Molecular mechanisms of glucocorticoid resistance in childhood acute lymphoblastic leukemia

Financial support for the publication of this thesis was provided by:

Amgen B.V.
Merck Sharp & Dohme B.V.
Novartis Oncology
Pfizer B.V.
Sanofi Aventis
Zambon Nederland B.V.

ISBN: 9056770861

Cover design: Rob Davelaar, The Netherlands.
Page layout: Peter van der Sijde, Groningen, The Netherlands.
Printed by: Febodruk, Enschede, The Netherlands.

Molecular Mechanisms of Glucocorticoid Resistance in Childhood Acute Lymphoblastic Leukemia

Moleculaire mechanismen van glucocorticoid resistentie in acute lymfatische leukemie bij kinderen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
donderdag 27 april 2006 om 11.00 uur

door
Willem Jan Egbert Tissing
geboren te Lisse

Promotiecommissie

Promotor:	Prof.dr. R. Pieters
Overige leden:	Prof.dr. P. Sonneveld Prof.dr. H.A. Büller Prof.dr. W.A. Kamps
Copromotoren:	Dr. J.P.P. Meijerink Dr. M.L. den Boer

CONTENTS

Chapter 1

General introduction

General introduction

Acute lymphoblastic leukemia (ALL) is the most common form of cancer in children, with 110 – 120 newly diagnosed children in the Netherlands each year. ALL is a haematological malignancy of lymphoid precursor cells and can be divided into two sub-groups: B-cell precursor ALL and T-cell precursor ALL. These two subgroups can be differentiated using light microscopy (morphology and cytochemical staining) and immunophenotypic classification. More recently, other subgroups have been identified based on genetic abnormalities in the leukemic cells such as chromosomal translocations (BCR-ABL, TEL-AML1, E2A-PBX1, MLL-gene rearranged) and chromosome copy number (hyperdiploidy). Both a favorable (TEL-AML1, hyperdiploidy) and unfavorable (BCR-ABL, MLL-gene rearranged ALL) prognosis have been linked to these genetic subtypes. The prognosis for children with ALL has increased significantly over the past 35 years, with a 5-year event-free survival of 75-80% in most current treatment protocols. (Figure 1) However, still 20-25% of the children relapses, with a significantly worse prognosis for survival. In most international treatment protocols, patients are divided into three risk-groups, i.e. standard risk, intermediate risk and poor risk. Traditional risk factors for this stratification are: white blood cell count at diagnosis ($<50 \times 10^9$/l or $\geq 50 \times 10^9$/l), age (< 1 year, 1-9 year or ≥10 year), central nervous system

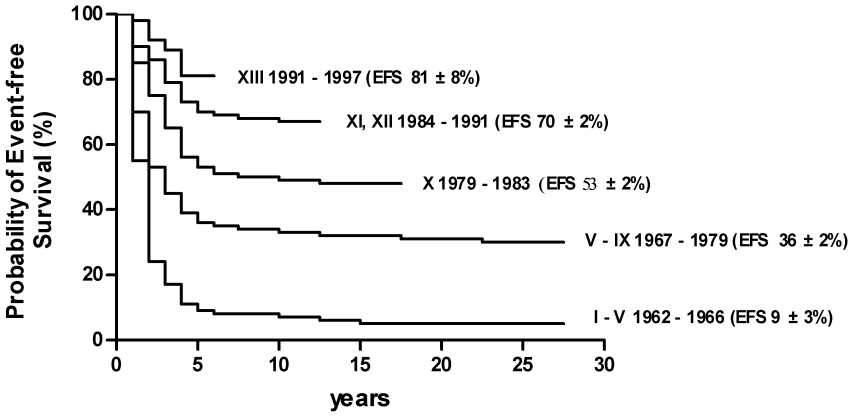


Figure 1. Event free survival in childhood ALL
Kaplan-Meier analysis of event-free survival (EFS) in 2255 children with ALL who were enrolled in 13 consecutive treatment protocols conducted at the St Jude Children's Research Hospital from 1962 – 1997. Treatment protocol numbers and 5 yr. EFS are depicted.
Adapted from Pui C.H. and Evans W.E., NEJM 1998, 339: 605-15

involvement, immunophenotype (pro-B ALL, common/pre-B ALL or T-cell) and chromosomal abnormalities (BCR-ABL translocation or MLL-gene rearrangement). However, the most important risk factors that are associated with a poor outcome are: (1) a poor early response to initial prednisone treatment as defined by the BFM (Berlin-Frankfurt-Munster) study group (*in vivo* prednisone response) and (2) a poor response to the initial multi-chemotherapy regimen, as determined by the level of detectable minimal residual disease (MRD). These two clinical response parameters and the absence or presence of a BCR-ABL translocation or rearranged MLL-gene in the leukemic cells are nowadays used to stratify patients into standard, medium and high risk groups. Another important risk factor is the *in vitro* response to prednisolone, as measured using a total cell kill assay. *In vitro* prednisolone resistance is an important adverse risk factor that can even identify patients with an unfavorable long-term clinical outcome despite the fact that these patients initially had a good *in vivo* response to prednisone.

Knowing that *in vitro* and *in vivo* prednisone resistance is such an important risk factor for adverse outcome, it is important to understand the mechanisms underlying prednisone (glucocorticoid) resistance in childhood ALL. However, not much is known about these mechanisms. **Chapter 2** reviews the literature on this topic and the **chapters 3 to 7** describe the research project undertaken to study the mechanisms involved in prednisone resistance in childhood ALL. In **chapter 3** we describe the study testing whether polymorphisms or mutations of the glucocorticoid receptor gene are related to prednisone sensitivity. The study described in **chapter 4** analyses whether the expression level of the glucocorticoid receptor and its splice variants are related to prednisone sensitivity. In **chapter 5** we study a possible relationship between prednisone sensitivity and the regulation of the glucocorticoid receptor upon prednisolone exposition. In **chapter 6** we tested the hypothesis that expression levels of the (co)chaperone molecules, needed by the glucocorticoid receptor to bind glucocorticoids, are related to prednisone sensitivity. In **chapter 7** we describe a study looking for genes and transcription pathways regulated upon prednisolone exposure. The results of the different studies are summarized and discussed in the **chapters 8** (English) and **9** (Dutch), including suggestions for further research.

Chapter 2

Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia

Leukemia, 2003; **17**: 17-25

Wim J.E. Tissing, Jules P.P. Meijerink, Monique L. den Boer, Rob Pieters

University Hospital Rotterdam / Sophia Children's Hospital, Department of Paediatric Oncology/Hematology

ABSTRACT

Glucocorticoids (GCs) are probably the most important drugs in the treatment of ALL. Despite the extensive use of GCs for many years, little is known about the molecular mechanisms of sensitivity and resistance. This review summarizes the knowledge on GC cytotoxicity in leukemia. The relevance of polymorphisms, splice variants and the number and regulation of the GC receptor is discussed. The role of multidrug resistance proteins, glutathione and glutathione S-transferase is evaluated, as well as the influence of the different heat-shock chaperone (HSP-90 and -70) and co-chaperone proteins (BAG-1 and others) which form a complex together with the GC receptor. At last the transactivation and transrepression (via NF-κB and AP-1 binding) of a wide range of genes (like c-*myc*) which initiates the final apoptosis pathway are discussed. Finally, suggestions for future directions of research in ALL patients are given.

INTRODUCTION

Glucocorticoids (GCs) are involved in many biological processes, including metabolism, development, differentiation, immunity, reproduction and neural activity. The diverse actions of GCs have led to their use as therapeutic agents in the treatment of many diseases. GCs can act anti-proliferative in specific cell types, which is the reason why GCs are used in immunosuppressive, anti-inflammatory and oncolytic therapy. The effect on lymphoid cells is dramatic and includes the induction of G1 cell cycle arrest and apoptosis. In newly diagnosed acute lymphoblastic leukemia (ALL), prednisone and dexamethasone have significant antileukemic effect in the majority of children.[1] Although GCs are the most important drugs used in the treatment of ALL for more than 50 years, the molecular basis of GC sensitivity and resistance remains largely unknown. Understanding of the molecular mechanisms related to GC cytotoxicity is crucial for understanding a major part of treatment success or failure in childhood ALL and is crucial for the exploration of possibilities to modulate GC resistance. This review summarizes the current knowledge on molecular determinants of glucocorticoid sensitivity and resistance in ALL.

