a different country and according to a different protocol. In a multivariate analysis that included the patient's age, ALL genetic subtype, ALL lineage, and leukocyte count, the combined gene-expression score remained significantly related to the risk of relapse in both the training and validation populations (Table 1). This indicates that the expression of genes associated with drug resistance has an independent influence on the outcome of treatment in ALL. Because genes associated with sensitivity or resistance differ for each antileukemic agent, our findings point to strategies whereby one could modulate specific components of therapy to which an individual patient is resistant.

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Chapter 7

# Expression of the Outcome Predictor in Acute Leukemia 1 (*OPAL1*) gene is not related to outcome in patients treated on contemporary COALL or St. Jude protocols

[1]Amy Holleman, [1]Monique L. den Boer, [2]Meyling H. Cheok, [1]Karin M. Kazemier, Deqing Pei, [4]James R. Downing, [5,6]Gritta E. Janka-Schaub, [5,7]Ulrich Göbel, [5,8]Ulrike B. Graubner, [3]Ching-Hon Pui, [2]William E. Evans, and [1]Rob Pieters.

*[1]Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Department of Pediatric Oncology and Hematology, Rotterdam, the Netherlands, [2]Department of Pharmaceutical Sciences, [3]Hematology-Oncology and [4]Pathology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA, [5]COALL study group, [6]University Children's Hospital, Hamburg, Germany, [7]Department of Pediatric Hematology and Oncology, University Medical Center, Heinrich-Heine-University, Düsseldorf, Germany, [8]Department of Pediatric Oncology, Dr. von Haunersches Children's Hospital, University of Munich, Munich, Germany.*

*Submitted*

**ABSTRACT**

The discovery of new prognostic genes may result into better risk classification and improve treatment of children with acute lymphoblastic leukemia (ALL). Recently, high expression of a novel gene named *OPAL1* (Outcome Predictor in Acute Leukemia) was reported as a new risk factor associated with favorable prognosis in ALL. Therefore, we investigated whether *OPAL1* was of prognostic importance in two independent cohorts of children treated on COALL-92/-97 (N=180) and on St. Jude (SJCRH) Total 13 protocols (N=257). We observed a 2.8-fold higher expression of *OPAL1* in *TEL-AML1*-positive compared to *TEL-AML1*-negative B-lineage ALL in both cohorts (P<0.0001). High *OPAL1* expression was not consistently associated with other favorable subtypes defined by age and white blood cell count at diagnosis (WBC), gender, immunophenotype or genetic abnormalities. *OPAL1* expression was not associated with increased *in vitro* sensitivity to prednisolone, vincristine, L-asparaginase or daunorubicin. In addition, *OPAL1* expression was not independently related to induction failure or long-term clinical outcome (DFS) in the total group of patients or in specific subgroups, such as T-lineage, *TEL-AML1*-positive and *TEL-AML1*-negative B-lineage ALL in either cohort. In conclusion, *OPAL1* gene expression is not an independent prognostic marker for childhood ALL treated on contemporary COALL and St. Jude protocols, and its previously reported prognostic relevance therefore appears to be treatment-specific.

**INTRODUCTION**

The prognosis of childhood acute lymphoblastic leukemia (ALL) has improved remarkably over the past four decades due to the introduction of effective risk-adapted combination chemotherapies. Conventional factors used to stratify patients are clinical and biological parameters such as age at diagnosis, initial white blood cell count (WBC), immunophenotype, the presence of specific genetic abnormalities[1] and early response to treatment.[2] Newer approaches include *in vitro* drug resistance profiles,[3] and measurement of minimal residual disease after induction of initial remission.[4,5]

The use of DNA microarrays enables investigators to simultaneously assess the expression of thousands of genes. In previous studies in childhood ALL, microarray analysis was successfully applied to identify known genetic and phenotypic subtypes,[6-8] as well as treatment-specific changes in gene expression[9] and genes related to drug resistance.[10] Recently, investigators used this technology to identify three novel genes, referred to as G0, G1 and G2, that were highly predictive of outcome in 254 patients with childhood ALL enrolled in Pediatric Oncology Group (POG) treatment protocols.[11-13] The top discriminating

gene, G0, was fully cloned and named *OPAL1* (Outcome Predictor in Acute Leukemia 1). The function of *OPAL1* is currently unknown, although the presence of a cytochrome c-like heme-binding site and a transmembrane domain suggests *OPAL1* may be involved in the mitochondrial electron transport chain.[14] We initially identified this gene as one of the top ranked class discriminating genes that was over-expressed in ALL cells positive for the *TEL-AML1* gene fusion.[7,8] In the POG study, *OPAL1* was expressed at higher levels in ALL subgroups with a favorable prognosis (i.e., ALL with t(12;21)/*TEL-AML1*, normal and hyperdiploid karyotypes) compared to a subgroup with an unfavorable prognosis (i.e., ALL with t(9;22)/*BCR-ABL*) and another subgroup previously associated with an unfavorable prognosis in (i.e., ALL with t(1;19)/*E2A-PBX1*).[12] High *OPAL1* expression was shown to be highly predictive of a favorable outcome in the total ALL group, but also in ALL subgroups, such as T-lineage ALL and t(12;21)/*TEL-AML1*-positive B-lineage ALL. Finally, low *OPAL1* was significantly related to induction failures.[12]

These provocative results based on one single gene prompted us to analyze in depth the expression pattern of *OPAL1* in independent cohorts of children with newly diagnosed ALL treated on protocols of the Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia (COALL, N=180) and St. Jude Children's Research Hospital (St. Jude, N=257). *OPAL1* expression was extensively investigated in T-lineage ALL, *TEL-AML1*-positive and hyperdiploid B-lineage ALL as well as other prognostic factors were considered. In addition, *OPAL1* expression was tested as a predictor of clinical outcome as well as its relation to *in vitro* resistance to four widely used drugs in the treatment of childhood ALL, i.e. prednisolone, vincristine, L-asparaginase and daunorubicin.

## MATERIALS AND METHODS

### Patient samples

Bone marrow and peripheral blood samples were obtained after informed consent from children with newly diagnosed ALL who were enrolled on either the Cooperative Study Group for Childhood Acute Lymphoblastic Leukemia protocols COALL-92/97 (N=180)[10,15] or the St. Jude Children's Research Hospital (St. Jude) protocols Total Therapy 13A (N=99) and B (N=158).[7,8,16] Leukemic blasts were enriched from peripheral blood or diagnostic bone marrow aspirates as previously described.[7,8] For *in vitro* drug sensitivity assays, mononuclear cells were isolated by sucrose density gradient centrifugation (Lymphoprep, density 1.077 g/ml; Nycomed Pharma, Oslo, Norway) within 24 hours after sampling. Cells were resuspended in culture medium consisting of RPMI 1640 (Dutch

modification without L-glutamine; Gibco BRL, Life Technologies, Breda, The Netherlands) supplemented with 20% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 2 mM L-glutamine, 200 μg/ml gentamycin (Gibco BRL) 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.125 μg/ml fungizone (Gibco BRL), and 5 μg/ml insulin, 5 μg/ml transferrin and 5 ng/ml sodium selenite (ITS media supplement; Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands). If necessary, leukemic samples were further enriched to more than 90% leukemic blasts by removing non-malignant cells with immunomagnetic beads (DynaBeads, Dynal Inc., Norway).[17]

***In vitro* drug resistance assay**

Responsiveness of leukemia cells to prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands), vincristine (VCR; TEVA Pharma, Mijdrecht, The Netherlands), L-asparaginase (ASP; Paronal, Christiaens, Breda, The Netherlands), and daunorubicin (DNR; Cerubidine, Rhône-Poulenc Rorer, Amstelveen, The Netherlands) was determined by the 4-day *in vitro* MTT drug resistance assay.[3] The concentration ranges tested for these drugs were: PRED: 0.008-250 μg/ml; VCR: 0.05-50 μg/ml; ASP: 0.003-10 IU/ml and DNR: 0.002-2.0 μg/ml. The drug concentration lethal to 50% of the ALL cells ($LC_{50}$ value) was used as the measure of cellular drug resistance. The cut-off $LC_{50}$ values used to assign cases as sensitive or resistant to each agent, were those previously shown to be associated with a good or poor treatment outcome in children with ALL.[3,18]

**Real-time quantitative PCR**

The mRNA expression levels of *OPAL1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference, were quantified using real-time PCR analysis on the ABI Prism 7700 sequence detection system as previously described.[19] PCR reactions were performed with an amplification efficiency of more than 95%. The comparative cycle time ($C_t$) value of the target PCR was normalized by subtracting the $C_t$ value of GAPDH ($\Delta C_t$).[20] Primer sequences used for *OPAL1* (NM_017787, chromosome 10) were: sense, 5'-TCCTTTTGGG TCTTAGACAG-3' and antisense, 5'-TTGGCAAAAACCTGAAAT-3' and the probe sequence: 5'-ACAGTCTCAGTGCTGCAACTACTACTATGA-3'. We observed a significant correlation between *OPAL1* mRNA expression assessed by real-time quantitative PCR and microarray ($r_s$=0.59, P=0.03, N=14).

**Microarray analysis**

Total RNA was hybridized to U133A (COALL) and U95Av2 (St. Jude) GeneChip® oligonucleotide microarrays, according to manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Data analysis was performed as described before and the full gene-expression profiles of the leukemic samples included in this present study were previously published.[8,10] Briefly, gene expression values were scaled to the target intensity of 2500, using Affymetrix Microarray Suite® (MAS) 5.0 software and $\log_2$-transformed. We used the U133A probe set 202808_at, which covers the same DNA sequence (99.8% sequence identity) as the Affymetrix U95A.v2 GeneChip® probe set 38652_at used by Mosquera-Caro *et al*.,[8,12] to analyze the expression of *OPAL1*. More information on these probe sets and its target sequences is available at Affymetrix® NetAffx https://www.affymetrix.com *OPAL1* expression determined with the U133A and the U95Av2 chip was available for a subset of 92 St. Jude patients ($r_s$=0.58, P<0.0001, Spearman rank correlation).

**Statistics**

The duration of disease-free survival (DFS) was defined as the time from diagnosis until the date of leukemia relapse (event), the last follow-up or secondary events other than relapse (censored). DFS curves were calculated according to the Kaplan-Meier method or a modification thereof in the presence of competing events (St. Jude)[21,22] Since no cut-off for *OPAL1* expression was provided by Mosquera-Caro *et al*.,[12] we performed survival analysis in two different ways; *OPAL1* was treated either as a continuous variable or as a categorical variable (*OPAL1* expression was divided into 3 groups by the 33$^{rd}$ and 67$^{th}$ percentile of expression (i.e., low [bottom third], intermediate [intermediate third] and high [top third]). Univariate analysis of the predictive value of *OPAL1* expression in three groups (2 degrees of freedom, 2 d.f.) was analyzed by log-rank test (COALL) and adjusted for competing events (St. Jude) whereas a Cox proportional hazard regression model (adjusted for competing events)[23] was used in univariate and multivariate analyses to assess the association of *OPAL1* expression as a continuous variable with DFS and other known prognostic factors. The model for multivariate analysis included conventional risk factors (i.e., WBC, age, immunophenotype and genetic abnormalities). Differences in *OPAL1* expression between ALL subgroups were tested using the Mann-Whitney U test. Spearman's correlation test was used to compare the expression of *OPAL1* by microarray with the expression data obtained by real-time quantitative-PCR and to relate *OPAL1* expression to *in vitro* drug resistance. All statistical tests were performed at a two-tailed significance level of 0.05.

## RESULTS

The association of *OPAL1* expression with prognostic features in ALL (i.e., age, white blood cell count (WBC), immunophenotype and genetic subtype) was tested.

**Table 1. *OPAL1* expression in prognostic subgroups of ALL at diagnosis** *OPAL1* expression was compared in prognostic ALL subgroups defined by age, white blood cell count (WBC), gender, immunophenotype and genetic subtype in children treated on **A.** COALL and **B.** St. Jude protocols. Indicated are the number of patients (N), median $\log_2$ transformed scaled *OPAL1* expression (*OPAL1*) in arbitrary units, the ratio of *OPAL1* expression in non-reference versus reference (*) group (fold-difference in scaled linear *OPAL1* expression signal) and P-values determined by the Mann-Whitney *U* test.

| | A. COALL/DCOG cohort | | | | B. St. Jude cohort | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **N** | ***OPAL1*** | **Ratio** | **P-value** | **N** | ***OPAL1*** | **Ratio** | **P-value** |
| **Age** | | | | | | | | |
| <10 years | 131 | 10.96 | 1.0* | | 181 | 12.29 | 1.0* | |
| >10 years | 49 | 10.75 | 0.86 | 0.39 | 76 | 12.08 | 0.87 | *0.001* |
| **White-cell count** | | | | | | | | |
| <10/nL | 43 | 10.92 | 1.0* | | 90 | 12.23 | 1.0* | |
| 10-49/nL | 67 | 10.78 | 0.91 | 0.97 | 90 | 12.38 | 1.11 | 0.42 |
| 50-100/nL | 28 | 10.97 | 1.04 | 0.93 | 40 | 12.29 | 1.05 | 0.88 |
| >100/nL | 42 | 10.97 | 1.04 | 0.76 | 47 | 11.99 | 0.85 | *0.023* |
| **Gender** | | | | | | | | |
| Female | 104 | 10.92 | 1.0* | | 95 | 12.10 | 1.0* | |
| Male | 76 | 10.93 | 1.00 | 0.74 | 162 | 12.23 | 1.09 | 0.53 |
| **ALL subtype** | | | | | | | | |
| B-other | 44 | 10.57 | 1.0* | | 71 | 11.90 | 1.0* | |
| *BCR-ABL* | 4 | 10.45 | 0.92 | 0.65 | 9 | 11.92 | 1.01 | 0.68 |
| *E2A*[£] | 9 | 10.78 | 1.16 | 0.58 | 14 | 11.83 | 0.96 | 0.95 |
| Hyperdiploid[#] | 40 | 10.52 | 0.97 | 0.37 | 51 | 12.12 | 1.17 | *0.006* |
| *MLL*[£] | 4 | 10.91 | 1.28 | 0.90 | 5 | 12.06 | 1.12 | 0.47 |
| *TEL-AML1* | 44 | 12.04 | 2.77 | *<0.001* | 71 | 13.38 | 2.79 | *<0.001* |
| T-lineage | 35 | 10.79 | 1.16 | 0.44 | 36 | 12.08 | 1.13 | 0.06 |

N: number of patients, HR: hazard ratio, CI: confidence interval, *: this group served as the reference group to calculate the ratio, [#]: Cytogenetic analysis revealed more than 50 chromosomes, [£]: COALL: *MLL* and *E2A* rearranged, St. Jude: *MLL-AF4* and *E2A-PBX1*

However, *OPAL1* expression was not related to age, WBC or gender nor did it differ between B-lineage without genetic markers (B-other) and T-lineage ALL among COALL and St. Jude patients (Table 1) but its lower expression was related to age >10 years and leukocyte count >100/nL among St. Jude patients (Table 1B).