Clinical aspects of glucocorticoid sensitivity

Glucocorticoid sensitivity is a major prognostic factor in childhood ALL. In BFM

trials blast count after 7 days of monotherapy with prednisone (including one intrathecal dose of methotrexate) was shown to be a strong and independent prognostic factor.[1-3] Subgroups with a poor prognosis like infants, T-ALL patients and patients with a Philadelphia chromosome positive ALL, more often show a poor clinical response to prednisone.[2, 4, 5] *In vitro* resistance to glucocorticoids at initial diagnosis is related to an unfavorable event free survival in childhood ALL as well.[6-9] Leukemic cells from the risk groups associated with a poor prognosis (T-ALL, proB-ALL, infant ALL) are relatively *in vitro* resistant to prednisolone.[10] Leukemic cells from adults with ALL, who have an unfavorable outcome as compared with children, are more resistant to GCs *in vitro* too.[11, 12] Leukemic cells of patients with relapsed ALL are 300 fold more *in vitro* resistant to prednisolone than the cells taken at diagnosis: in paired initial / relapse samples resistance to GCs is increased in the majority of patients at the time of relapse compared to initial diagnosis.[13]

The glucocorticoid receptor gene and activation by glucocorticoids

The glucocorticoid receptor gene (GR) is located on chromosome 5 (5q31) and consists of 9 exons encoding for 3 characteristic domains of the protein.[14] (Figure 1) The N-terminal region contains a transactivation domain (AF-1) that is involved in transcriptional activation of target genes.[15] An internal DNA binding domain consisting of two highly conserved "zinc fingers" is crucial for the binding to the Glucocorticoid Response Elements (GRE) sequence. This domain contains a nuclear localization signal (NLS1). The first zinc finger (exon 3) encodes for domains necessary for binding NF-κB and AP-1[16] and is therefore important for

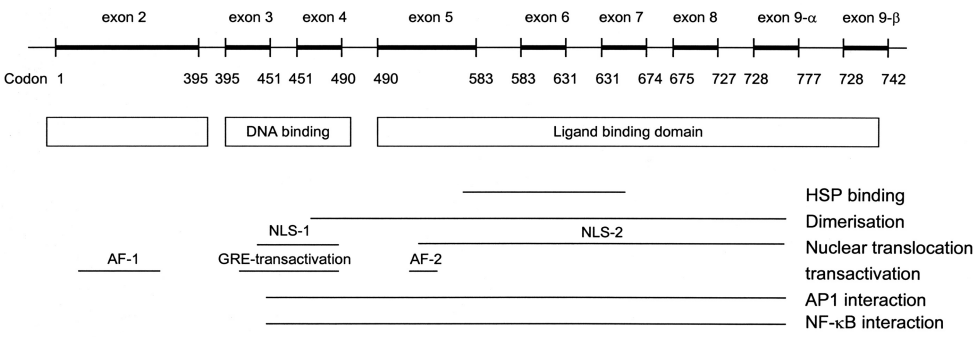


Figure 1 Schematic overview of the GR gene
The GR gene, localized on chromosome 5, consists of 9 coding exons. The functional parts of the receptor are indicated.

the transrepression mode of the receptor. The second zinc finger domain (exon 4) encodes for receptor dimerisation and GRE mediated transactivation.[17-20] The C-terminal part of the protein contains the ligand binding domain that also binds heat shock proteins (hsp) and is involved in receptor dimerisation. This domain contains a second nuclear localization signal (NLS2) and transcription activation (AF-2) site.[15, 19] The two transactivation domains (AF-1 and AF-2) interact with other nuclear proteins such as CBP (CREB binding protein) and P300 that are important for stabilization and activation of the transcription initiation complex in the promoters of glucocorticoid responsive genes.[21] GCs enter the cell by passive diffusion and bind to the GR, which is located in the cytoplasm as a homodimer. (Figure 2)

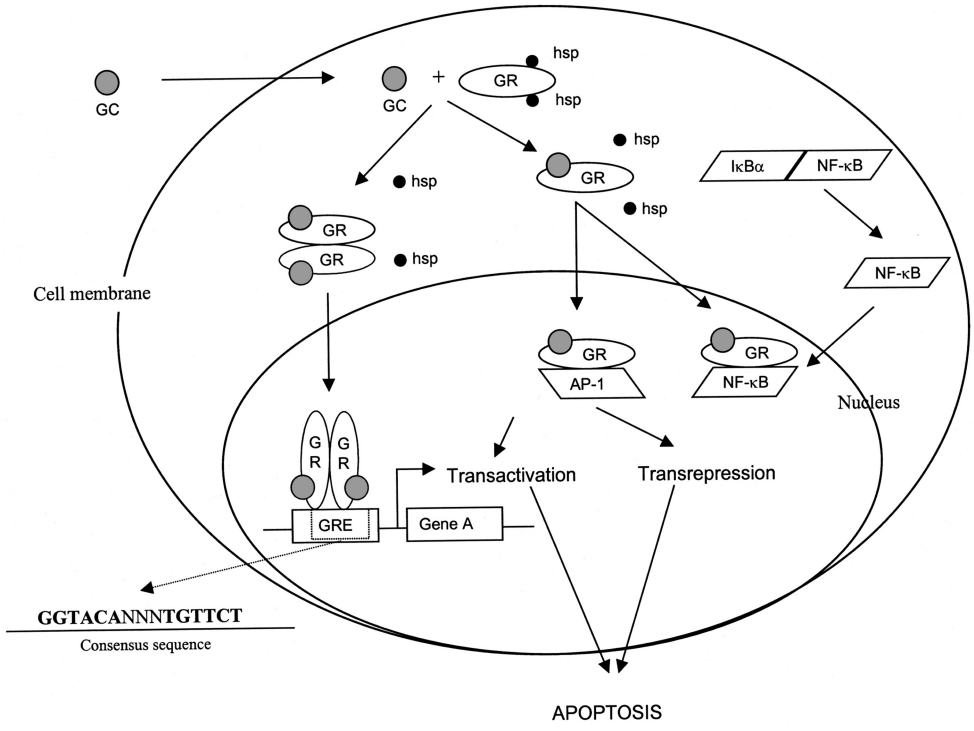


Figure 2. Model of GC induced apoptosis in leukemic cells
Glucocorticoids (GC) enter the cell by passive transport, and bind to the glucocorticoid receptor (GR). The unbound GR forms heterocomplexes consisting of heat-shock chaperone molecules HSP-90 and 70, co-chaperone molecules HSP-40, HOP (p60), P23 and immunophillins FKBp52 and CYP40, required for optimal configuration of the GR to be able to bind GC. As a homodimer the GC-GR complex translocates to the nucleus. There it interacts with either a GRE (consensus sequence (**GGT ACA** NNN **TGT TCT**) of a target gene (transactivation), or it interacts with other transcription factors such as AP-1 and NF-κB (transrepression). Both processes may finally result in the induction of cell death (apoptosis). NF-κB is kept in the cytoplasm in a complex with IκBα. Upon dissociation it can translocate to the nucleus.

The GR belongs to the nuclear hormone receptor superfamily, and is highly homologous to the mineralocorticoid, progesterone and androgen receptor. Binding of GCs to the GR triggers the dissociation of proteins bound to the receptor such as HSPs and BAG-1.[22] This discloses and activates the nuclear localization signal (NLS) domains of the receptor. The GC-GR complex then translocates to the nucleus as a homodimer, where it can interact with GRE's (consensus sequence **GGT ACA** NNN **TGT TCT**).[23] These are located in promoter regions of GC-responsive genes, leading to transcriptional activation or inactivation of these genes (the latter hardly ever occurs).[24] The GC-GR complex can also directly interact with transcription factors like activating protein-1 (AP-1) or nuclear factor κB (NF-κB), by forming transrepression complexes. The formation of these complexes represses the transcriptional activity of both the GR, AP-1 and NF-κB.[25] Both transactivation (via GRE-binding) and transrepression (via interaction with AP-1 or NF-κB) processes can induce apoptosis of GC-sensitive cells, but it is yet unknown if one or both are important in ALL.[16, 26-30]

MOLECULAR MECHANISMS OF SENSITIVITY AND RESISTANCE IN LEUKEMIC CELLS

Number of glucocorticoid receptors

The relationship between the number of GRs per cell and the response to GCs in patients with ALL has been subject of many studies, as reviewed earlier.[31] Most authors report a significant correlation between low numbers of receptor per cell and a higher rate of induction failure or relapse. However, a high receptor number does not necessarily predict a good response to a GC containing regimen. All studies about GR numbers used dexamethasone binding assays in which only functional receptors are measured (i.e. probably the α- and the γ-isoform -see below).

In contrast to basal expression levels of the receptor, upregulation of the number of GRs upon GC exposure may be more important for GC induced apoptosis. In non-lymphoid cell lines and in peripheral blood mononuclear cells of children with auto-immune disorders, with no apoptotic response upon GC exposure, a decrease in GR mRNA expression and receptor number was observed after exposure to GCs.[32-37] Contrary to the down-regulation in those non-lymphoid cell lines, upregulation of the GR upon GC exposure is reported in leukemic and lymphoid cell lines. In the leukemic T-cell line CCRF-CEM an upregulation of GR mRNA was shown in the first few hours after exposure to GCs.[38] Ramdas *et al.* showed that the

basal level of GR expression is inadequate to mediate GC-induced apoptosis in a leukemic human T cell line 6TG1.1.[39] An increase in GR number by autoregulation is required to induce apoptosis in these cells. Recently, Breslin *et al*. studied the hypothesis that activation of different promoters of the GR gene may regulate the expression of GR under the influence of GCs in different tissues.[40] Exon 1A3 mRNA (one of at least five transcripts from three different promoters) is expressed most abundantly in hematological cancer cell lines that are sensitive to GC-induced apoptosis. Furthermore, GC exposure causes upregulation of exon 1A3-containing GR transcripts in (hematological) CEM-C7 cells.

No data exist proving the hypothesis that leukemic cells that are sensitive to GCs are able to upregulate their receptor number more pronounced than resistant leukemic cells.

Membrane bound receptor

Whereas the GR is generally described as a cytosolic receptor, Gametchu *et al*. described a membrane bound variant (mGR).[41] The expression of the mGR is reported to be cell cycle regulated in the CCRF-CEM cell line. The highest expression is found during the late S-G_2/M phase when the cells are most sensitive to the apoptotic effect of GC. The mGR expression varied among leukemic patients, whereas no mGR was found in the membrane of lymphocytes of healthy individuals, the latter being highly resistant to GC.[41, 42] In a pilot study no correlation was found between the expression of mGR and *in vitro* sensitivity to GCs in childhood ALL samples. (B. Gametchu, personal communication)

Polymorphisms / somatic mutations of the glucocorticoid receptor gene

A number of endocrinological glucocorticoid resistance syndromes has been described that are associated with genetic mutations in the GR.[43-47] *(A genetic mutation is defined as an inheritable germline mutation present in a limited number of individuals (most commonly within a family) and associated with a higher risk to develop a malignancy (e.g. Li Fraumeni syndrome). A polymorphism is defined as an inheritable genetic germline variant of a single locus (most frequently a single nucleotide variation) that is present in at least 1% of the population. The opposite of polymorphisms are somatic mutations that are associated with a specific type of disease, and thereby restricted to the malignant cells only.)* These mutations result in a lower number of functional receptors and interfere with GC binding or transactivational capacity. Besides genetic mutations, several polymorphisms have been described. Although most polymorphisms do

not effect receptor function[48, 49], one polymorphism has been described which is associated with increased sensitivity to GC. This N363S polymorphism is present in 3-6% of the population.[49-51] Table 1 summarizes the literature references on polymorphisms and mutations in the GR as found in healthy individuals, a patient with childhood ALL and patients with a glucocorticoid resistance syndrome.

The role of polymorphisms or somatic mutations in the GR in relation to GC cytotoxicity in childhood leukemia is largely unknown. Hillmann *et al*. showed that the GC-resistant CCRF-CEM cell line contains one GR allele with the L753F mutation. Analysis of the original biopsy material also revealed the same mutation, but in a substantial lower frequency than expected, concordant with the hypothesis that this mutation was only limited to a leukemic subclone.[52]

The different mutations can cause a decreased sensitivity to GCs in various ways. Decreased sensitivity to GCs may be related to the location of the mutation (N-terminal, DNA-binding region or ligand binding region) or to the preferential degradation of mutated GRs. The GR transcript can be truncated by a mutation, which introduces a premature stop codon, resulting in loss of mRNA expression and loss of GR number and might be associated with decreased GC sensitivity.[43, 52]

Table 1. Polymorphisms and mutations in the glucocorticoid receptor gene described in healthy individuals, a patient with childhood ALL and patients with a glucocorticoid resistance syndrome.

Identity of genetic alteration	GC Sensitivity	Reference
Polymorphisms		
Healthy individuals		
Double mutation codon 22+23	Unchanged	49
F29L	Unchanged	48
L112F	Unchanged	48
D233N	Unchanged	48
K293K	Unchanged	48
N363S	Increased	43
Intron 3: 46 nucleotides upstream from exon 4: G – C	Unchanged	49
Intron 4: 16 nucleotides upstream from exon 5: G – C	Unchanged	49
D677D	Unchanged	48
Codon 766: Asn – Asn	Unchanged	48/49
Somatic Mutations		
Patients with GC resistance syndromes		
R477H	Decreased	47
I559N	Decreased	45
Δ4 deletion 3' boundary exon 6	Decreased	43
D641V	Decreased	44
G679S	Decreased	47
V729I	Decreased	46
Patient with ALL		
L753F	Decreased	52/120

Other mutations may interfere with correct splicing of the pre-mRNA transcript. Karl *et al*. showed that a 4-basepair deletion on the boundary of exon 6 resulted in the absence of transcripts of that allele, so GR is only encoded by one wild type allele.[43]

The relevance of polymorphisms and genetic or somatic mutations in the GR gene for GC sensitivity and prognosis in childhood leukemia is subject of ongoing studies in our laboratory[53] and of others. If these genetic aberrations are important for the cellular response to GCs, the presence of these polymorphisms / mutations can be used to identify patients at risk for a poor response to GC treatment. In addition, patients with a polymorphism that is associated with increased GC sensitivity like the N363S polymorphism may benefit from GCs in their treatment. However, these patients may also suffer from severe side effects, such as Cushing syndrome, overweight, depression and avascular necrosis of bone. These patients may still have an excellent prognosis when treated with a lower dosage of GCs.

Splice variants of the glucocorticoid receptor

Five different splice variants of the GR gene have been described, formed by alternative splicing, i.e. the α, β, γ, GR-P and GR-A isoform. (see Figure 3)

1 - The α isoform is the functional receptor and is encoded for by exon 2 to 9α.[54] It is located in the cytoplasm in the absence of GCs, but migrates to the nucleus upon GC binding.[55] The only study of the relation between splice variants and GC sensitivity in leukemic patients was done by Longui *et al*.[56] They described a reduced GR-α expression in the leukemic blasts of 13 ALL patients in comparison with EBV transformed lymphocytes of 9 normal controls. The GR-α expression was lowest in T-ALL samples and no concomitant decrease in GR-β expression in the leukemic cells of these patients was observed. The authors suggest that decreased GR-α expression levels may explain the lower GC sensitivity in T-ALL patients as compared to pre B-ALL. However, the proof for the relevance of the alpha splice variant is indirect as there was no *in vitro* or *in vivo* assessment of GC sensitivity and the patient number is very small.

2 - The β isoform is the result of alternative splicing of exon 9β instead of 9α, resulting in 15 unique C-terminal amino acids. As a consequence, GR-β cannot bind GCs.[54] At mRNA level, GR-β expression is 0.2 – 1 % of the total GR expression.[57, 58] Strikingly, at protein level, the data are controversial and vary from levels comparable to mRNA levels[59, 60] to 5-fold higher expression levels compared with GR-α.[61] The reason for these conflicting data might be lack of specificity of the

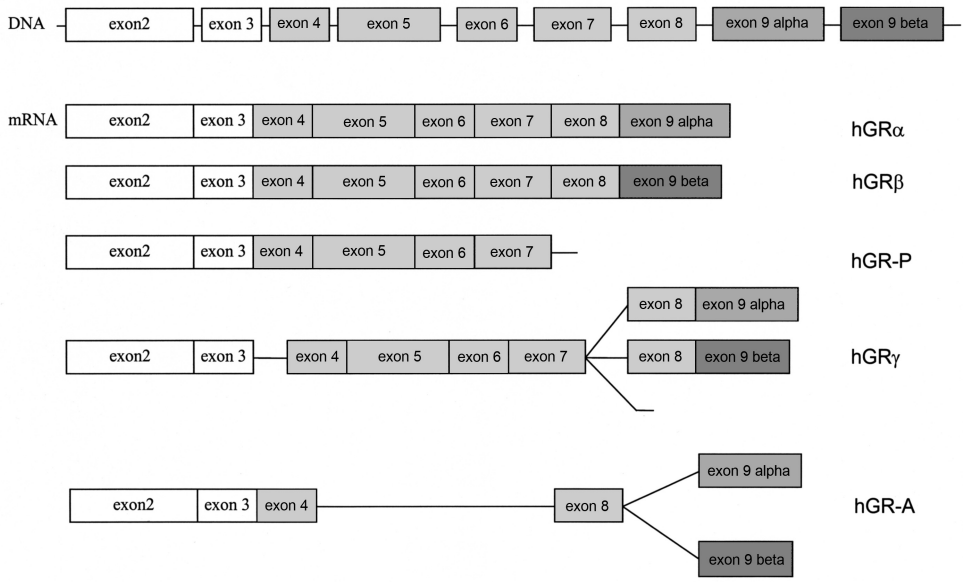


Figure 3. The glucocorticoid receptor gene and 5 different splice variants

antibodies used in these studies.