We observed a 2.8-fold higher expression of *OPAL1* in *TEL-AML1*-positive compared to *TEL-AML1*-negative B-lineage ALL in both the COALL and St. Jude cohorts (P<0.0001; Figure 1). *OPAL1* expression did not consistently differ in both study groups for the other ALL subtypes examined (i.e., hyperdiploid, *E2A*-rearranged, *MLL*-rearranged or *BCR-ABL*-positive B-lineage ALL).

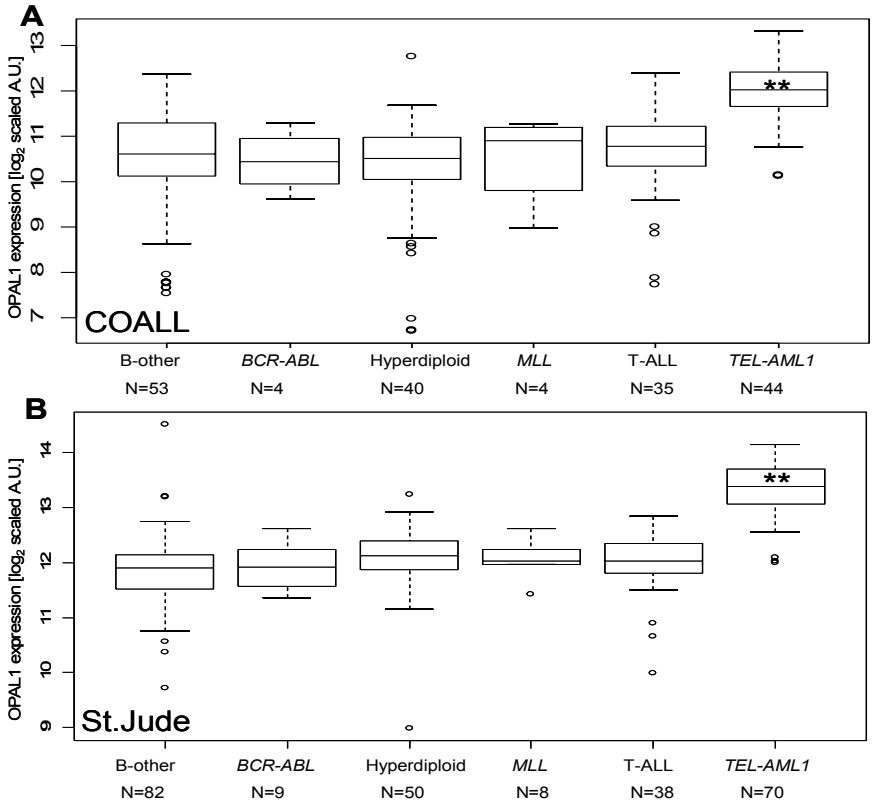


**Figure 1. *OPAL1* expression in different ALL subtypes.** *OPAL1* expression was compared in a total of 180 children with ALL treated according to COALL protocols (**A**), and in a total of 257 children with ALL treated according to St. Jude protocols (**B**). *OPAL1* expression is expressed in $log_2$-transformed scaled arbitrary units (AU), the medians (horizontal lines), the 25$^{th}$ and 75$^{th}$ percentiles (boxes), the ranges (bars) and the outliers (open circles) are shown. **Indicates P<0.0001, determined by the Mann-Whitney U test.

Because drug resistance is a major cause of treatment failure, we investigated whether *OPAL1* expression was related to *in vitro* drug resistance to any of four drugs that form an integral component of contemporary chemotherapeutic protocols for children with ALL. No correlation was observed between *in vitro* drug resistance and *OPAL1* expression for prednisolone ($r_s$=0.00, P=0.99), L-asparaginase ($r_s$=-0.02, P=0.81) and daunorubicin ($r_s$=0.05, P=0.54). By contrast, vincristine resistance showed a significant correlation with *OPAL1* expression ($r_s$=0.34, P<0.0001) which was opposite from expected. This observation was concordant with 3.6-fold increased VCR-resistance of *TEL-AML1*-positive ALL compared to non*TEL-AML1* precursor B-lineage ALL (P<0.0001; $LC_{50}$=0.697 ug/ml and 0.193 µg/ml, respectively).

The relation between *OPAL1* expression and treatment outcome was subsequently investigated in both cohorts. Of the 180 patients that were part of the COALL studies 92 and 97, 42 had disease-related events and 6 had competing events (2 secondary malignancies and 4 deaths in remission). Of the 257 patients included in the St. Jude study Total Therapy 13A and B, 42 had disease-related events, 20 had competing events (16 secondary malignancies and 4 deaths in remission). In the group of COALL patients, *OPAL1* expression was significantly associated with disease-free survival (DFS) when OPAL1 expression was divided into 3 equally sized groups based on the individual rank in expression (2 d.f., P=0.01; Figure 2A) but not when *OPAL1* expression was treated as continuous variable (P=0.45). In contrast to COALL patients, but as reported previously,[11-13] *OPAL1* expression was significantly associated with DFS of St. Jude treated patients both when divided into three equally sized groups (2 d.f.: P=0.002; Figure 2B) and when used as continuous variable (P<0.0001). However, from the order of both DFS-curves of COALL and St. Jude it becomes clear that the predictive value of *OPAL1* is not biological relevant since the order for favorable, intermediate and unfavorable outcome is high-low-intermediate expression of *OPAL1*, respectively (Figure 2).

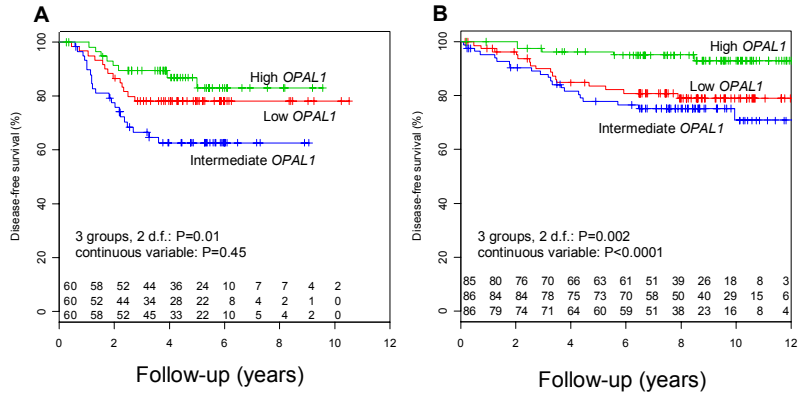


**Figure 2. *OPAL1* versus prognosis in children with newly diagnosed ALL.** Disease-free survival (DFS) of **A.** 180 children with newly diagnosed ALL treated on COALL 92/97 and **B.** 257 newly diagnosed children with ALL treated on St. Jude Total 13 protocols was estimated according to Kaplan-Meier analysis adjusted for competing events. Patients were grouped based on their rank in *OPAL1* expression in the total group of each cohort (i.e., low [bottom third], intermediate [intermediate third] and high [top third]).

The clinical value of *OPAL1* expression was further studied within major prognostically important ALL subtypes (Table 2). Firstly, within T-lineage ALL and B-lineage ALL negative for the *TEL-AML1* fusion transcript, we were unable to confirm an association between high

*OPAL1* expression and a favorable DFS in either treatment cohort. In both cohorts even the opposite (although statistically non-significant) correlation was observed in T-ALL, i.e. a low *OPAL1* expression correlated with a favorable DFS (Table 2). This result is in disagreement with our results for the total group of patients (Figure 2) as well as those obtained for the initial POG cohort[11-13] where high *OPAL1* expression was found to be a favorable outcome predictor in T-ALL patients.

**Table 2**. **Predictive value of *OPAL1* in pediatric ALL.** Overview of 4-year disease-free survival rates for low, intermediate and high *OPAL1* expression (division based on cut-offs as established in COALL and St. Jude study cohorts) in genetic subgroups of pediatric ALL. **A**. COALL patients and **B**. St Jude patients. P-values are derived from Cox univariate analysis using *OPAL1* expression as continuous variable.

| | **A. COALL** | | | **B. St. Jude** | | |
|---|---|---|---|---|---|---|
| **Variable** | **N** | **4-yr DFS with SE (%)** | **P-value*** | **N** | **4-yr DFS with SE (%)** | **P-value*** |
| **Total group** | | | 0.45 | | | <0.0001 |
| low | 60 | 78 ± 6 | | 85 | 86 ± 4 | |
| intermediate | 60 | 63 ± 7 | | 86 | 82 ± 4 | |
| high | 60 | 87 ± 5 | | 86 | 96 ± 2 | |
| **Non-*TEL-AML1*** | | | 0.84 | | | 0.35 |
| low | 48 | 75 ± 5 | | 70 | 87 ± 4 | |
| intermediate | 36 | 67 ± 9 | | 63 | 90 ± 4 | |
| high | 17 | 77 ± 14 | | 17 | 94 ± 6 | |
| ***TEL-AML1*** | | | 0.61 | | | 0.006 |
| low | 2 | n.d. | | 1 | n.d. | |
| intermediate | 6 | 83 ± 15 | | 3 | 67 ± 33 | |
| high | 36 | 97 ± 3 | | 67 | 97 ± 2 | |
| **Hyperdiploid** | | | 0.16 | | | 0.0005 |
| low | 20 | 95 ± 5 | | 15 | 93 ± 7 | |
| intermediate | 15 | 92 ± 8 | | 27 | 89 ± 6 | |
| high | 5 | 100 | | 9 | 100 ± 0 | |
| **B-other** | | | 0.55 | | | 0.0001 |
| low | 21 | 67 ± 14 | | 70 | 87 ± 4 | |
| intermediate | 12 | 42 ± 16 | | 65 | 89 ± 4 | |
| high | 11 | 60 ± 22 | | 84 | 96 ± 2 | |
| **T-lineage** | | | 0.06 | | | 0.61 |
| low | 10 | 90 ± 12 | | 14 | 79 ± 12 | |
| intermediate | 18 | 47 ± 14 | | 20 | 60 ± 11 | |
| high | 7 | 57 ± 37 | | 2 | n.d. | |

*: P-value Cox univariate analysis adjusted for competing events, *OPAL1* as continuous variable, n.d.=not detected

Among *TEL-AML1*-positive B-lineage ALL cases treated in the COALL protocols, *OPAL1* expression was not significantly associated with DFS (continuous variable: P=0.61). When all patients treated on the St. Jude protocols (Total Therapy 13A and 13B) were analyzed, the association between high *OPAL1* expression and higher DFS was significant when the expression was analyzed as a continuous variable (P=0.006). Interestingly, *OPAL1* expression treated as continuous variable had no prognostic significance among the 37 patients treated in study 13A (P=0.32), but was significant among the 34 patients treated in study 13B (P=0.0002). Notably, all 37 patients except one

in 13A, as compared to only 10 of the 34 in 13B *TEL-AML1* positive patients were treated according to the high-risk arm of the respective protocol, suggesting that the prognostic impact of *OPAL1* expression may be treatment dependent.