The β isoform might have a dominant negative effect over the α isoform. Vottero *et al*. and Carlstedt-Duke reviewed the controversial data on this hypothesis.[62, 63] In GC resistant asthma patients, Leung *et al* found a relatively higher expression of GR-β in peripheral blood mononuclear cells as compared to GC sensitive asthma patients[64], but Gagliardo *et al* could not confirm this.[65] Transfection studies in cell lines are also inconclusive. Some transfection studies showed a negative effect of GR-β over GR-α[66, 67], whereas others did not.[60, 68, 69] As the expression level of the beta isoform is very low compared to the alpha isoform, the relevance of the beta expression or resistance in childhood leukemia is unlikely.

3 - The γ isoform has only recently been described.[70] In this splice variant exon 4 is alternatively spliced to exon 3 thereby including 3 basepairs of the intron region resulting in an additional arginine residue. This isoform is expressed at 3.8 – 8.7 % of total GR mRNA in different human tissues but it is unknown whether this variant is a separate splice variant or part of the α, β and GR-P isoforms.[70, 71] Ray *et al*. reported that the biological activity of the γ isoform is reduced to 50% of the wild-type receptor.[72] Gerdes *et al*. reported preliminary results showing a possible role for the γ isoform in poor prednisone response in childhood ALL.[71]

4 - The GR-P isoform is encoded by exons 2-7 plus several basepairs from the subsequent intron region.[73] As a consequence, this isoform lacks the ligand binding domain and therefore can not bind GCs. The GR-P transcripts account for up to 10-20% of total GR mRNA, but has been reported to be upregulated in a small group of hematological malignancies (ALL, non Hodgkin's lymphoma and multiple myeloma, up to 54 % of total GR mRNA).[57, 74-76] In a study of De Lange *et al*.[77], transfection of GR-P receptor increased the activity of the GR-α receptor. In the same study no relation was found between sensitivity to GCs and the ratios of the GR-α, GR-β and GR-P isoforms in a group of multiple myeloma patients.

5 - The fifth variant described, the GR-A variant, has an excision of exons 5,6 and 7, resulting in the in frame juxtaposition of exon 8 to 4. No further information is known about the expression levels and function of this variant.[73]

Studying the relevance of the alpha, beta and hGR-P splice variants for GC sensitivity in childhood ALL, preliminary data from our laboratory did not show a relation between mRNA levels of the 3 splice variants and *in vivo* or *in vitro* sensitivity to GC. Further work is needed to define the importance of the different splice variants in relation to GC sensitivity in ALL and to delineate the mechanisms of GR mRNA splicing regulation.

Phosphorylation of the GR

Phosphorylation of receptors is a general regulation mechanism in cells. Phosphorylation of the GR modulates the GR function as reviewed by Bodwell.[78] The phosphorylation rate is maximal in the S-phase and enhances the transactivating and transrepressing activities of the GR. Furthermore the GR is destabilized by phosphorylation, resulting in a shorter half-life of the protein. The clinical relevance of phosphorylation of the GR is unknown.

Multidrug resistance and GC sensitivity

P-glycoprotein (P-GP) is a drug-efflux pump responsible for multidrug resistance (MDR) and encoded for by the mdr-1 gene. In a murine thymoma cell line it was shown that increased resistance to dexamethasone was linked to an increased expression of the mdr1 gene of P-GP. Verapamil, able to restore intracellular drug concentrations by blocking the drug-efflux pump, was able to increase the intracellular level of dexamethasone.[79, 80] However, contrary to these cell line studies, in a report on leukemic cells of 112 children with ALL no correlation between GC resistance *in vitro* and the expression or function of P-GP activity

was found.[81] Furthermore, functional P-GP activity was not related to *in vivo* prednisone response in a group of 90 ALL patients.[82] In addition, GC-resistance was not associated with an increased expression of other multi-drug resistance related proteins such as major vault protein / lung resistance protein (MVP/LRP) and multidrug resistance-associated protein (MRP-1).[81]

Glutathione and Glutathione S-transferase

Increased glutathione (GSH) levels and glutathione S-transferase (GST) overexpression contribute to resistance to various cytostatic drugs. Maung *et al*. found in their study of 21 newly diagnosed patients a positive correlation between GSH level and prednisolone resistance.[83] However, in a recent report concerning 62 newly diagnosed children with ALL, this relationship was not confirmed.[84] In addition, the expression levels of GST (α, μ and π-class) were not related to *in vitro* prednisolone sensitivity in childhood ALL.[85] Anderer *et al*. reported about the implication of polymorphisms in the GST genes for GC sensitivity in childhood ALL. In a case-control study of 45 *in vivo* poor responders and 90 good responders there was a reduced risk of prednisone poor response in patients with a homozygous deletion of GSTT1 (null genotype), although this difference was not statistically significant (p=0.071).[86]

Protein interaction with the GR in the cytoplasm

The unstimulated GR is sequestered within the cytoplasm where it can bind into heterocomplexes consisting of heat-shock chaperone molecules HSP-90 and -70, co-chaperone molecules HSP-40, HOP (p60), P23 and immunophillins FKBP-51 and CYP40. This heterocomplex is required for optimal configuration of the GR to be able to bind GC.[87] In a recent study only HSP 90 and HSP 70 were found to be required and sufficient for GC binding to its receptor.[88] A second function of the heat shock chaperone molecules is the formation of a heterocomplex with the GR, which induces a conformational change in the ligand binding domain of the receptor, facilitating nuclear transport of the receptor.[22] Kojika *et al* reported about the adverse effect of an abnormal expression of HSP-90 and a low expression of HSP-70 on sensitivity to GCs in 2 human leukemic cell lines.[89]

Another co-chaperone molecule BAG-1 (also named RAP46) has been found to bind to many different proteins including HSP-70. This molecule may inhibit HSP-70 function and GR-heterocomplex formation thereby interfering with GC binding to the GR.[90, 91] It is tempting to speculate that overexpression of BAG-1 may result

in cellular resistance to GCs, and indeed, cell line studies have demonstrated that BAG-1 overexpression resulted in cellular resistance against dexamethasone-induced apoptosis.[91]

Glucocorticoids and transcription factors

Besides from binding to the GRE, the GC-GR complex is thought to recruit NF-κB and AP-1 into a transrepression complex. This complex abrogates the transcriptional activation of GR, NF-κB and AP-1 responsive genes (transrepression). Besides physical interaction and inactivation of NF-κB by the GC-GR complex[92], NF-κB is also inactivated by a second mechanism. Since IκBα is a GR responsive gene[93-95], induction of GR leads to higher levels of IκBα which decreases the level of nuclear NF-κB. Enhanced expression of IκBα may thereby stimulate GC induced apoptosis.[95, 96] Contrary to this hypothesis are the results from Heck *et al*. who conclude by dimerisation-defective GR mutant studies that IκBα synthesis is neither required nor sufficient for the hormone mediated downregulation of NF-κB activity.[97] The only study using leukemic blasts from children with ALL did not find a difference in NF-κB level in a group of 42 good and poor *in vivo* responders to GC.[98]

The second transcription factor interacting with the GR is AP-1, a heterodimer of Fos and Jun proteins. Since AP-1 target genes are involved in cell proliferation, a reduction of functional AP-1 levels may therefore facilitate apoptosis. However, Bailey *et al*.[99] did not find a relationship between levels of AP-1 and *in vitro* prednisolone resistance in ALL and chronic lymphoid leukemia blasts, nor between prednisolone-induced changes in AP-1 binding activity and *in vitro* GC resistance.

It is not known whether GR-GRE binding, GR interactions with transcription factors or both are of importance for GC induced apoptosis in ALL. Favoring the importance of GR-GRE binding are the results from a study by Reichardt *et al*.[27] An A458T point mutation was introduced in the DNA binding region of mouse GR, thereby abolishing GRE-binding. T-lymphocytes of these mutant mice were refractory to dexamethasone induced apoptosis, whereas wild type T-lymphocytes were highly sensitive to dexamethasone induce apoptosis.

Genes regulated by GC

Many genes have been proposed to be regulated by GCs.[29, 100] GCs induce G0/G1 arrest of proliferating cells. This suggests that GCs reduce the expression of genes related to cell growth and cell viability. An important gene is c-*myc,* a known target

gene of NF-κB. Thompson *et al*. concluded in their studies that negative regulation of c-*myc* expression is a significant step in the initial pathway leading to apoptosis in the CEM cell line.[101-103] Non-obese diabetic (NOD) mice with T lymphocytes that are resistant to GC-inducible apoptosis show no reduced c-*myc* levels after GCs.[104] On the contrary, Löffler *et al* reported that c-*myc* downregulation may not be critical for induction of cell-death by GCs since transfection of c-*myc* into the human T-cell leukemia CEM cell line increased the sensitivity to GC-induced cell death.[105] One of the reasons for these conflicting results may be that increased c-*myc* expression sensitizes the CEM cell line to apoptosis in a similar way as shown by Evan *et al*, i.e. an enhancement of apoptosis by enforced c-*myc* expression following growth-factor deprivation which was observed in the absence of GC treatment.[106]

Another important target gene, known to be upregulated in a leukemic cell line but downregulated in cells not undergoing apoptosis after GC exposure is the GR gene itself.[29, 32-34, 36, 39] The relationship between the potency to upregulate the GR number and sensitivity to GCs in ALL is currently subject of study in different laboratories.