**Table 3. Prognostic value of *OPAL1* expression in ALL** Cox multivariate proportional hazards analysis computed with known prognostic factors (i.e., age, white blood cell count (WBC), immunophenotype and genetic subtype). *OPAL1* expression was treated as continuous variable. Hazard ratios (HR), 95% confidence intervals (95% CI) and P-values are shown.

| | COALL cohort | | | | St. Jude cohort | | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **N** | **HR** | **95% CI** | **P-value** | **N** | **HR** | **95% CI** | **P-value** |
| **Age** | | | | | | | | |
| <10 years | 131 | 1.0* | | | 181 | 1.0* | | |
| >10 years | 49 | 1.24 | 0.64-2.41 | 0.52 | 76 | 1.27 | 0.63-2.54 | 0.50 |
| **White-cell count** | | | | | | | | |
| <10/nL | 43 | 1.0* | | | 90 | 1.0* | | |
| 10-49/nL | 67 | 0.55 | 0.20-1.54 | 0.25 | 80 | 1.02 | 0.43-2.38 | 0.97 |
| 50-100/nL | 28 | 0.97 | 0.33-2.88 | 0.96 | 40 | 0.64 | 0.18-2.29 | 0.50 |
| >100/nL | 42 | 1.59 | 0.58-1.35 | 0.36 | 47 | 1.06 | 0.38-3.00 | 0.92 |
| **ALL subtype** | | | | | | | | |
| B-other | 44 | 1.0* | | | 71 | 1.0* | | |
| *BCR-ABL* | 4 | 2.38 | 0.51-11.14 | 0.27 | 9 | 8.99 | 2.33-34.70 | *0.001* |
| *E2A*[£] | 9 | 0.97 | 0.28-3.40 | 0.96 | 14 | 0.53 | 0.07-4.17 | 0.55 |
| Hyperdiploid[#] | 40 | 0.15 | 0.03-0.65 | *0.010* | 51 | 0.75 | 0.26-2.18 | 0.60 |
| *MLL*[£] | 4 | 9.28 | 2.37-36.4 | *0.001* | 5 | 3.92 | 0.59-26.2 | 0.16 |
| *TEL-AML1* | 44 | 0.18 | 0.06-0.68 | *0.010* | 71 | 0.83 | 0.20-3.35 | 0.79 |
| T-lineage | 35 | 0.79 | 0.33-1.93 | 0.61 | 36 | 3.40 | 1.25-9.28 | *0.020* |
| ***OPAL1*** | | | | | | | | |
| expression | 180 | 1.02 | 0.77-1.34 | 0.90 | 257 | 0.68 | 0.42-1.12 | 0.13 |

N: number of patients, HR: hazard ratio, CI: confidence interval, *: this group served as the reference group to calculate the ratio, [#]: Cytogenetic analysis revealed more than 50 chromosomes, [£]: COALL: *MLL* and *E2A* rearranged, St. Jude: *MLL-AF4* and *E2A-PBX1*

Among hyperdiploid cases, higher expression of *OPAL1* was significantly related to a favorable prognosis in St Jude ($P$ = 0.0005; Table 2) but not in COALL patients (P=0.16).

No association with induction failure was found in either COALL or St. Jude cohort. When known risk factors (i.e. age, WBC, immunophenotype and genetic subtypes) were included in a multiple regression model, *OPAL1* expression was no longer predictive of prognosis in ALL in the COALL as well as in the St. Jude study groups (P>0.1, continuous variable; Table 3).

## DISCUSSION

Recently, we identified expression signatures associated with cellular drug resistance and outcome in B-lineage ALL.[10] In this study, *OPAL1* was not among the top 124 most discriminating genes for cellular drug resistance that were associated with outcome. This

per se does not exclude a predictive role for *OPAL1* in childhood ALL, as this gene may be significant at a lower level than the cut-off P-values used for the construction of the resistance signature models, or may directly be related to treatment outcome as the selection of genes in our earlier study was focused on *in vitro* drug resistance profiles and not directly on outcome. Therefore, we analyzed the expression patterns of *OPAL1* in leukemic cells of two independent groups of 180 COALL and 257 St. Jude patients at initial diagnosis of ALL and correlated the expression of this gene to age, WBC, gender, immunophenotype, genetic subtype, *in vitro* drug resistance and clinical outcome.

We observed a 2.8-fold higher *OPAL1* expression in children with *TEL-AML1*-positive B-lineage ALL uniformly in both cohorts. This is consistent with the higher *OPAL1* levels observed in *TEL-AML1*-positive cases as initially reported by our study[8] and by Mosquera-Caro *et al*.[12] *OPAL1* expression was not consistently elevated in ALL with normal karyotypes or in hyperdiploid B-lineage ALL samples which was in disagreement with the previous POG report.[12] In addition, we were not able confirm the low levels of *OPAL1* in ALL containing the *BCR-ABL* gene fusion, but the numbers of patients may be too small to detect a difference (N=4 [COALL], N=9 [St. Jude]). For the St. Jude patient group higher levels of *OPAL1* were found in hyperdiploid ALL cells, and a relation was found for lower *OPAL1* expression in patients older than 10 years and WBC greater than 100/nL. *OPAL1* expression was not increased in subgroups of COALL patients with a favorable prognosis (i.e., age less than 10 years, WBC less than 25/nL, female sex, hyperdiploidy, normal karyotype) except for *TEL-AML1*. Taken together, these data suggest that, in contrast to the observation made by Mosquera-Caro *et al*., *OPAL1* was not consistently related to all subgroups with favorable prognosis.

*In vitro* sensitivity to several drugs are related to favorable outcome.[3,18,24,25] Based on the previously observed relation between high *OPAL1* expression and favorable prognosis[12,13] we tested the relation between high *OPAL1* expression and *in vitro* drug sensitivity. However, in the present study we observed no relation between a high *OPAL1* expression and sensitivity to prednisolone, L-asparaginase and daunorubicin and a weak relation with vincristine resistance, which is more likely explained by *TEL-AML1*-positive ALL being more resistant to vincristine than *OPAL1* expression being related to vincristine resistance. This indicates that *OPAL1* is not a major determinant of cellular drug sensitivity, suggesting that its prognostic value (as seen in St. Jude treated patients only) may be associated with regrowth capacity of leukemic cells. More insight into the biological function of *OPAL1* is needed to address this issue.

We found no evidence of an association between low *OPAL1* expression and worse outcome in COALL and St. Jude-protocol treated ALL patients of T-lineage and *TEL-AML1*-negative B-lineage ALL. In T-lineage ALL the prognostic value of *OPAL1* expression even was reversed (i.e., low [instead of high] expression of *OPAL1* was linked to a favorable prognosis). The significant association for high *OPAL1* expression and favorable disease-free survival in *TEL-AML1*-positive and hyperdiploid ALL patients treated at St. Jude was not consistent with these subgroups treated at COALL centers. In addition, the association was only significant for *TELAML1*-positive patients treated on the Total 13B protocol, where *TEL-AML1*-positive ALL were largely under-treated using low-risk therapy. These data suggest that *OPAL1* expression may be prognostic in ALL containing the *TEL-AML1* gene fusion, but that this association is not independent of the treatment patients receive. Most importantly, the relationship of *OPAL1* expression with disease-free survival was not independent of known risk factors (i.e., age, WBC and ALL subtype) in two independent cohorts of patients.

In conclusion, *OPAL1* expression is expressed at a higher level in *TEL-AML1*-positive B-lineage ALL compared to other leukemic subtypes. However, we found no major evidence of elevated *OPAL1* expression in other subgroups with a favorable prognosis nor did we consistently observe decreased *OPAL1* expression in subgroups with an unfavorable prognosis. In addition, increased *OPAL1* expression was not independently associated with *in vitro* drug sensitivity of COALL-treated children with ALL. Not considering other known risk factors, a predictive value of *OPAL1* expression was only found in St. Jude treated patients and not in COALL patients. However, this predictive value was highly dependent of other known risk factors (as shown in multivariate analysis). This suggests that the predictive value previously reported for *OPAL1* expression in childhood ALL may be related to the treatment given. The present data suggest that *OPAL1* expression is not suitable as treatment-independent marker for risk stratification of children with ALL.

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Chapter 8

# Sensitizing effect of glycolysis inhibition on prednisolone resistance in acute lymphoblastic leukemia

[1]Amy Holleman, [2]David J. VanderWeele, [1]Monique M.C.J. Passier, [1]Karin M. Kazemier, [3]Meyling H. Cheok, [3]William E. Evans, [2]Charles M. Rudin, [1]Monique L. den Boer, and [1]Rob Pieters

*[1]Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Department of Pediatric Oncology and Hematology, Rotterdam, the Netherlands,*

*[2]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Department of Oncology, Baltimore, Maryland, USA,*

*[3]St. Jude Children's Research Hospital, Department of Pharmaceutical Sciences, Memphis, Tennessee, USA.*

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**ABSTRACT**

Glucocorticoids are a key component of chemotherapeutic protocols used to treat childhood acute lymphoblastic leukemia (ALL) and both *in vitro* and in vivo glucocorticoid sensitivity are important prognostic factors in childhood ALL. We recently observed that enhanced expression of the glycolytic enzymes GAPDH and GLUT3 is related to *in vitro* prednisolone (PRED) resistance in pediatric B-lineage ALL.. In the present study, we demonstrate that PRED-resistance is linked to a higher glycolytic rate in ALL cell lines. Moreover, we show that the glycolysis inhibitor 2-deoxy-D-glucose (2-DG) selectively sensitized PRED-resistant but not PRED-sensitive ALL cell lines to PRED-induced apoptosis. In conclusion, these data suggest that resistance to PRED is associated with an enhanced glucose metabolism in ALL. Chemotherapeutic intervention by targeting the glucose metabolism with agents such as 2-DG may offer a new strategy to sensitize leukemic cells to glucocorticoids.

**INTRODUCTION**

Prednisolone (PRED) is a member of the glucocorticoid class of hormones, which form a standard component of the treatment of childhood acute lymphoblastic leukemia (ALL).[1] Sensitivity of leukemic blasts to glucocorticoids is an important prognostic factor in childhood ALL. Children with a good *in vivo* PRED response, i.e. a reduction in peripheral blast count below 1,000 blasts/µl after 1-week prednisone monotherapy, have a significant better outcome than patients with a higher number of circulating blasts.[1-4] In addition, various groups demonstrated a relation between *in vitro* PRED resistance at initial diagnosis and short- and long-term clinical outcome.[5-7] Despite its clinical significance, the molecular basis of glucocorticoid resistance in childhood ALL is still poorly understood.

After administration to the patient's blood, glucocorticoids enter the leukemic cells by passive diffusion and bind to the cytoplasmic glucocorticoid receptor. The resulting complex subsequently translocates to the nucleus where it triggers transactivation or transrepression of a wide array of target genes. Collectively this altered gene-expression is though to result in the induction of apoptosis in the leukemic cell.[8,9] The causes of glucocorticoid resistance in pediatric ALL have been investigated at various levels between the entry of the glucocorticoids in the cell and the final induction of apoptosis.[10] Current data suggest that the level of glucocorticoid receptor expression is lower in PRED resistant leukemic cells of patients, but this observation is presumably not the only explanation for PRED resistance in ALL.[11-16] We previously observed less PRED-induced phosphatidyl serine exposure, mitochondrial membrane depolarization, caspase-3 activation and PARP

inactivation in PRED-resistant ALL cells, suggesting that PRED resistance is associated with a defect at the mitochondrial level in childhood ALL.[17] Baseline expression of Bcl-2 family members such as Bcl-2, Bcl-Xl, Bax so far have not been significantly been associated with glucocorticoid resistance in pediatric ALL.[13,18-20] A notable exception is the anti-apoptotic Bcl-2 family member MCL1, which was overexpressed in PRED-resistant pediatric B-lineage ALL.[21] Besides altered MCL1 expression, we recently demonstrated that both the glucose transporter 3 (*GLUT3)* and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were relatively overexpressed in PRED resistant leukemic cells of children with B-lineage ALL.[21] Since drug resistance in malignant cells has previously been linked with an altered glucose metabolism[22-24], we hypothesized that the high level expression of two glycolysis-associated genes may be indicative for increased glucose consumption in PRED resistant leukemic cells.

In the present study we show that PRED-resistant ALL cell lines have an increased glycolytic rate compared to PRED-sensitive ALL cell lines. Inhibition of glycolysis selectively sensitized PRED -resistant but PRED-sensitive ALL cell lines to PRED-induced apoptosis. We conclude from these data that resistance to PRED is functionally linked to an increased glycolytic rate in leukemic cells. This knowledge may point to new strategies to sensitize leukemic cells to glucocorticoids in clinical practice.

## MATERIALS AND METHODS

### Cell lines and culture

The human T-lineage leukemic cell line Jurkat (ACC 282), and the human T-lineage leukemic cell line Molt4 (ACC 362) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). The human precursor B-lineage leukemic cell line RS4;11 (CRL 1873) was obtained from the American Type Cell Culture Collection (ATCC, Manassas, VA, USA). The human precursor B-lineage leukemic cell line TOM-1 was a kind gift of Dr. H.B. Beverloo (Department of Clinical Genetics, Erasmus MC Rotterdam, the Netherlands).[25] All cell lines were maintained in RPMI 1640 (Gibco BRL, Breda, the Netherlands) supplemented with 10% fetal calf serum (FCS; Integro, Zaandam, The Netherlands), 100 IU/ml penicillin, 100 µg/ml streptomycin and 0.125 µg/ml fungizone (PSF; Gibco BRL).