Using gene chip arrays, many genes have been found to be regulated under the influence of GCs. A notable cluster of the repressed genes seemed to be of importance for the processes of transcription, mRNA splicing and protein synthesis. It remains to be shown whether GCs trigger one specific apoptotic pathway or that a more general suppression of macromolecule synthesis is the cause of apoptosis.[107]

Apoptosis pathway

GCs induce apoptosis in leukemic cells. Both transactivation of GRE containing genes and transrepression of genes via NF-κB and AP-1 interactions are probably essential.[16, 26-28, 30] In rat thymocytes it was shown that glucocorticoid induced apoptosis depends on protein synthesis and de novo gene expression.[108] There are two major apoptosis pathways: the first starting with a disruption of the mitochondrial membrane potential and cytochrome c and SMAC release after which caspase 9 is activated. This pathway seems to be important for GC induced apoptosis.[109, 110] The second apoptosis pathway, induced by membrane death receptors as reviewed by Ashkenzi[111], is not critically involved in the apoptotic response to GC.[29] Brady *et al*. showed that the apoptosis pathway induced by GCs is p53 independent.[112]

An important protein studied in relation to GC induced cell death is the anti-apoptotic protein BCL-2. Hartmann *et al*. reported that BCL-2 protects cells against GC induced apoptosis in the human T-ALL cell line (CCRF-CEM) up to 48 hours. But, when cultured for another 24 hours, these cells undergo massive apoptosis. BCL-2 did not affect GC-mediated growth arrest, thereby separating the anti-proliferative effect of GCs from the apoptosis-inducing effect.[113] This was confirmed in other studies, as GCs in the presence of high levels of BCL-2 only has an anti-proliferative effect but not an apoptotic effect, while in the presence of low BCL-2 expression, GC induced apoptosis.[114-116] In different studies in children with ALL, expression of BCL-2 or BCL-2 family members could not be related to *in vitro* or *in vivo* GC resistance.[117-119] Contrary to the hypothesis that the anti-apoptotic protein BCL-2 is related to resistance to GC induced apoptosis are the results of a study of 110 patients treated according to the German BFM protocols. Good responders to initial prednisone therapy had higher BCL-2 expression levels than poor responders. The authors speculate that this high BCL-2 expression might be due to upregulation by cytokines, which may also be important for *in vivo* tumor cell survival.[82] BCL-2 is a member of a larger BCL-2 family, consisting of both pro- as well as anti-apoptotic proteins. It could be that not the absolute expression of BCL-2, but the ratio of all family members are the most important in determining sensitivity to GC treatment in ALL. However Salomons *et al*. did not find a relation between the BAX:BCL-2 ratio and the response to 7 days monotherapy of prednisone in a group of 76 patients.[117]

The final activation of caspases seems to be a downstream effector event during apoptosis, but the trigger for this pathway remains unknown. Kofler suggests that the continuous repression of metabolic pathways by GR upregulation contributes to cell cycle arrest, ultimately leading to apoptosis. Particularly critical in this context might be the downregulation of lactate dehydrogenase, an enzyme controlling glycolysis in cells.[29, 38] The suggestion that metabolic events have to take place before the induction of apoptosis may be in accordance with our own unpublished *in vitro* observations that induction of apoptosis in ALL cells derived from patients is a late event starting only after 24 hours from the start of exposure to GCs.

PERSPECTIVES

In vivo and *in vitro* sensitivity to GCs are associated with prognosis in childhood ALL. This review has summarized the knowledge on genes and proteins that regulate GC sensitivity. Future studies should focus on polymorphisms, splice

variants and regulation of the GR, on transcription factors activated or repressed by GCs and on downstream molecules that control the apoptotic cascade. Based upon knowledge derived from these studies, strategies to modulate GC resistance in ALL can be developed.

References

1. Reiter A, Schrappe M, Ludwig WD*, et al.* Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86*.* Blood 1994;84:3122-33.
2. Riehm H, Reiter A, Schrappe M*, et al.* Die corticosteroid-abhangige dezemierung der leukamiezellzahl im blut als prognose-faktor bei der akuten lymphoblastischen leukemie im kindesalter (therapiestudie ALL-BFM 83)*.* Klin Padiatr 1986;199:151-60.
3. Arico M, Basso G, Mandelli F*, et al.* Good steroid response in vivo predicts a favorable outcome in children with T-cell acute lymphoblastic leukemia. The Associazione Italiana Ematologia Oncologia Pediatrica (AIEOP)*.* Cancer 1995;75:1684-93.
4. Dordelmann M, Reiter A, Borkhardt A*, et al.* Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia*.* Blood 1999;94:1209-17.
5. Schrappe M, Arico M, Harbott J*, et al.* Philadelphia chromosome-positive (Ph+) childhood acute lymphoblastic leukemia: good initial steroid response allows early prediction of a favorable treatment outcome*.* Blood 1998;92:2730-41.
6. Pieters R, Huismans DR, Loonen AH*, et al.* Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia*.* Lancet 1991;338:399-403.
7. Kaspers GJ, Veerman AJ, Pieters R*, et al.* In vitro cellular drug resistance and prognosis in newly diagnosed childhood acute lymphoblastic leukemia*.* Blood 1997;90:2723-9.
8. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. *In vitro* drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia*.* Blood 1997;89:2959-65.
9. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs*.* Blood 1998;92:259-66.
10. Pieters R, den Boer ML, Durian M*, et al.* Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants*.* Leukemia 1998;12:1344-8.
11. Maung ZT, Reid MM, Matheson E, Taylor PR, Proctor SJ, Hall AG. Corticosteroid resistance is increased in lymphoblasts from adults compared with children: preliminary results of in vitro drug sensitivity study in adults with acute lymphoblastic leukaemia*.* Br J Haematol 1995;91:93-100.
12. Styczynski J, Pieters R, Huismans DR, Schuurhuis GJ, Wysocki M, Veerman AJ. In vitro drug resistance profiles of adult versus childhood acute lymphoblastic leukaemia*.* Br J Haematol 2000;110:813-8.
13. Klumper E, Pieters R, Veerman AJ*, et al.* In vitro cellular drug resistance in children with relapsed/refractory acute lymphoblastic leukemia*.* Blood 1995;86:3861-8.
14. Theriault A, Boyd E, Harrap SB, Hollenberg SM, Connor JM. Regional chromosomal assignment of the human glucocorticoid receptor gene to 5q31*.* Hum Genet 1989;83:289-91.
15. Hollenberg SM, Evans RM. Multiple and cooperative trans-activation domains of the human glucocorticoid receptor*.* Cell 1988;55:899-906.

16. Tao Y, Williams-Skipp C, Scheinman RI. Mapping of glucocorticoid receptor DNA binding domain surfaces contributing to transrepression of NF-{kappa}B and induction of apoptosis. J Biol Chem 2001;276:2329-32.
17. Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J. Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. J Biol Chem 1991;266:3107-12.
18. Zilliacus J, Wright AP, Carlstedt-Duke J, Gustafsson JA. Structural determinants of DNA-binding specificity by steroid receptors. Mol Endocrinol 1995;9:389-400.
19. Picard D, Yamamoto KR. Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. Embo J 1987;6:3333-40.
20. Hollenberg SM, Giguere V, Segui P, Evans RM. Colocalization of DNA-binding and transcriptional activation functions in the human glucocorticoid receptor. Cell 1987;49:39-46.
21. Barnes PJ. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. Clin Sci (Colch) 1998;94:557-72.
22. Defranco DB. Role of molecular chaperones in subnuclear trafficking of glucocorticoid receptors. Kidney Int 2000;57:1241-9.
23. Beato M. Gene regulation by steroid hormones. Cell 1989;56:335-44.
24. Tsai SY, Carlstedt-Duke J, Weigel NL*, et al.* Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell 1988;55:361-9.
25. Cidlowski JA, Bellingham DL, Powell-Oliver FE, Lubahn DB, Sar M. Novel antipeptide antibodies to the human glucocorticoid receptor: recognition of multiple receptor forms in vitro and distinct localization of cytoplasmic and nuclear receptors. Mol Endocrinol 1990;4:1427-37.
26. Chapman MS, Askew DJ, Kuscuoglu U, Miesfeld RL. Transcriptional control of steroid-regulated apoptosis in murine thymoma cells. Mol Endocrinol 1996;10:967-78.
27. Reichardt HM, Kaestner KH, Tuckermann J*, et al.* DNA binding of the glucocorticoid receptor is not essential for survival. Cell 1998;93:531-41.
28. Helmberg A, Auphan N, Caelles C, Karin M. Glucocorticoid-induced apoptosis of human leukemic cells is caused by the repressive function of the glucocorticoid receptor. Embo J 1995;14:452-60.
29. Kofler R. The molecular basis of glucocorticoid-induced apoptosis of lymphoblastic leukemia cells. Histochem Cell Biol 2000;114:1-7.
30. Nazareth LV, Thompson EB. Leukemic cell apoptosis caused by constitutively active mutant glucocorticoid receptor fragments. Recent Prog Horm Res 1995;50:417-21.
31. Kaspers GJ, Pieters R, Veerman AP. Glucocorticoid resistance in childhood leukemia. International Journal of Pediatric Hematology/Oncology 1997;4:583-96.
32. Burnstein KL, Bellingham DL, Jewell CM, Powell-Oliver FE, Cidlowski JA. Autoregulation of glucocorticoid receptor gene expression. Steroids 1991;56:52-8.
33. Webster JC, Cidlowski JA. Downregulation of the glucocorticoid receptor. A mechanism for physiological adaptation to hormones. Ann N Y Acad Sci 1994;746:216-20.
34. Shimojo M, Hiroi N, Yakushiji F, Ueshiba H, Yamaguchi N, Miyachi Y. Differences in down-regulation of glucocorticoid receptor mRNA by cortisol, prednisolone and dexamethasone in HeLa cells. Endocrine Journal 1995;42:629-36.
35. Andreae J, Tripmacher R, Weltrich R*, et al.* Effect of glucocorticoid therapy on glucocorticoid receptors in children with autoimmune diseases. Pediatr Res 2001;49:130-5.
36. Silva CM, Powell-Oliver FE, Jewell CM, Sar M, Allgood VE, Cidlowski JA. Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid. Steroids 1994;59:436-42.

37. Pujols L, Mullol J, Perez M*, et al.* Expression of the human glucocorticoid receptor alpha and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone. Am J Respir Cell Mol Biol 2001;24:49-57.
38. Tonko M, Ausserlechner MJ, Bernhard D, Helmberg A, Kofler R. Gene expression profiles of proliferating vs. G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. Faseb J 2001;15:693-9.
39. Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res 1999;59:1378-85.
40. Breslin MB, Geng CD, Vedeckis WV. Multiple promoters exist in the human gr gene, one of which is activated by glucocorticoids. Mol Endocrinol 2001;15:1381-95.
41. Gametchu B, Chen F, Sackey F, Powell C, Watson CS. Plasma membrane-resident glucocorticoid receptors in rodent lymphoma and human leukemia models. Steroids 1999;64:107-19.
42. Sackey FN, Watson CS, Gametchu B. Cell cycle regulation of membrane glucocorticoid receptor in CCRF-CEM human ALL cells: correlation to apoptosis. Am J Physiol 1997;273: E571-83.
43. Karl M, Lamberts SWJ, Detera-Wadleigh SD*, et al.* Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. journal of clinical endocrinology and metabolism 1993;76:683-9.
44. Hurley DM, Accili D, Stratakis CA*, et al.* Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 1991;87:680-6.
45. Karl M, Lamberts SW, Koper JW*, et al.* Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assoc Am Physicians 1996;108:296-307.
46. Malchoff DM, Brufsky A, Reardon G*, et al.* A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 1993;91:1918-25.
47. Ruiz M, Lind U, Gafvels M*, et al.* Characterization of two novel mutations in the glucocorticoid receptor gene in patients with primary cortisol resistance. Clin Endocrinol 2001;55:363-71.
48. Feng J, Zheng J, Bennett WP*, et al.* Five missense variants in the amino-terminal domain of the glucocorticoid receptor: No association with puerperal psychosis or schizophrenia. Am J Med Genet 2000;96:412-7.
49. Koper JW, Stolk RP, de Lange P*, et al.* Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Hum Genet 1997;99:663-8.
50. Huizenga NA, Koper JW, De Lange P*, et al.* A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab 1998;83:144-51.
51. Lin RCY, Wang WYS, Morris BJ. High penetrance, overweight, and glucocorticoid receptor variant: case-controll study. BMJ 1999;319:1337-8.
52. Hillmann AG, Ramdas J, Multanen K, Norman MR, Harmon JM. Glucocorticoid receptor gene mutations in leukemic cells acquired in vitro and in vivo. Cancer Res 2000;60:2056-62.
53. Tissing WJE, Meijerink JPP, den Boer ML*, et al.* Polymorphisms in the glucocorticoid receptor gene in childhood leukemia [abstract]. Leukemia 2001;15:511.
54. Hollenberg SM, Weinberger C, Ong ES*, et al.* Primary structure and expression of a functional human glucocorticoid receptor cDNA. Nature 1985;318:635-41.
55. Oakley RH, Webster JC, Jewell CM, Sar M, Cidlowski JA. Immunocytochemical analysis of the glucocorticoid receptor alpha isoform (GRalpha) using GRalpha-specific antibody.

Steroids 1999;64:742-51.
56. Longui CA, Vottero A, Adamson PC*, et al.* Low glucocorticoid receptor alpha/beta ratio in T-cell lymphoblastic leukemia. Horm Metab Res 2000;32:401-6.
57. Tissing WJE, Lauten M, Meijerink JPP*, et al.* Glucocorticoid receptor splice variants alpha, beta and GR-P and in vivo glucocorticoid resistance in childhood acute lymphoblastic leukemia [abstract]. Blood 2001;98:313a.
58. Oakley RH, Sar M, Cidlowski JA. The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. J Biol Chem 1996;271:9550-9.
59. Oakley RH, Webster JC, Sar M, Parker CR, Jr., Cidlowski JA. Expression and subcellular distribution of the beta-isoform of the human glucocorticoid receptor. Endocrinology 1997;138:5028-38.
60. Hecht K, Carlstedt-Duke J, Stierna P, Gustafsson J, Bronnegard M, Wikstrom AC. Evidence that the beta-isoform of the human glucocorticoid receptor does not act as a physiologically significant repressor. J Biol Chem 1997;272:26659-64.
61. de Castro M, Elliot S, Kino T*, et al.* The non-ligand binding beta-isoform of the human glucocorticoid receptor (hGR beta): tissue levels, mechanism of action, and potential physiologic role. Mol Med 1996;2:597-607.
62. Carlstedt-Duke J. Glucocorticoid Receptor beta: View II. Trends Endocrinol Metab 1999;10:339-42.
63. Vottero A, Chrousos GP. Glucocorticoid receptor beta: View I. Trends Endocrinol Metab 1999;10:333-8.
64. Leung DYM, Hamid Q, Vottero A*, et al.* Association of glucocorticoid insensitivity with increased expression of glucocorticoid receptor beta. J Exp Med 1997;186:1567-74.
65. Gagliardo R, Chanez P, Vignola AM*, et al.* Glucocorticoid receptor alpha and beta in glucocorticoid dependent asthma. Am J Respir Crit Care Med 2000;162:7-13.
66. Oakley RH, Jewell CM, Yudt MR, Bofetiado DM, Cidlowski JA. The dominant negative activity of the human glucocorticoid receptor beta isoform. Specificity and mechanisms of action. J Biol Chem 1999;274:27857-66.
67. Bamberger CM, Bamberger AM, de Castro M, Chrousos GP. Glucocorticoid receptor beta, a potential endogenous inhibitor of glucocorticoid action in humans. J Clin Invest 1995;95:2435-41.
68. de Lange P, Koper JW, Brinkmann AO, de Jong FH, Lamberts SW. Natural variants of the beta isoform of the human glucocorticoid receptor do not alter sensitivity to glucocorticoids. Mol Cell Endocrinol 1999;153:163-8.
69. Brogan IJ, Murray IA, Cerillo G, Needham M, White A, Davis JR. Interaction of glucocorticoid receptor isoforms with transcription factors AP-1 and NF-kappaB: lack of effect of glucocorticoid receptor beta. Mol Cell Endocrinol 1999;157:95-104.
70. Rivers C, Levy A, Hancock J, Lightman S, Norman M. Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing. J Clin Endocrinol Metab 1999;84:4283-6.
71. Gerdes K, Beger C, Lauten M*, et al.* Quantification of the glucocorticoid receptor and its splice variant gamma in childhood acute lymphoblastic leukemia using real-time PCR [abstract]. Blood 2001;98:113a.
72. Ray DW, Davis JRE, White A, Clark AJL. Glucocorticoid receptor structure and function in glucocorticoid-resistant small cell lung carcinoma cells. Cancer research 1996;56:3276-80.
73. Moalli PA, Pillay S, Krett NL, Rosen ST. Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells. Cancer Res 1993;53:3877-9.
74. Krett NL, Pillay S, Moalli PA, Greipp PR, Rosen ST. A variant glucocorticoid receptor