**Drug-resistance assay**

The *in vitro* sensitivity of leukemia cells to prednisolone (PRED; Bufa Pharmaceutical Products, Uitgeest, The Netherlands) was determined in the MTT assay. Briefly, round-bottomed 96-well microculture plates were filled with 20 μl of different dilutions of a drug and stored at –20ºC. Six concentrations of each drug were tested in duplicate. The range of final concentration ranged between 22 and 69,000 ug/ml for the PRED resistant (Jurkat and Molt4) and between 0.0073 and $2\times10^{-7}$ μg/ml for PRED sensitive cell lines (RS4;11 and TOM-1). To determine the effect of 2-deoxy-D-glucose (2-DG; Sigma Aldrich, Zwijndrecht, the Netherlands) on *in vitro* PRED cytotoxicity, the ALL cell lines were pre-incubated for 1 hour prior to PRED exposure with 2-DG or an equivalent volume of culture medium (control) at 37ºC in a humidified incubator in 5% $CO_2$. Aliquots of 80 μl cell suspension (2 x $10^6$ cells/ml) were added to each well. Four wells contained 100 μl culture medium without drugs or cells for blanking the plate reader and 8 wells contained 100 μl culture medium with cells and without drug for measuring control cell viability. After incubating plates for 4 days at 37˚C in a humidified incubator in 5% $CO_2$, 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT, 5mg/ml; Sigma Aldrich) was added and the plates were incubated for an additional 6 hours. During these 6 hours, the living cells present in each well will reduce the yellow MTT tetrazolium salt to purple-blue formazan crystals. The formazan crystals were dissolved with 100 μl of 0.04 N HCl-isopropanyl alcohol. The optical density (OD) of the wells, which is linearly related to cell number,[28] was determined spectrophotometrically at a wavelength of 562 nm. Leukemic cell survival (LCS) was calculated by the equation: LCS = ($OD_{day4}$ treated well/mean $OD_{day4}$ control wells) x 100%. MTT-Assay results were only used if the drug-free control wells contained ≥70% leukemic cells after 4 days of culture.

**Measurement of glycolytic rate**

Glycolytic rate was measured as conversion of [5-$^3$H]glucose to $^3H_2O$, as described previously.[26,27] Briefly, $10^6$ cells were washed in PBS and resuspended in 500 µl Krebs buffer [25 nM $NaHCO_3$, 115 mM NaCl, 2mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.25% BSA (pH 7.4)] containing 10 mM glucose and 1 µCi [5-$^3$H]glucose, and incubated for 1 hour in 5% $CO_2$ at 37°C. For each sample an open tube containing 100 µl of cells and 50 µl 0.2 N HCl was placed upright in a scintillation vial containing 1 ml $H_2O$. The vials were sealed and $^3H_2O$ produced as a result of glycolysis was allowed to equilibrate with $H_2O$ outside the tube for 24 hours at room temperature. The amount of $^3$H retained within the tube, and the

amount that had diffused into the surrounding $H_2O$ by evaporation and condensation, were determined separately. [5-$^3$H]glucose and $^3H_2O$ standards were included in each experiment, allowing calculation of the rate of conversion of [5-$^3$H]glucose to $^3H_2O$.[26]

**Measurement of the doubling time**

Logarithmically growing cells were exposed to low dose 2-DG (0.2 mM), high dose 2-DG (4.0/1.0 mM) or an equivalent volume of culture medium (control). Subsequently, the cell concentration was measured every 24 hours for 8 days by a Coulter Particle Analyzer (Beckman-Coulter, Inc., Fullerton, CA, USA). Cell viability was assessed by propidium iodide exclusion on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and analyzed using CellQuest Pro software (Becton Dickinson). The cells lines were passed every 48 hrs back to a density of $1\times10^6$ cells/ml during the course of the experiment to prevent overgrowth. The doubling time was estimated with the numbers of viable cells from day 0 to day 8. All experiments were performed in duplicate.

## RESULTS

**Enhanced expression of glycolytic enzymes in PRED-resistant ALL cell lines**

ALL cell lines were selected for relative sensitivity and resistance towards prednisolone (PRED; Figure 1). Jurkat (median $LC_{50}$: 7791 µg/ml) and Molt4 (median $LC_{50}$: 8436 µg/ml) are *in vitro* both $2\times10^6$-fold more resistant to PRED than RS4;11 (median $LC_{50}$: 0.00394 µg/ml) and $1\times10^7$-fold more resistant to PRED than TOM-1 (median $LC_{50}$: 0.00081 µg/ml).

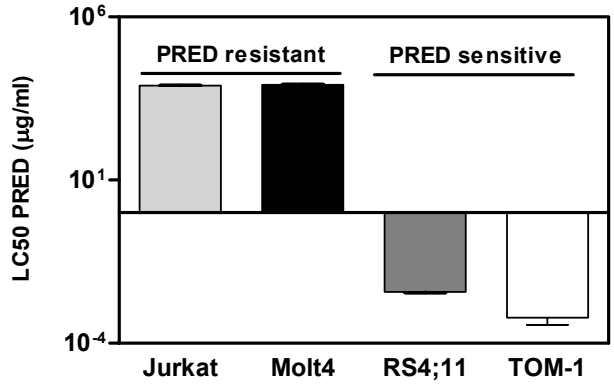


**Figure1. *In vitro* PRED responsiveness of 2 PRED-resistant and 2 PRED-sensitive ALL cell lines** *In vitro* responsiveness to PRED was assessed by the MTT assay in 4 ALL cell lines as described in materials and methods. The presented data are the result of three independent experiments.

The rate of conversion of 5-$^{3}$H glucose to $^{3}H_2O$ was examined in these ALL cell lines. The rate of glycolysis was enhanced in both PRED-resistant (129 and 132 nmol glucose/$10^6$ cell/hr for Jurkat and Molt4 respectively) compared to both PRED-sensitive ALL cell lines (38 and 87 nmol glucose/$10^6$ cell/hr for RS4;11 and TOM-1 respectively; Figure 2).

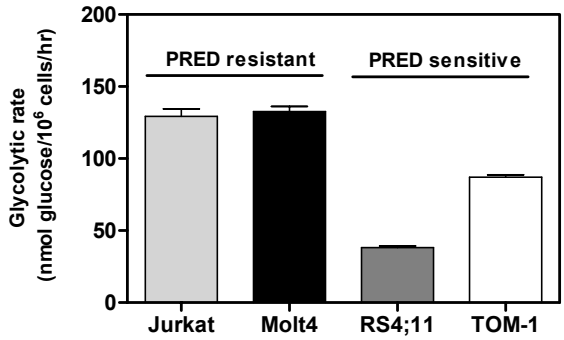


**Figure 2. Glycolytic rate in glucocorticoid (GC) resistant and sensitive ALL cell lines.** All cells lines were maintained at log-phase during culture and the baseline glycolytic rate was measured in cells incubated with a 5-$^{3}$H-glucose tracer by the production of $[^{3}H]_2O$ as described in "Materials and methods". Measurements of glycolytic rate were performed in triplicate. Individual bars represent the mean ± SD.

To further establish the causal relation between an enhanced glycolytic rate and PRED resistance, we investigated whether the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) has the potential to sensitize PRED-resistant cell lines to PRED-induced apoptosis. 2-DG is a non-metabolizable glucose analog that interferes with glycolysis by competing with glucose for key enzymes in the glycolysis pathway.[28] The effect of 2-DG is considered to be sensitizing when the observed effect of the combination of 2-DG and PRED is greater than the product of the effects of each individual agent. As shown in Figure 3, inhibition of glycolysis by 2-DG synergistically sensitized PRED-resistant but not PRED-sensitive ALL cells to PRED-induced apoptosis.

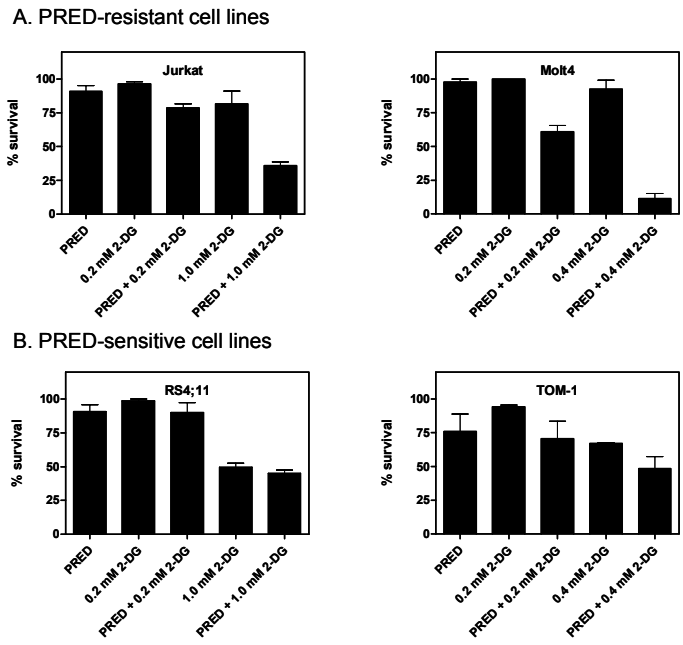


**Figure 3. The effect of 2-DG on PRED-induced cytotoxicity.** ALL cell lines were pre-incubated for 1 hour in 5% $CO_2$ at 37°C with the indicated doses of 2-DG. The % of surviving cells was subsequently determined by the MTT assay as described in "Materials and methods". MTT results are show for 2760 µg/ml PRED for (A) the PRED-resistant and 0.00011 µg/ml PRED and (B) for the PRED-sensitive ALL cell lines. Values shown are the mean ± SD of three independent experiments.

### The effect of 2-DG on doubling time in PRED-resistant cell lines

Theoretically, the influence of 2-DG is expected to be the largest in rapidly dividing cell lines due to their higher energy consumption. As indicated in Figure 4 both PRED-resistant cell lines divide more rapidly than the PRED-sensitive cell lines. Since this might bias our results, we determined the effect of 2-DG on the doubling time of PRED-sensitive and PRED-resistant cell lines. Exposure to 2-DG increases the doubling time for the PRED-sensitive RS4;11 cell line by ~2-fold (Figure 4). No doubling time could be determined for TOM-1 treated with 0.4 mM 2-DG due to the toxic effect of this 2-DG dosage on this cell line. In contrast to the PRED-sensitive cell lines, 2-DG exposure hardly affected the doubling time of both PRED-resistant cell lines (Figure 4). This excludes the possibility that the sensitizing effect of 2-DG in PRED-resistant ALL cell lines was due to interference of this compound with the proliferation rate of these cells.

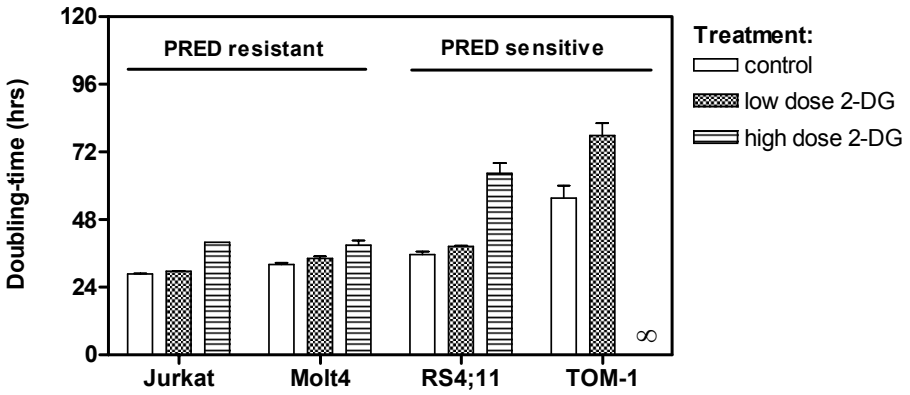


**Figure 4. The effect of 2-DG on doubling time.** Cells lines were treated with low dose 2-DG (0.2 mM) or high dose 2-DG (0.4 or 1.0 mM) or an equivalent volume of culture medium (control). The number of cells was counted every 24 hrs for 8 days and the doubling time was based on the number of viable cells as described in "Materials and methods". Since TOM-1 stopped dividing after treatment with 0.4 mM 2-DG, this doubling time could not be determined (indicated by a ∞ in the graph).

## DISCUSSION

Sensitivity of leukemic blasts to glucocorticoids is an important determinant of treatment response in childhood ALL. Identification of the possibilities to circumvent glucocorticoid resistance is therefore of clinical importance. We recently demonstrated overexpression of the glucose membrane transporter *GLUT3* and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) in PRED-resistant childhood B-lineage ALL.[21] These data suggested that an enhanced glycolytic rate may be involved in PRED-resistance in childhood ALL. Glycolysis is the primary source of ATP generation in malignant cells under both hypoxic and normoxic conditions (the Warburg effect)[29], suggesting that glycolysis plays a crucial role in the maintenance of the cancer cell.[30,31] Enhanced glycolysis as demonstrated by increased accumulation of the glucose analog $^{18}$fluorodeoxyglucose is observed in virtually all invasive human tumors.[30]

In the present study, we demonstrated that PRED-resistant ALL cell lines have an increased glycolytic rate compared to PRED-sensitive cell lines. The non-hydrolysable glucose analog 2-DG selectively sensitized PRED-resistant leukemic cells to PRED. These findings imply enhanced glycolysis as one of the factors underlying PRED resistance in ALL. How can an increased glycolytic rate hamper PRED-induced apoptosis? The facts that glycolysis is the primary source of ATP in malignant cells and exposure of leukemic cell lines to genotoxic agents, including glucocorticoids, led to down-regulation of glycolytic

metabolism[10,32-34] suggests that a decrease in cellular ATP levels is required for drug-induced apoptosis to occur. In agreement with this hypothesis, maintenance of ATP production after treatment with genotoxic agents was associated with resistance to apoptosis in Fanconi anemia.[35] Conversely, depletion of ATP by inhibition of glycolysis potently induced apoptosis in both solid[36] and leukemic cell lines[34,37] A recent study shed light on the mechanisms of ATP depletion-induced apoptosis in multidrug-resistant leukemic cells; inhibition of glycolysis led to a rapid dephosphorylation of the glycolysis-apoptosis integrating molecule BAD[38], relocalization of BAX to mitochondria, and massive cell death.[39] Taken together these data suggest that the decreased apoptosis we previously observed in resistant ALL cells after exposure to PRED[17] may be explained by their enhanced glycolytic rate. This higher glycolytic rate may facilitate ATP production under conditions were mitochondrial functionality is normally inhibited (e.g. upon exposure to PRED).

An attractive explanation for the augmented glycolytic rate as observed in PRED-resistant ALL cell lines may be the activity of the serine-threonine kinase Akt.[40] Constitutive activation of Akt is commonly observed in malignant cells and is directly responsible for the enhanced glycolytic rate of cancer cells.[41] The serine-threonine kinase mammalian target of rapamycin (mTOR) is a downstream target of Akt. The mTOR-inhibitor rapamycin has been shown to inhibit Akt-mediated maintenance of glycolysis[22] and sensitized primary multiple myeloma cells to dexamethasone-induced apoptosis.[42] One of the factors responsible for the Akt-induced increase in glycolysis is the hypoxia-inducible factor (HIF). The HIF transcriptional complex is a heterodimer that consists of a constitutively β-subunit (HIF-1β) and a α-subunit (HIF-1α, HIF-2α, or HIF-3α) that accumulates rapidly under hypoxic conditions or after activation of mTOR.[43] Activation of the HIF pathway leads to an enhanced glycolytic rate by inducing the expression of a wide array of key genes regulating glycolysis, including *GLUT3* and *GAPDH*.[43-45] Interestingly, it has been recently demonstrated that the oxygen-regulated component of HIF-1 (HIF-1α) is overexpressed in bone marrow samples of childhood ALL and absent in biopsies of normal bone marrow.[46] These data imply that PRED resistance may be explained by constitutive activation of Akt and HIF signaling pathways resulting into an increased glycolytic rate.

In conclusion, PRED resistance in ALL cells is linked to a higher glycolytic rate which can be selectively modulated by the glycolysis inhibitor 2-DG. Historically, enhanced glycolysis was thought of as an adaptation to the hypoxic conditions that occur in the core of solid tumors.[30] Here we show that enhanced glycolysis also occurs in leukemias. At the moment, a phase I clinical trail is being conducted that evaluates the effectiveness of 2-DG

as a single agent or in combination with docetaxel in patients with solid tumors.[31] Our present data as well as the recent observation that HIF-1a is overexpressed in bone marrow samples of children with ALL suggest that 2-DG may also be applied to sensitize resistant leukemic cells to glucocorticoids in pediatric ALL.

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Chapter 9

# General discussion

Chapter 9

## 9.1 Summary

The treatment of pediatric acute leukemia has greatly improved over the past 4 decades, resulting in long-term disease-free survival of approximately 80% for ALL[1-3] and 60% for AML.[4] Despite this progress, a considerable number of children ultimately relapse with a disease that is highly refractory to further treatment. A high proportion of the contemporary treatment failures can be contributed to cellular drug resistance. However, relatively little is known about the causes of cellular drug resistance in childhood acute leukemia.

In the last years it has become clear that most, if not all, chemotherapeutic agents ultimately induce programmed cell death or apoptosis in their target cells.[5] Defects in the apoptosis route allow genetically instable cells to survive and are thought of as one of the major driving forces behind leukemogenesis.[6,7] These observations led to the hypothesis that aberrations in the apoptosis pathway contribute to cellular drug resistance in children with acute leukemia. Therefore, a thorough knowledge of the aberrations in the apoptosis route is critical for understanding the causes of treatment failure and for a rational approach to drug design and therapy.

In **chapter 2** a literature overview is given of the current knowledge on apoptosis and defects in the execution phase of apoptosis contributing to cellular drug resistance in childhood ALL and AML. Each cell possesses two major pathways which can induce apoptosis upon activation, the mitochondrial or “intrinsic” pathway and the death receptor or “extrinsic” pathway. Activation of both pathways leads to cellular disassembly by the activity of a family of cysteine proteases called caspases.[8] There is general agreement that the intrinsic apoptosis pathway is involved in drug-induced apoptosis. The role of the extrinsic apoptosis pathway in drug-induced apoptosis, however, remains controversial.[9,10,11,12]

Functional blocks in both apoptosis pathways have been identified in childhood acute leukemia. Most reports failed to demonstrate an association between expression levels of apoptotic proteins and cellular drug resistance. Moreover, the studies that did show an association were contradictory. This may be explained by the fact that most studies examined a small number of apoptosis proteins in rather heterogeneous groups of children with acute leukemia. In addition, aside from the Bcl-2 and IAP family, almost none of the large numbers of known apoptosis regulators has been examined in clinical specimens of childhood acute leukemia until now. In the subsequent chapters we describe our studies that investigate whether aberrations, either in or outside the apoptosis pathway, contribute to cellular drug resistance and treatment failure in childhood acute leukemia.

We started by testing the hypothesis that cellular drug resistance is associated with decreased functional apoptosis in childhood ALL. To this aim, drug-induced apoptosis was compared between sensitive and resistant ALL cells to 4 chemotherapeutic drugs which form an integral part of contemporary treatment protocols, i.e. prednisolone, vincristine, L-asparaginase and daunorubicin, in **chapter 3**. Flow cytometry was used to assess functional apoptosis at various levels of the apoptosis route, i.e. phosphatidylserine externalization, collapse of mitochondrial transmembrane potential ($\Delta\Psi_m$), caspase-3 activation and PARP inactivation. Exposure to each drug resulted in early induction of phosphatidylserine externalization and $\Delta\Psi_m$ depolarization followed by caspase-3 activation and PARP inactivation in the majority of patients. For all four drugs, a significant inverse correlation was found between cellular drug resistance and (1) the percentage of cells with phosphatidylserine externalization and (2) the percentage of cells with $\Delta\Psi_m$ depolarization. However, the percentage of cells with caspase-3 activation and the percentage of cells with PARP inactivation showed a significant inverse correlation with cellular resistance for prednisolone and L-asparaginase only. This suggests that caspase-3 activation and PARP inactivation are not essential for vincristine and daunorubicin -induced apoptosis. In conclusion, resistance to 4 unrelated drugs is associated with defect(s) upstream or at the level of phosphatidylserine externalization and $\Delta\Psi_m$ depolarization. This leads to decreased activation of apoptotic parameters in resistant cases of pediatric ALL.

We next investigated whether the decreased apoptosis observed in drug resistant ALL samples was associated with defects in the effector apoptosis route itself. To this aim we used micro-array technology to analyze the expression of 70 key apoptosis-associated genes in leukemic cells of 190 children with newly diagnosed ALL in **chapter 4**. Expression of MCL1 and DAPK1 as well as BCL2L13, HRK and TNF was significantly associated with prednisolone and L-asparaginase resistance respectively. No single apoptosis-related gene was associated with resistance to all four unrelated drugs. High Bcl2L13 expression was not only associated with L-asparaginase resistance, but also with unfavorable clinical outcome. Multivariate analysis including known risk factors revealed that BCL2L13 is an independent prognostic factor in pediatric ALL. The same trend was observed in a validation group of 92 children with newly diagnosed ALL treated on a different protocol.

Childhood ALL is a heterogeneous disease consisting of various genetic subtypes such as t(1;19)/[E2A-PBX1], t(9;22)/[BCR-ABL], t(12;21)/[TEL-AML1], 11q23/[MLL] rearrangements, hyperdiploidy (>50 chromosomes) and T-lineage ALL, that differ markedly in their treatment response.[13] Leukemia subtypes with a relatively unfavorable prognosis have been associated with *in vitro* drug resistance[14-16] and subtypes with a favorable

prognosis with *in vitro* drug sensitivity.[17,18] Therefore, we also investigated in **chapter 4** whether these subtypes are associated with differential expression of any of these 70 apoptosis-associated genes. Differential expression of 44 out of 70 apoptosis genes was observed in T-lineage ALL, 22 in hyperdiploid, 16 in TEL-AML1 positive, 13 in E2A-rearranged B-lineage ALL. The simultaneous upregulation of several NF-κB target genes in T-lineage ALL may point to enhanced NF-kB activity in T-lineage compared to B-lineage ALL. The TNF receptor ligand (TNF) is expressed higher in hyperdiploid, TEL-AML1 positive B-lineage ALL *and* in L-asparaginase sensitive patients. Since both hyperdiploid and TEL-AML1 positive B-lineage ALL are *in vitro* sensitive to L-asparaginase,[17,18] these data point to novel insights in the apoptotic features underlying L-asparaginase cytotoxicity. In conclusion, ALL subtypes have a unique expression pattern of apoptosis genes, which can be used to generate new hypotheses regarding the origin of cellular drug resistance in different leukemic subtypes.

Decreased mRNA levels or increased mRNA levels of anti-apoptotic of pro-apoptotic genes may not necessarily reflect the actual activity at the protein level.[19-24] Therefore, we used quantitative Western blotting to investigate protein levels of Apaf-1, procaspase-2, -3, -6, -7, -8, -10 and PARP in children with newly diagnosed ALL and AML in **chapter 5**. PARP expression was absent in 4/15 T-ALL and 3/10 AML cases. The absence of PARP protein was associated with decreased PARP mRNA levels, which were not caused by genomic deletions. PARP expression was 7-fold lower in T-lineage ALL and 10-fold lower in AML compared to B-lineage ALL. In addition, PARP expression was lower in prednisolone, vincristine and L-asparaginase (PVA) resistant compared to sensitive patients. Not only PARP, but also procaspase-2 expression was decreased in T-lineage ALL (3-fold) and AML (3-fold) compared to B-lineage ALL and in PVA resistant patients compared to sensitive patients (2-fold). No relation between Apaf-1, procaspase-3, -6, -7, -8, -10 and drug resistance was found. In conclusion, expression of PARP and procaspase-2 is lower in leukemic subtypes with an unfavorable prognosis and low expression of these proteins is related to cellular drug resistance in childhood acute leukemia.

In **chapter 6** a genome-wide approach was used to identify gene-expression patterns associated with cellular drug resistance in leukemic cells of children with newly diagnosed ALL. We identified 42, 59, 54 and 22 gene probes that were differentially expressed in B-lineage leukemia cells that were either *in vitro* sensitive or resistant to prednisolone, vincristine, L-asparaginase or daunorubicin, respectively. These include carbohydrate metabolism-associated genes (e.g., *CS*, *GAPDH* and *GLUT3*) and transcription associated genes (e.g., *SMARCB1*, *PRPF18*, and *CTCF*) for prednisolone,

translation-associated genes (e.g., *RPS3*, *RPL3*, EIF3S7 and *EEF1G*) for L-asparaginase and cytoskeleton- and extracellular matrix-associated genes (e.g., *TMSB10*, *PDLIM1*, and *DSG3*) for vincristine. The set of genes associated with daunorubicin resistance formed a heterogeneous group, where no single pathway was overrepresented. The expression pattern of these 124 genes and 28 ESTs was significantly related to treatment outcome, in a multivariate analysis with other known prognostic variables. Furthermore, the expression of these genes discriminated treatment outcome in an independent population of patients treated on a different protocol at a different institution. Notably, 121 of the 124 discriminating genes have not been previously associated with resistance to these anticancer agents. So, differential expression of a relatively small number of genes confers drug resistance and alters treatment outcome in childhood acute lymphoblastic leukemia.

Children with acute lymphoblastic leukemia (ALL) may benefit from the discovery of new prognostic factors to improve risk group stratification. Recently, high expression of a novel gene named *OPAL1* (Outcome Predictor in Acute Leukemia) was reported as a new risk factor associated with favorable prognosis in childhood ALL.[25-27] In **chapter 7** we investigated whether *OPAL1* was of prognostic importance in two independent cohorts of children treated on COALL-92/-97 (n=180) and on St. Jude Total 13 protocols (n=257). We observed a 2.8-fold higher expression of *OPAL1* in *TEL-AML1*-positive compared to *TEL-AML1*-negative B-lineage ALL in both cohorts. In contrast to the original report[25-27], we found no consistent association between high *OPAL1* expression and favorable subtypes defined by age and white blood cell count at diagnosis (WBC), gender, immunophenotype or genetic abnormalities. In addition, *OPAL1* expression was not associated with increased *in vitro* sensitivity to prednisolone, vincristine, L-asparaginase or daunorubicin and was not independently related to induction failure or long-term clinical outcome (DFS) in the total group of patients or in specific subgroups, such as T-lineage, *TEL-AML1*-positive and *TEL-AML1*-negative B-lineage ALL in either cohort. In conclusion, *OPAL1* gene expression is not an independent prognostic marker for childhood ALL treated on contemporary COALL and St. Jude protocols, and its previously reported prognostic relevance therefore appears to be treatment-specific.

The large advantage of micro-array technology is that the findings provide new insights into the basis of treatment failure and may point to novel targets for developing strategies to overcome drug resistance. In the chapter 6, we observed that prednisolone resistance in childhood B-lineage ALL was associated with concomitant upregulation of genes involved in glycolysis, i.e. the glucose transporter 3 (GLUT3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Therefore, we investigated in **chapter 8** whether an

enhanced glycolytic rate was associated with prednisolone resistance and whether inhibition of the glycolysis augmented prednisolone-induced cytotoxicity in human leukemia cell lines. We observed an enhanced glycolytic rate in 2 prednisolone-resistant compared to 2 prednisolone-sensitive cell lines. The glycolytic inhibitor 2-deoxy-D-glucose markedly sensitized the prednisolone-resistant cell lines to prednisolone-induced apoptosis, but had no effect in the prednisolone-sensitive cell lines.