messenger RNA is expressed in multiple myeloma patients. Cancer Res 1995;55:2727-9.
75. Segeren CM, de Lange P, Wiemer E*, et al.* Preferential expression of a non-functional glucocorticoid receptor in hematological malignancies [abstract]. Blood 1998;92:283b.
76. Segeren CM, Sonneveld P, de Lange P*, et al.* Molecular mechanisms of therapy resistance in multiple myeloma: the role of the non-functional glucocorticoid receptor delta [abstract]. Blood 1999;94:593a.
77. de Lange P, Segeren CM, Koper JW*, et al.* Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res 2001;61:3937-41.
78. Bodwell JE, Webster JC, Jewell CM, Cidlowski JA, Hu JM, Munck A. Glucocorticoid receptor phosphorylation: overview, function and cell cycle-dependence. J Steroid Biochem Mol Biol 1998;65:91-9.
79. Bourgeois S, Gruol DJ, Newby RF, Rajah FM. Expression of an mdr gene is associated with a new form of resistance to dexamethasone-induced apoptosis. Mol Endocrinol 1993;7:840-51.
80. Ueda K, Kino K, Taguchi Y*, et al.*, *Role of P-Glycoprotein in the transport of hormones and peptides*, in *Multidrug resistance in cancer cells*, S. Gupta and T. Tsuruo, Editors. 1996, John Wiuley & sons Ltd: Chicester. p. 303-19.
81. den Boer ML, Pieters R, Kazemier KM*, et al.* Relationship between major vault protein/lung resistance protein, multidrug resistance-associated protein, P-glycoprotein expression, and drug resistance in childhood leukemia. Blood 1998;91:2092-8.
82. Wuchter C, Karawajew L, Ruppert V*, et al.* Constitutive expression levels of CD95 and Bcl-2 as well as CD95 function and spontaneous apoptosis in vitro do not predict the response to induction chemotherapy and relapse rate in childhood acute lymphoblastic leukaemia. Br J Haematol 2000;110:154-60.
83. Maung ZT, Hogarth L, Reid MM, Proctor SJ, Hamilton PJ, Hall AG. Raised intracellular glutathione levels correlate with in vitro resistance to cytotoxic drugs in leukaemic cells from patients with acute lymphoblastic leukemia. Leukemia 1994;8:1487-91.
84. Kearns PR, Pieters R, Rottier MM, Pearson AD, Hall AG. Raised blast glutathione levels are associated with an increased risk of relapse in childhood acute lymphocytic leukemia. Blood 2001;97:393-8.
85. Den Boer ML, Pieters R, Kazemier KM*, et al.* Different expression of glutathione S-transferase alpha, mu and pi in childhood acute lymphoblastic and myeloid leukaemia. Br J Haematol 1999;104:321-7.
86. Anderer G, Schrappe M, Brechlin AM*, et al.* Polymorphisms within glutathione S-transferase genes and initial response to glucocorticoids in childhood acute lymphoblastic leukaemia. Pharmacogenetics 2000;10:715-26.
87. Pratt WB. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. J Biol Chem 1993;268:21455-8.
88. Rajapandi T, Greene LE, Eisenberg E. The molecular chaperones Hsp90 and Hsp70 are both necessary and sufficient to activate hormone binding by glucocorticoid receptor. J Biol Chem 2000;275:22597-604.
89. Kojika S, Sugita K, Inukai T*, et al.* Mechanisms of glucocorticoid resistance in human leukemic cells: implication of abnormal 90 and 70 kDa heat shock proteins. Leukemia 1996;10:994-9.
90. Kanelakis KC, Morishima Y, Dittmar KD*, et al.* Differential effects of the hsp70-binding protein BAG-1 on glucocorticoid receptor folding by the hsp90-based chaperone machinery. J Biol Chem 1999;274:34134-40.
91. Kullmann M, Schneikert J, Moll J*, et al.* RAP46 is a negative regulator of glucocorticoid

receptor action and hormone-induced apoptosis. J Biol Chem 1998;273:14620-5.
92. Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS. Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. Mol Cell Biol 1995;15:943-53.
93. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M. Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science 1995;270:286-90.
94. Scheinman RI, Cogswell PC, Lofquist AK, Baldwin AS. Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. Science 1995;270:283-6.
95. Wang W, Wykrzykowska J, Johnson T, Sen R, Sen J. A NF-kappa B/c-myc-dependent survival pathway is targeted by corticosteroids in immature thymocytes. J Immunol 1999;162:314-22.
96. Ramdas J, Harmon JM. Glucocorticoid-induced apoptosis and regulation of NF-kappaB activity in human leukemic T cells. Endocrinology 1998;139:3813-21.
97. Heck S, Bender K, Kullmann M, Gottlicher M, Herrlich P, Cato AC. I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. Embo J 1997;16:4698-707.
98. Kordes U, Krappmann D, Heissmeyer V, Ludwig WD, Scheidereit C. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. Leukemia 2000;14:399-402.
99. Bailey S, Hall AG, Pearson AD, Redfern CP. The role of AP-1 in glucocorticoid resistance in leukaemia. Leukemia 2001;15:391-7.
100. Geley S, Fiegl M, Hartmann BL, Kofler R. Genes mediating glucocorticoid effects and mechanisms of their regulation. Rev Physiol Biochem Pharmacol 1996;128:1-97.
101. Thulasi R, Harbour DV, Thompson EB. Suppression of c-myc is a critical step in glucocorticoid-induced human leukemic cell lysis. J Biol Chem 1993;268:18306-12.
102. Thompson EB, Medh RD, Zhou F, *et al.* Glucocorticoids, oxysterols, and cAMP with glucocorticoids each cause apoptosis of CEM cells and suppress c-myc. J Steroid Biochem Mol Biol 1999;69:453-61.
103. Medh RD, Wang A, Zhou F, Thompson EB. Constitutive expression of ectopic c-Myc delays glucocorticoid-evoked apoptosis of human leukemic CEM-C7 cells. Oncogene 2001;20:4629-39.
104. Martins TC, Aguas AP. Involvement of c-myc in the resistance of non-obese diabetic mice to glucocorticoid-induced apoptosis. Immunology 1998;95:377-82.
105. Loffler M, Ausserlechner MJ, Tonko M, *et al.* c-Myc does not prevent glucocorticoid-induced apoptosis of human leukemic lymphoblasts. Oncogene 1999;18:4626-31.
106. Evan GI, Wyllie AH, Gilbert CS, *et al.* Induction of apoptosis in fibroblasts by c-myc protein. Cell 1992;69:119-28.
107. Obexer P, Certa U, Kofler R, Helmberg A. Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA-, protein- and nucleotide synthesis. Oncogene 2001;20:4324-36.
108. Mann CL, Hughes FM, Jr., Cidlowski JA. Delineation of the signalling pathways involved in glucocorticoid- induced and spontaneous apoptosis of rat thymocytes. Endocrinology 2000;141:528-38.
109. Hakem R, Hakem A, Duncan GS, *et al.* Differential requirement for caspase 9 in apoptotic pathways in vivo. Cell 1998;94:339-52.
110. Kuida K, Haydar TF, Kuan CY, *et al.* Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell 1998;94:325-37.
111. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. Science 1998;281:1305-8.

112. Brady HJ, Salomons GS, Bobeldijk RC, Berns AJ. T cells from baxalpha transgenic mice show accelerated apoptosis in response to stimuli but do not show restored DNA damage-induced cell death in the absence of p53. gene product in. Embo J 1996;15:1221-30.
113. Hartmann BL, Geley S, Loffler M*, et al.* Bcl-2 interferes with the execution phase, but not upstream events, in glucocorticoid-induced leukemia apoptosis. Oncogene 1999;18:713-9.
114. Alnemri ES, Fernandes TF, Haldar S, Croce CM, Litwack G. Involvement of BCL-2 in glucocorticoid-induced apoptosis of human pre-B- leukemias. Cancer Res 1992;52:491-5.
115. Caron-Leslie LA, Evans RB, Cidlowski JA. Bcl-2 inhibits glucocorticoid-induced apoptosis but only partially blocks calcium ionophore or cycloheximide-regulated apoptosis in S49 cells. Faseb J 1994;8:639-45.
116. Smets LA, Van den Berg J, Acton D, Top B, Van Rooij H, Verwijs-Janssen M. BCL-2 expression and mitochondrial activity in leukemic cells with different sensitivity to glucocorticoid-induced apoptosis. Blood 1994;84:1613-9.
117. Salomons GS, Smets LA, Verwijs-Janssen M*, et al.* Bcl-2 family members in childhood acute lymphoblastic leukemia: relationships with features at presentation, in vitro and in vivo drug response and long-term clinical outcome. Leukemia 1999;13:1574-80.
118. Coustan-Smith E, Kitanaka A, Pui CH*, et al.* Clinical relevance of BCL-2 overexpression in childhood acute lymphoblastic leukemia. Blood 1996;87:1140-6.
119. Haarman EG, Kaspers GJ, Pieters R*, et al.* BCL-2 expression in childhood leukemia versus spontaneous apoptosis, drug induced apoptosis, and in vitro drug resistance. Adv Exp Med Biol 1999;457:325-33.
120. Ashraf J, Thompson EB. Identification of the activation-labile gene: a single point mutation in the human glucocorticoid receptor presents as two distinct receptor phenotypes. Mol Endocrinol 1993;7:631-42.