In summary, we identified various aberrations, both at mRNA, protein and activation level, within the apoptosis route that were associated with cellular drug resistance in childhood acute leukemia. In addition, by applying a genome-wide analysis, we observed that aberrations in drug-specific pathways upstream of the mitochondria (glycolysis, protein synthesis) were associated with cellular drug resistance. The lack of apoptosis genes within the selected genes suggests that, at least at mRNA levels, defects upstream of the effector apoptosis pathway are more important than defects within the apoptosis pathway in cellular drug resistance in children with ALL. Alleviation of these aberrations may be a valuable tool to enhance the efficacy of chemotherapy in childhood acute leukemia in the future.

## 9.2 General discussion and future perspectives.

In this thesis, we investigated whether defects in the apoptosis route were associated with cellular resistance to 4 drugs that form an integral part of anti-leukemic therapy in children, i.e. prednisolone, vincristine, L-asparaginase and daunorubicin. Induction of apoptosis by chemotherapeutic agents can be subdivided into three general phases: insult generation, signal transduction and execution (Figure 1).

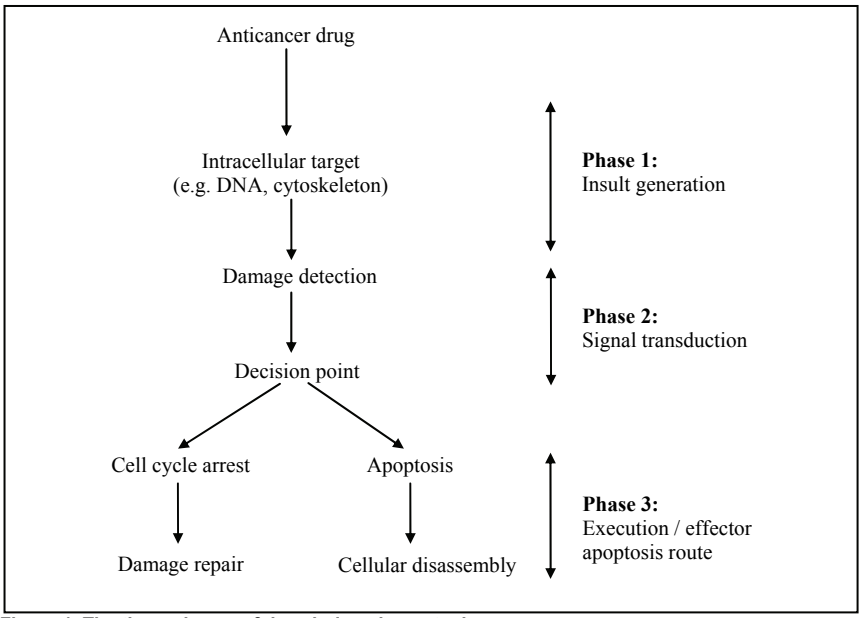


**Figure 1. The three phases of drug-induced apoptosis**

The main results of the conducted studies were summarized in the previous paragraph. We observed no single apoptosis-associated gene which expression was associated with resistance to all 4 antileukemic agents examined. This is in agreement with our recent study, where no apoptosis-associated genes were among the genes discriminative for *de novo* crossresistance to these 4 agents in newly diagnosed ALL.[28] Table 1 provides an overview of the aberrations in the apoptosis pathway we described in this thesis for each individual antileukemic agent. Since the apoptotic defects associated with sensitivity or resistance differ for each drug, our findings point to strategies whereby one could modulate specific components of therapy. In this paragraph, we will address the most promising modulation targets to modulate for each drug.

**Prednisolone**

We identified aberrations within the effector apoptosis route at mRNA (chapter 4), protein (chapter 3 and 4) and functional level (chapter 3 and 5) in prednisolone resistant ALL samples.

We observed increased expression of the anti-apoptotic Bcl-2 family member Mcl-1 in ALL cells resistant to prednisolone (Table 1). Interestingly, a recent report described *BCR-ABL*-dependent upregulation of Mcl-1 in *BCR-ABL* positive primary chronic myeloid leukemia (CML) cells.[29] This suggests Mcl-1 may be involved in prednisolone resistance commonly observed in *BCR-ABL* positive childhood ALL.[30] Depletion of Mcl-1 levels by antisense Mcl-1 oligonucleotides sensitized lung cancer cells lines to apoptosis induced by cytotoxic agents as well as by ionizing radiation.[31]

**Table 1. Drug-specific defects with the apoptosis pathway**

| Gene involved | Mechanism in resistant samples | Chapter |
|---|---|---|
| **PRED resistance** | | |
| - | Decreased drug-induced PS externalization | 3 |
| - | Decreased drug-induced $\Delta\Psi_m$ externalization | 3 |
| CASP2 | Decreased protein expression in untreated cells | 5 |
| CASP3 | Decreased drug-induced cleavage | 3 |
| DAPK1 | Increased mRNA expression in untreated cells | 6 |
| MCL1 | Increased mRNA expression in untreated cells | 6 |
| PARP | Decreased protein expression in untreated cells | 5 |
| PARP | Decreased drug-induced cleavage | 3 |
| **VCR resistance** | | |
| - | Decreased drug-induced PS externalization | 3 |
| - | Decreased drug-induced $\Delta\Psi_m$ externalization | 3 |
| PARP | Decreased drug-induced cleavage | 3 |
| **ASP resistance** | | |
| - | Decreased drug-induced PS externalization | 3 |
| - | Decreased drug-induced $\Delta\Psi_m$ externalization | 3 |
| BCL2L13 | Decreased mRNA expression in untreated cells | 6 |
| CASP3 | Decreased drug-induced cleavage | 3 |
| HRK | Decreased mRNA expression in untreated cells | 6 |
| **DNR resistance** | | |
| - | Decreased drug-induced PS externalization | 3 |
| - | Decreased drug-induced $\Delta\Psi_m$ externalization | 3 |

A promising new strategy to inhibit Mcl-1 is the administration of the oral multi-kinase inhibitor Sorafenib or BAY 43-9006. This compound has broad-spectrum antitumor activity

in colon, breast, and non-small-cell lung cancer xenograft models. Its activity in various malignancies, including CML and AML, is currently assessed in clinical trials.[32] Treatment of various cell lines, including leukemic cells lines, with BAY 43-9006 diminished Mcl-1 levels in a dose-dependent fashion without affecting other Bcl-2 family members.[33] Taken together, these data suggest that BAY 43-9006 is an attractive candidate for modulation of prednisolone resistance in childhood ALL.

Enzymes of the carbohydrate metabolism were relatively overrepresented in the set of genes associated with prednisolone resistance. The simultaneous upregulation of the glucose transporter *GLUT3* and the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) suggested that prednisolone resistance is associated with an enhanced glycolytic rate (chapter 6). Indeed, we observed an increased glycolytic rate in prednisolone-resistant compared to prednisolone-sensitive ALL cell lines (chapter 8). Moreover, administration of the glycolysis inhibitor 2-deoxy-G-glucose (2-DG) sensitized prednisolone-resistant ALL cell lines towards prednisolone-induced apoptosis (chapter 9). The clinical potential of 2-DG was recently underlined in a study where administration of 2-DG increased the efficacy of standard chemotherapeutic drugs *in vivo* when applied in nude mouse xenograft tumor model[34]. The toxicity of 2-DG is currently assessed in a phase I clinical trial for solid tumors and lymphomas.[35] The glycolytic inhibitor 3-BrPA was able to substantially increase the cytotoxic effect of doxorubicin, vincristine, or Ara-C in multidrug resistant AML cell lines.[36] A disadvantage of the use of glycolytic inhibitors is that its effectiveness is significantly affected by the presence of its natural counterpart glucose.[37] Further research is required to determine whether the use of glycolysis inhibitors may lead to clinical benefit in childhood acute leukemia.

**Vincristine**

Besides defects within the apoptosis pathway (Table 1), vincristine resistance was associated with cytoskeleton- and extracellular matrix-associated genes (chapter 6). The association between vincristine resistance and aberrant expression of cytoskeleton proteins is in line with microtubule alterations previously observed in vincristine-resistant ALL cell lines[38]. As mentioned in chapter 1, vinca alkaloids like vincristine exert its cytotoxic effect by inhibiting tubulin polymerization and disrupting overall cytoskeletal integrity. Vincristine has been found to work synergistically with the actin depolymerizing agent cytochalasin B[39]. These data suggest that modulation of cytoskeleton proteins other than tubulin, such as *TMSB10* or actin, may offer a strategy to sensitize leukemia cells to vinca alkaloids. A recent report described aberrant expression of multiple cytoskeleton proteins in

vincristine resistant ALL cell lines, i.e. tubulin-associated genes, actin, actin-associated genes and the intermediate filament lamin B.[40] It should be noted that vincristine resistance was induced in this study by culturing ALL cell lines in the continuous presence of vincristine. The cytoskeletal defects that accumulated in these cell lines may not necessarily reflect the *in vivo* situation. Therefore, it remains to be determined whether modulation of cytoskeletal proteins will increase the therapeutic efficacy of leukemia therapy in vincristine-resistant children with acute leukemia.

**L-asparaginase**

Besides defects within the apoptosis pathway (Table 1), L-asparaginase resistance in childhood ALL was associated with overexpression of a large group of ribosomal proteins, initiation and elongation factors (chapter 6). We hypothesized that his may be caused by a constitutively activated mTOR pathway in L-asparaginase resistant ALL samples. Mutations leading to overexpression of eIF-4E/4E-BP1 and $p70^{s6k}$ have been previously described to induce cellular transformation[41-43] and are commonly observed in several types of human cancers[44] Rapamycin (sirolimus) is an anti-proliferative and immunosuppressive *streptomyces* derivative which inhibits the protein kinase activity of mTOR and blocks downstream signaling events, including the transcription of TOP-containing mRNAs, which encode many components of the translational apparatus such as ribosomal proteins, initiation and elongation factors (Fig 2).

In our hands, the use of rapamycin did not sensitize primary ALL and AML cells to L-asparaginase-induced apoptosis (unpublished data). However, a recent report showed that rapamycine induced apoptosis in primary leukemic blasts in children with ALL and, more importantly, increased doxorubicin-induced apoptosis even in non-responder samples.[45] A closer look at the data described in that report learned that rapamycin did not induce significant apoptosis in 11 of the 25 leukemia samples (44%). Interestingly, 11 of the 14 good *in vitro* responders to rapamycine had a good *in vivo* prednisone response and 8 of the 11 *in vitro* poor responders to rapamycin had a poor *in vivo* prednisone response.[45] This suggests that the effectiveness of rapamycine may be limited in drug resistant ALL samples. Moreover, caution must be taken when using ribosomal protein inhibitors to overcome L-asparaginase resistance. We demonstrated that overexpression of the same group of ribosomal proteins was associated with L-asparaginase resistance but also with vincristine sensitivity in newly diagnosed ALL.[28] Therefore, although ribosomal protein inhibitors may have the potential to sensitize ALL cells to L-asparaginase-induced apoptosis they may actually increase vincristine resistance.

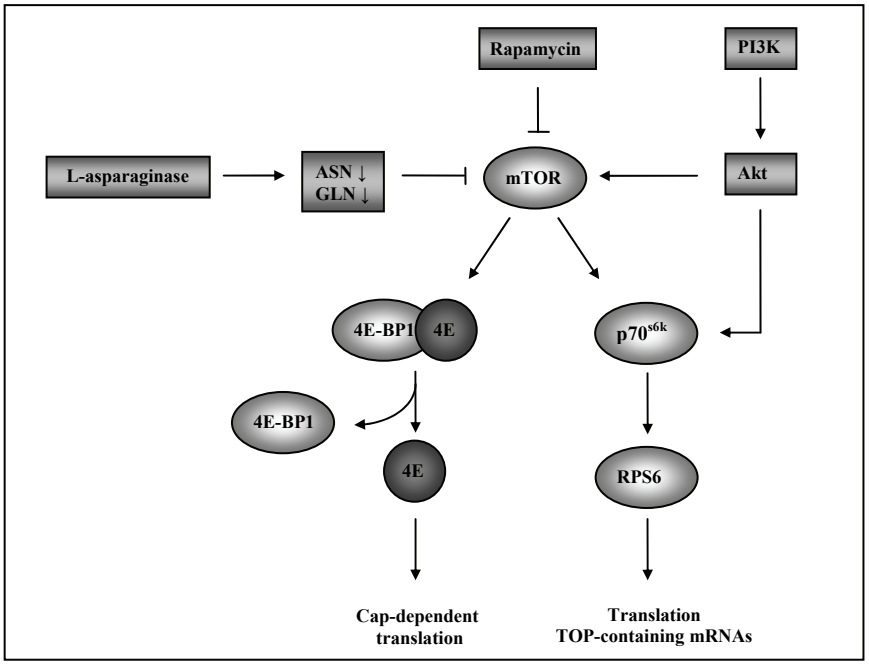


**Figure 2. Overview of mTOR-mediated signaling in mammalian cells.** *Abbreviations:* ASN: asparagine, GLN: glutamate, mTOR: mammalian target of rapamycin, PI3K: phosphatidylinositol-3-kinase, $p70^{S6K}$: p70 S6 kinase, RPS6: ribosomal protein S6, TOP: oligonucleotide tract rich in pyrimidines juxtaposed to the 5'-cap of particular transcripts, 4E: eukaryotic initiation factor 4E, 4E-BP1: 4E-binding protein

**Daunorubicin**

The set of genes associated with daunorubicin resistance formed a heterogeneous group, where no single pathway was overrepresented (chapter 6). One of the genes that was over-expressed in DNR resistant ALL was chromodomain helicase DNA-binding protein 4 (CHD4). CHD4 is a central component of the nucleosome remodeling and histone deacetylation (NRD) complex, which leads to transcriptional repression[46]. Indeed, the histone deacetylase inhibitor (HDAC) AN-9 has been shown to sensitize non-leukemic cell lines to the cytotoxicity of anthracyclines[47,48]. The synergy between doxorubicin and AN-9 is caused by the ability of AN-9 metabolites to facilitate the formation of DNA-doxorubicin adducts[48,49]. This suggests that targeting CHD4 and/or HDACs may be new strategies to circumvent DNR resistance in pediatric ALL. The clinical benefit of several HDAC inhibitors is currently evaluated in patients with various malignancies, including leukemia, in phase I clinical trails.

**Gene-expression signatures profiling and cellular drug resistance**

As discussed in the previous paragraphs, gene expression profiling has revealed new important clues for causes of resistance to 4 classes of drugs in children with ALL. The relationship between affected genes and drug resistance is currently being functionally validated as well as ways to modulate drug resistance by targeting the affected genes and/or pathways. It should be noted that the prediction accuracy of drug resistance-associated gene expression signatures was lower (i.e. ~70-75%) compared to the prediction accuracy found for signatures that can be used to classify ALL[50-53] or AML[54-56] in immunophenotypic and genetic subgroups (i.e. ~80-100%). This may be explained by the fact that the gene expression signatures predictive for immunophenotype and genetic subgroups are more discriminative than the signature associated with drug resistance. For example, we found that unsupervised hierarchical clustering clustered patients according to immunophenotype rather than according to their resistance to any of the four antileukemic agents in children with ALL at initial diagnosis.[57] These data suggest that the relatively lower prediction accuracies of ~70-75% for drug resistance may be caused by the strong influence of genetic subtypes on the expression signatures of individual leukemic samples.

**Gene-expression profiling and treatment outcome**

The gene-expression signatures that were associated with resistance to individual agents were also related to treatment outcome in two independent cohorts of children with ALL. In 2002, Van 't Veer *et al*. applied micro-array technology to identify a set of 70 genes able to predict clinical outcome in women with breast cancer with 90% accuracy.[58] This has led to the development of a chip carrying these 70 discriminative genes, which will be used in the near future to select patient with an unfavorable prognosis who will benefit from adjuvant chemotherapy. As mentioned above, the prediction accuracy of the expression signatures for drug resistance described in chapter 6 was ~70% in the total group and ~73%in the B-lineage ALL group.[57] This is prediction accuracy is too low to create a "prognostic microarray" containing only these drug resistance-predictive genes. It should be noted that the primary selection of genes in this study was based upon *in vitro* drug resistance profiles and not on treatment outcome and the relationship between this gene signature and outcome was done to confirm the clinical relevance of these resistance genes. A new selection of genes directly based upon treatment outcome most likely will produce expression signatures with higher outcome prediction accuracies. Advantages of the use of micro-arrays as treatment stratification tools are that they allow a fast screening. For instance gene expression profiles can be used to distinguish between ALL and AML

subtypes with different prognosis.[50-56] A micro-array combining genes associated with subtype classification and relapse would be a valuable diagnostic tool. The use of gene expression signatures for subtype classification and relapse prediction in pediatric ALL is prospectively evaluated in the new SKION ALL-10 treatment protocol, which has started in November 2004. Future generation whole-genome chips may help to identify additional genes and increase the prediction accuracy.

### 9.3 Conclusions

The data described in this thesis suggest that cellular drug resistance to four widely used chemotherapeutic agents in children with acute leukemia cannot be explained by one isolated defect. As illustrated in Figure 2, simultaneous defects upstream **and** in the effector apoptosis route were described in this thesis. These defects may contribute to cellular drug resistance in children with acute leukemia.

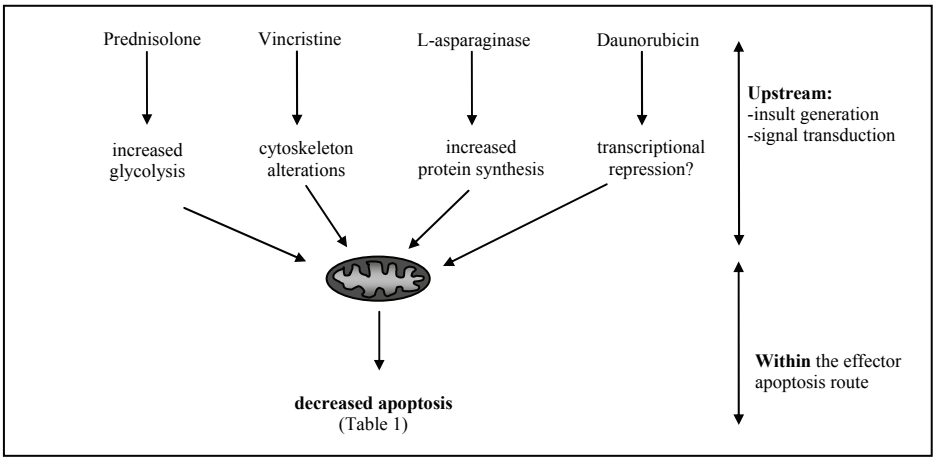


**Figure 2. Summary of drug-specific defects associated with cellular drug resistance in childhood acute leukemia**

Importantly, new mechanisms of drug resistance have been described in this thesis, which may lead to a better understanding of the underlying biology of cellular drug resistance in childhood acute leukemia. This knowledge may be applied in the future to develop rational treatment strategies in order to improve the efficacy of chemotherapy in childhood acute leukemia. This is exemplified by the rational strategy we used for augmenting the efficacy of prednisolone-induced cytotoxicity in prednisolone-resistant acute leukemia.

The majority of studies described in this thesis have been performed in untreated primary ALL and AML cells. The big advantage of this approach is that genes discovered in untreated samples can be used as a treatment stratification tool at the onset of treatment. Treatment-induced changes in the expression level of a small number of apoptotic proteins have been reported in leukemic cells.[59,60] The relation between treatment-induced changes in a large number of apoptosis-associated genes or proteins and cellular drug resistance has not been investigated. Screening of treatment-induced changes of gene expression both at mRNA and protein level will give additional insights in the mechanisms of cellular drug resistance.

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Chapter 10

# Nederlandse samenvatting

## INTRODUCTIE

### Wat is leukemie?

Bij een gezonde persoon worden in het beenmerg witte bloedcellen worden gevormd, die vervolgens afgegeven worden aan het bloed. In het bloed aangekomen spelen ze een belangrijke rol bij de bescherming van het lichaam tegen infecties. Leukemie, ook wel “bloedkanker” genoemd, begint met een foutje in een witte bloedcel, waardoor deze ongeremd gaat delen. Na een tijdje zitten er zo veel abnormale witte bloedcellen in het beenmerg dat er onvoldoende ruimte is voor andere cellen die ook in het beenmerg gevormd worden, zoals normale rode en witte bloedcellen en bloedplaatjes. Dit leidt tot de karakteristieke klinische symptomen van leukemie: bloedarmoede, bloedingen en infecties door onvoldoende afweer.

### De verschillende soorten leukemie

Afhankelijk van het type witte bloedcel waarin het foutje voorkomt, kan leukemie worden onderverdeeld in 2 groepen: myeloïde of lymfatische leukemie. Op basis van de snelheid waarmee er klinische symptomen ontstaan wordt leukemie nog verder ingedeeld in: acute of chronische leukemie. Chronische leukemie waarbij de klinische symptomen geleidelijk ontstaan komt vaker voor bij ouderen. Bij patiënten met acute leukemie ontwikkelen de symptomen zich snel. Als patiënten met acute leukemie niet behandeld worden, zal het merendeel daarom binnen een paar weken tot maanden aan de gevolgen van de ziekte overlijden. Bij patiënten met chronische leukemie duurt dit langer. Acute leukemie is de meest voorkomende vorm van kanker bij kinderen. Ongeveer 80% van de kinderen met leukemie heeft acute lymfatische leukemie (ALL) en 15-20% van de kinderen heeft acute myeloïde leukemie (AML).

### De behandeling van kinderen met acute leukemie

Kinderen met acute leukemie worden behandeld met cytostatica, dit zijn geneesmiddelen die de kwaadaardige leukemiecellen moeten vernietigen. Tijdens de chemotherapie worden er verschillende combinaties van cytostatica gebruikt. Door een continue verbetering van de chemotherapieprotocollen bereikt momenteel meer dan 98% van de kinderen met ALL en 80-90% van de kinderen met AML een complete remissie. Van de kinderen die complete remissie bereiken zal op de lange termijn echter bij 20-25% van de kinderen met ALL en 40-50% van de kinderen met AML de leukemie weer terug komen, dit noemt men een recidief. Bij kinderen die een recidief hebben gehad, is moeilijker om alle

leukemiecellen weer weg te krijgen. Van deze kinderen geneest uiteindelijk minder dan 50% met ALL en 30-35% met AML.

**Cellulaire drug resistentie**

ALL is de meest voorkomende kanker op de kinderleeftijd met 120 nieuwe patiënten in Nederland per jaar. Daarom is het van groot belang dat uitgezocht wordt waarom sommige kinderen met acute leukemie niet genezen. De algemene gedachte is dat bij de kinderen waarbij de leukemie niet verdwijnt de leukemiecellen niet adequaat op cytostatica kunnen reageren. Dit verschijnsel wordt cellulaire drug resistentie genoemd.

Uit laboratoriumonderzoek is gebleken dat alle cytostatica die gebruikt worden in de behandeling van de acute leukemie de cellen aanzetten tot zelfmoord, ook wel apoptose genoemd. Dit heeft geleid tot de gedachte dat de leukemiecellen van kinderen die niet genezen fouten hebben in de apoptose route. Er is echter maar weinig bekend over eventuele defecten in de apoptose route in de cellen van kinderen met acute leukemie en de rol daarvan bij cellulaire drug resistentie. Daarom hebben we in dit proefschrift de mechanismen van cellulaire drug resistentie bestudeerd en ons vooral toegespitst op de identificatie van mogelijke fouten in de apoptose route in leukemiecellen die resistent zijn voor cytostatica.

## NIEUWE GENOMISCHE DETERMINANTEN VAN APOPTOTISCHE DEFECTEN IN ACUTE LEUKEMIE

In **hoofdstuk 2** van dit proefschrift wordt een uitgebreid overzicht gegeven van de huidige kennis op het gebied van apoptose en de defecten in de apoptose route die tot op dat moment beschreven waren in cellen van kinderen met leukemie. Zodra een leukemiecel cytostatica vanuit het bloed opneemt, wordt normaliter een “zelfmoordprogramma” ofwel apoptose geactiveerd. Zodra dit programma aan gaat, worden er een heleboel eiwitten in de cel actief, waaronder caspases. Actieve caspases knippen als een soort schaartjes de cel van binnenuit kapot. Caspases kunnen behalve door cytostatica (de intrinsieke apoptose route) ook door cytokines zoals TNFα (de extrinsieke apoptose route) geactiveerd worden.

Uit de literatuur blijkt dat er wel al eerder defecten zijn gevonden in met name de intrinsieke apoptose route bij kinderen met acute leukemie. Het bleek echter een stuk moeilijker om een consistent verband te vinden tussen deze defecten en cellulaire drug

resistentie. Vaak zijn in deze studies slechts enkele van de vele mogelijke eiwitten bestudeerd die een rol spelen in de apoptose route.

**Apoptose en cytostatica resistentie**

In **hoofdstuk 3** hebben we een aantal apoptose kenmerken onderzocht in de resistente cellen van kinderen met ALL wanneer ze in het laboratorium worden blootgesteld aan verschillende cytostatica. De 4 cytostatica die we getest hebben (prednisolon, vincristine, L-asparaginase en daunorubicine) vormen een belangrijke component van de chemotherapieprotocollen die gebruikt worden voor de behandeling van kinderen met ALL.

Nadat de leukemiecellen waren bloot gesteld aan een van de 4 cytostatica hebben we de activatie van diverse apoptose-parameters gemeten. Daarnaast werden voor dezelfde cellen gemeten of ze gevoelig of juist resistent waren voor deze 4 cytostatica met behulp van de *in vitro* MTT test. Met deze test wordt bepaald hoeveel cellen doodgaan na blootstelling aan deze cytostatica.

Behandeling met ieder van de 4 cytostatica leidde tot activatie van 4 belangrijke fases in de apoptose route, d.w.z. phosphatidylserine externalisatie en depolarisatie van de mitochondriale transmembraan-potentiaal ($\Delta\Psi_m$), activatie van caspase-3 en inactivatie van PARP. Uit het onderzoek bleek echter dat ALL cellen die *in vitro* resistent waren voor prednisolon of L-asparaginase verminderde phosphatidylserine externalisatie, $\Delta\Psi_m$ depolarisatie, caspase-3 activatie én PARP inactivatie vertoonden vergeleken met de *in vitro* gevoelige cellen. ALL cellen die *in vitro* resistent waren voor vincristine en daunorubicine vertoonden ook een sterk verminderde apoptotische activiteit, maar dan alleen op het niveau van phosphatidylserine externalisatie en $\Delta\Psi_m$ depolarisatie. Dit suggereert dat caspase-3 activatie en PARP inactivatie niet noodzakelijk zijn voor apoptose geïnduceerd door vincristine en daunorubicine. Concluderend kunnen we stellen dat er in *in vitro* resistente cellen van kinderen met ALL iets fout gaat voordat er phosphatidylserine externalisatie en $\Delta\Psi_m$ depolarisatie optreedt. Dit leidt tot een verminderde activiteit op alle fases van de apoptose route in resistente kinderen met ALL cellen.

**mRNA expressie van apoptose genen en cytostatica resistentie**

*In vitro* resistente ALL cellen gaan minder snel dood na behandeling met cytostatica. Onbekend is echter of dit komt omdat resistente cellen defecten hebben in de uiteindelijke

uitvoer van het apoptose programma of dat dit komt doordat resistente cellen over een mechanisme beschikken waardoor het cytostaticum zijn werk niet kan doen en daardoor ook nooit tot apoptose kan aanzetten . In een cel worden allerlei eiwitten actief als een cel in apoptose gaat. Of die eiwitten wel of niet in de cel aanwezig zijn, wordt bepaald door de genen die in het DNA van de cel liggen. Als een gen “aanstaat” wordt er eerst messenger RNA (mRNA) gemaakt, wat vervolgens in de cel wordt omgezet tot eiwit. In **hoofdstuk 4** hebben we van 70 genen die een rol spelen bij apoptose gekeken of ze “aan” of “uitstonden” in de cellen van 190 kinderen met nieuw gediagnosticeerde ALL. De gedachte hierbij was dat als er apoptose genen “uit” staan die eigenlijk “aan” horen te staan, dit kan leiden tot cellulaire drug resistentie, omdat de cellen gewoonweg niet in apoptose *kunnen* gaan.

We vonden een afwijkende hoeveelheid mRNA van 2 apoptose genen (MCL1 en DAPK1) in prednisolon-resistente ALL cellen en van 3 apoptose genen (BCL2L13, HRK en TNF) in L-asparaginase-resistente ALL cellen. Een hoge expressie van BCL2L13 op mRNA niveau was niet alleen geassocieerd met L-asparaginase resistentie, maar ook met een langere overlevingskans . Als we andere bekende risicofactoren in overweging namen, bleek BCL2L13 de enige onafhankelijke prognostische factor voor overleving bij kinderen met ALL. Dat betekent dat we op basis van de mate van BCL2L13 expressie bij diagnose uitspraken kunnen over de kans dat een kind geneest of niet.

Leukemiecellen vertonen bijna altijd genetische afwijkingen. ALL bij kinderen is daarom een heterogene groep die is opgebouwd uit vele verschillende genetische subtypes (zie ook Tabel 2 van hoofdstuk 1). Bij ALL cellen met een translocatie hebben er 2 chromosomen een stukje DNA uitgewisseld. De meest voorkomende genetische subtypes zijn ALL met de t(12;21) translocatie en ALL waarin de celkern meer dan 50 chromosomen bevat: hyperdiploïde ALL. Verder zijn vinden er ook herschikkingen van het MLL gen op chromosoom 11q23, t(1;19) translocaties en t(9;22) translocaties plaats in ALL cellen. Deze genetische afwijkingen komen allen voor in ALL die uitgaat van “foute” B-lymfocyten, ofwel B-cel ALL. Daarnaast is er ook nog ALL die uitgaat van T-lymfocyten: T-cel ALL. Omdat deze types allemaal verschillend op chemotherapie reageren en dus een verschillende prognose hebben, hebben we in **hoofdstuk 4** ook onderzocht of dit te maken heeft met de expressie van 70 verschillende apoptose genen. We hebben ontdekt dat ieder van deze ALL subtypes inderdaad een eigen expressiepatroon heeft van apoptose genen.

**Eiwit expressie van apoptose genen en cytostatica resistentie**

Over het algemeen geldt dat er van genen die hoog “aanstaan” en dus veel mRNA produceren ook veel eiwit in de cel aanwezig is. Er zijn echter uitzonderingen. Daarom hebben we in **hoofdstuk 5** voor verschillende apoptose genen ook de hoeveelheid eiwit gekwantificeerd en vergeleken tussen *in vitro* gevoelige en resistente cellen van kinderen met nieuw gediagnosticeerde ALL (N=43) en AML (N=10). De genen waarvan we het eiwit hebben gekwantificeerd zijn: Apaf-1, procaspase-2, -3, -6, -7, -8, -10 en PARP. In 5 van de 15 kinderen met T-cel ALL en in 3 de van de kinderen met AML was geen PARP detecteerbaar. De afwezigheid van PARP eiwit ging in dit geval gepaard met een sterk verminderde aanwezigheid van mRNA. Bij de patiënten die wel PARP eiwit hadden, viel op dat binnen die groep de patiënten met T-cel ALL en AML een significant verlaagde PARP expressie vertoonden. Daarnaast hadden de cellen die resistent waren voor prednisolon, vincristine én L-asparaginase (PVA) minder PARP eiwit dan de cellen die gevoelig waren voor deze 3 middelen. Niet alleen van PARP, maar ook van procaspase-2 was er minder eiwit aanwezig in T-cel ALL, AML en PVA-resistente cellen. De hoeveelheid eiwit die er van Apaf-1, procaspase-3, -6, -7, -8, en -10 aanwezig was, hing niet samen met gevoeligheid dan wel resistentie voor PVA. Van zowel T-cel ALL, AML als drug resistente cellen is bekend dat de prognose slechter is dan B-cel ALL. Concluderend kunnen we stellen dat er minder PARP en procaspase-2 eiwit aanwezig is in leukemie subtypes met een slechte prognose.

**Gen-expressie profielen en cytostatica resistentie**

Omdat het DNA van de mens tienduizenden verschillende genen bevat, was het vroeger onbegonnen werk om te kijken welke genen er precies “aan” en welke genen er “uit” stonden. Gelukkig maken micro-arrays dit tegenwoordig mogelijk voor een heel groot deel van de genen die in een menselijke cel aanwezig zijn. Het geheel aan genen wat in een cel “aan” en “uit” staan noemen we het gen-expressie profiel. In **hoofdstuk 6** hebben we micro-arrays gebruikt om genen te identificeren die betrokken zijn bij cellulaire chemotherapie resistentie. Dit hebben we bereikt door gen-expressie profielen van ALL cellen van patiënten die gevoelig voor een bepaald cytostaticum waren te vergelijken met die van patiënten die resistent waren voor hetzelfde cytostaticum.

Op deze manier hebben we 42, 59, 54 en 22 genen ontdekt die betrokken waren bij respectievelijk *in vitro* prednisolon, vincristine, L-asparaginase of daunorubicine

resistentie in B-cel ALL. Genen die geassocieerd waren met resistentie voor prednisolon coderen relatief vaak voor eiwitten die betrokken zijn bij het omzetten van glucose in energie (glycolyse) in de cel. Voor vincristine resistentie betrof dit genen die een functie hadden in de opbouw van RNA en DNA. Genen die betrekking hadden op L-asparaginase resistentie waren vooral betrokken bij de vorming van eiwitten in de cel. De gecombineerde expressiescore van alle 124 genen bleek onafhankelijk van andere bekende prognostische factoren de kans op een recidief van de leukemie te voorspellen. Dit was niet alleen het geval bij de eerste studiegroep van kinderen met ALL, maar ook bij een tweede onafhankelijke groep kinderen met ALL die met een ander chemotherapieprotocol waren behandeld in het St. Jude Children's Research Hospital in Amerika. Opvallend is dat 121 van de 124 genen nog nooit eerder aan cellulaire drug resistentie gelinkt waren. Concluderend kan gesteld worden dat een relatief beperkt aantal genen samenhangt met chemotherapie resistentie en genezingskans bij kinderen met ALL.

**De voorspellende waarde van *OPAL1* voor de uitkomst van therapie**

Als het mogelijk zou zijn om voor aanvang van chemotherapie te voorspellen wat de kans is dat een kind gaat genezen met reguliere chemotherapie, zouden de kinderen met een kleine kans op genezing zwaardere therapie kunnen krijgen en kinderen met een grote genezingskans juist een lichtere vorm, zodat ze minder last hebben van de bijwerkingen die gepaard gaan met chemotherapie. Therapie aangepast op het risicoprofiel van een individueel kind noemen we "tailored therapy" of therapie op maat. Omdat veel kinderen baat kunnen hebben van deze therapie op maat wordt veel energie gestoken in het vinden van prognostische factoren. *OPAL1* (Outcome Predictor in Acute Leukemia) is een nieuw gen waarvan recent is gesuggereerd dat hoge expressie sterk geassocieerd zou zijn met een grote kans op genezing in kinderen met ALL.

In **hoofdstuk 7** onderzochten wij of dit ook opgaat voor kinderen die behandeld werden met het Duitse COALL en het Amerikaanse St. Jude Total 13 protocol. We zagen een verhoogde mRNA expressie van *OPAL1* in t(12;21)-positieve B-ALL cellen. We waren echter niet in staat de verhoogde *OPAL1* expressie te bevestigen die men eerder zag in andere ALL subtypes met een gunstige prognose. Daarnaast was *OPAL1* expressie niet gerelateerd aan gevoeligheid voor prednisolone, vincristine, L-asparaginase of daunorubicin. Opvallend was ook dat *OPAL1* expressie niet onafhankelijk van andere risicofactoren gerelateerd was aan de kans op genezing in de totale groep patiënten of in

ALL subtypes, zoals T-cel ALL, t(12;21)-positieve of -negatieve B-cel ALL in geen van beide cohorten. Onze resultaten tonen aan dat *OPAL1* expressie geen onafhankelijke prognostische marker is in kinderen behandeld met COALL en St. Jude protocollen en benadrukken dat de prognostische waarde van *OPAL1* protocolspecifiek is.

**Nieuwe behandelingsstrategieën**

Het grote voordeel van micro-array studies is dat ze tal van nieuwe data genereren die vervolgens gebruikt kunnen worden voor de identificatie van aangrijpingspunten voor nieuwe behandelingsstrategieën. Zoals in hoofdstuk 6 beschreven, waren genen die hoog aan stonden in prednisolon-resistente ALL cellen relatief vaak betrokken bij de glycolyse. Tijdens de glycolyse wordt glucose in de cel omgezet naar energie. De cel heeft deze energie nodig voor allerlei processen, bijvoorbeeld celdeling. Uit onderzoek was al bekend dat in vergelijking met gezonde cellen, de sneldelende kankercellen vaak een verhoogde glycolyse hebben. Verder was er bekend dat in cellen die in apoptose gaan de totale hoeveelheid energie binnen de cel drastisch afneemt. Dit bracht ons op het idee dat een verhoogde glycolyse wel eens betrokken zou kunnen zijn bij prednisolon resistentie in ALL.

In **hoofdstuk 8** tonen we aan dat ALL cellijnen die resistent zijn voor prednisolon daadwerkelijk een hogere glycolyse vertonen vergeleken met ALL cellijnen die gevoelig zijn voor prednisolon. Opvallend was dat het toedienen van de glycolyse remmer 2-deoxy-D-glucose de ALL cellijnen die resistent zijn voor prednisolon, maar niet de ALL cellijnen die gevoelig zijn voor prednisolon, gevoeliger maakte voor prednisolon.

Ten slotte volgt in **hoofdstuk 9** een discussie van de resultaten van het onderzoek zoals in dit proefschrift beschreven is. De bevindingen in dit proefschrift kunnen wellicht bijdragen aan betere behandelingen voor kinderen met ALL door (a) betere stratificatie van kinderen met ALL en hierop aangepaste therapie op maat en (b) de identificatie van aangrijpingspunten voor nieuwe behandelingsstrategieën die in vervolgonderzoek verder uitgewerkt zullen worden.

## Curriculum vitae

## Publications

## Dankwoord / Acknowledgements

**CURRICULUM VITAE**

Amy Holleman was born on April 4th, 1977 in Tilburg, the Netherlands. She finished Grammar School at the Mill Hill College in Goirle in 1995. In the same year, she started studying Biology at the University of Utrecht, which was continued with the study Fundamental Biomedical Sciences a year later. Her first doctoral training was followed at the Group for Comparative Endocrinology of the Department of Zoology under supervision of Dr. Jan Bogerd and Prof.dr. Henk J.Th. Goos. She followed her second doctoral training at the Target Discovery Unit of NV Organon under supervision of Dr. Anneke Sijbers. The study Fundamental Biomedical Sciences was successfully completed in august 2000.

On September 1st 2000 she started the PhD project described in this thesis at the Department of Pediatric Oncology/Hematology at Erasmus MC/Sophia Children's Hospital in Rotterdam. The PhD project was supervised by Dr. Monique L. Den Boer and Prof.dr. Rob Pieters. In 2002 she was awarded the Rene Vogels stipendium of the Nederlandse Vereninging voor Oncologie (NVvO). With this money she conducted 4 months of research in the laboratory of Prof.dr. William Evans at the Department of Pharmaceutical Sciences of the St. Jude Children's Research Hospital in Memphis, Tennessee, USA. While still working on her thesis she passed the admittance exam for the 4-year study Medicine at the School for Utrecht Medical Masters (SUMMA) at the University of Utrecht.

In November 2004 she started working as a data manager at the HOVON (Hemato-Oncology for Adults in the Netherlands) data center, Department of Trails & Statistics at the Erasmus MC, Rotterdam. In February 2005 she started SUMMA, and is a full-time student again.

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