Chapter 3

Genetic variations in the glucocorticoid receptor gene are not related to glucocorticoid resistance in childhood acute lymphoblastic leukemia

Clin Cancer Research, 2005; **11**: 6050-6056

Wim J.E. Tissing[1], Jules P.P. Meijerink[1], Monique L. den Boer[1], Bas Brinkhof[1], Elisabeth F.C. van Rossum[3], Elisabeth R. van Wering[2], Jan W. Koper[3], Pieter Sonneveld[4], Rob Pieters[1]

1- Dept of Pediatric Oncology / Hematology, Erasmus MC – Sophia Children's Hospital, Rotterdam, the Netherlands
2- Dutch Childhood Oncology Group, the Hague, the Netherlands
3- Dept of Endocrinology, Erasmus MC, Rotterdam, the Netherlands
4- Dept of Hematology, Erasmus MC, Rotterdam, the Netherlands

ABSTRACT

Glucocorticoid (GC) sensitivity is an important prognostic factor in pediatric acute lymphoblastic leukemia (ALL). For its antileukemic effect, GCs bind the intracellular GC receptor (GR), subsequently regulating transcription of downstream genes. We analyzed whether genetic variations within the *GR* gene are related to differences in the cellular response to GC.

Methods. In leukemic samples of 57 children, the *GR* gene was screened for nucleotide variations using a PCR - SSCP - sequencing strategy. Data were linked to *in vivo* and *in vitro* GC resistance.

Results. No somatic mutations were detected in the *GR* gene coding region, but six polymorphisms (i.e. ER22/23EK, N363S, *Bcl1*, intron mutation 16 basepairs upstream of exon 5, H588H and N766N) were identified. In 67% of ALL cases at least one minor allele of these polymorphisms was detected. Although only borderline significant, the incidence for the N363S polymorphism minor allele was higher (12% vs. 6%, p=0.06) and for the ER22/23EK minor allele lower (4% vs. 7.6%, p=0.1) than in a healthy, comparable population. The different genotypes of the polymorphisms were not related to prednisone resistance.

In conclusion, polymorphisms but not somatic mutations in the *GR* gene coding region occur in leukemic blasts of children with ALL. Our data suggest that these genetic variations are not a major contributor for differences in cellular response to GCs in childhood ALL. The higher incidence of the N363S minor allele and the lower incidence of the ER22/23EK minor allele in our ALL population as compared to a normal population warrants further research.

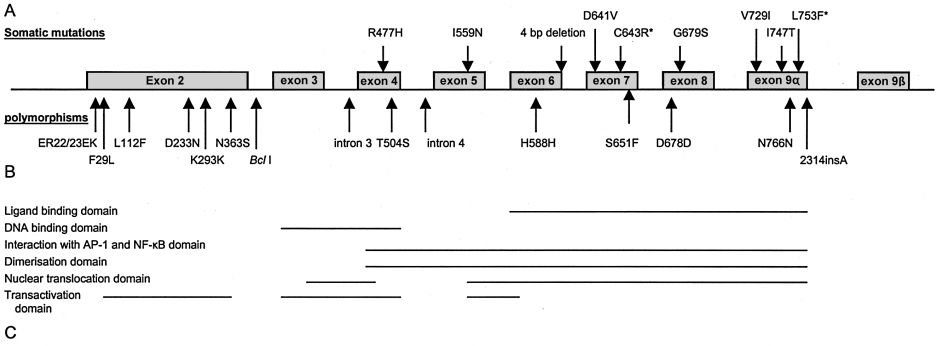
INTRODUCTION

Glucocorticoids (GC) are the keystone in the treatment of children with acute lymphoblastic leukemia (ALL). *In vivo* and *in vitro* GC response are important prognostic factors for outcome.[1-5]

GCs enter the cell by passive diffusion and bind to the intracellular glucocorticoid receptor (GR). The GC-GR complex is translocated to the nucleus, where it triggers transactivation as well as transrepression of GC responsive genes. The transactivation and/or transrepression of downstream genes finally result in the induction of programmed cell death (apoptosis). The presence of point mutations or particular polymorphisms in the *GR* gene may lead to an impaired formation of

the GC-GR complex or alter the transactivation or transrepression process.

In the last decade, patients with generalized glucocorticoid resistance syndromes were described, which were linked to somatic mutations in the *GR* gene localized within specific domains of the *GR* gene.[6-10] (Figure 1A and 1B) Besides somatic mutations, polymorphisms within the *GR* gene have been described in healthy populations (Figure 1C), of which some were associated with altered responsiveness to GC. The ER22/23EK polymorphism results in decreased sensitivity for GC[11], whereas two other polymorphisms (N363S and *Bcl1*) have been associated with an increased GC sensitivity as measured with a dexamethasone suppression test (i.e. the response of the serum cortisol level upon 1 mg dexamethasone given orally the evening before) in asymptomatic healthy adults.[12-14] The clinical relevance of the increased sensitivity for GCs in relation to the N363S polymorphism remains controversial[15-19], but the *Bcl1* polymorphism has been related to a higher body mass index, abdominal obesity and higher systolic blood pressure.[12, 20, 21] Besides



Polymorphism	Incidence in healthy population	GC sensitivity	Phenotype	reference
ER22/23EK	7.4% heterozygote	decreased	Better metabolic health profile	11, 22
N363S	6% heterozygote	increased	Possibly related to overweight and higher body mass index	13, 15-18, 38
Bcl1	48% heterozygote 12% homozygote	increased	Increased (abdominal) obesity, increased body mass index and increased blood pressure.	12, 20, 35-37
Intron mutation 46 basepairs upstream from exon 4	2.5% heterozygote	Normal		18, 22
Intron mutation 16 basepairs upstream from exon 5	42% heterozygote 10% homozygote	Normal		18, 22
N766N	22.5% heterozygote 2.5% homozygote	Normal		18, 22

Figure 1. The human glucocorticoid receptor (GR) gene with known mutations and polymorphisms
A. The gene structure of the GR. Known somatic mutations and polymorphisms are indicated with arrows. The C643R and L753F are mutations found in ALL cell lines.
B. Location of functional domains within the GR gene.
C. Polymorphisms in the GR as reported in the literature.

these three polymorphisms, various other polymorphisms have been described with no or an unknown relationship to GC responsiveness.[19, 22-24]

Till date, primary ALL patient cells have not been analyzed for genetic variations in the *GR* gene which might explain GC resistance. Somatic mutations in the *GR* gene have been described in 2 ALL cell lines (L753F, C643R) for which it is yet unclear whether these mutations alter the response to GC.[25-28]

In the present study we analyzed the incidence of genetic variations in the coding region of the *GR* gene and the *Bcl1* restriction site and whether such genetic variations are related to *in vitro* or *in vivo* GC resistance in childhood ALL.

MATERIALS & METHODS

Patients. All patients were treated according to the DCOG (Dutch Childhood Oncology Group) ALL treatment protocols 7 and 8. These protocols start with 7 days monotherapy of prednisone and 1 intrathecal dose of MTX. The prednisone response was determined on day 8: prednisone good response (PGR) is defined as less than 1000 leukemic blasts/µl peripheral blood and prednisone poor response (PPR) as ≥ 1000 blasts/µl.[29] Patient material was taken prior to initial therapy. To differentiate between (leukemic) somatic mutations and polymorphisms, we also collected normal peripheral blood mononuclear cells (PBMCs) of the same patients at complete remission (CR). The study has been approved by the medical ethical committee of the Erasmus Medical Center Rotterdam and written informed consent was obtained from the patients and/or their parents or guardians as appropriate. The mononuclear cell fraction was separated by Lymphoprep density gradient centrifugation (density 1.077 g/ml, Nycomed Pharma, Oslo, Norway), and, when necessary, non-leukemic cells were depleted by immunomagnetic beads to purify the samples to more than 90% of leukemic cells.

DNA isolation. DNA was extracted using Trizol reagent (Gibco BRL, Life Technologies), according to the protocol supplied by the manufacturer with some modifications. An additional protein degradation step was performed, using Proteinase K (1 mg/ml). DNA was purified in 2 separate steps. In the first step DNA was precipitated using 0.5M NaCl, 60 µg of glycogen and 2.5 volumes of 70% EtOH followed by an additional phenol extraction and a chloroform extraction. In the second purification step, DNA was precipitated in 0.3M NaAc and 2 volumes of EtOH 70%.

PCR amplification. 100 ng genomic DNA was used to amplify the *GR* gene using

the primers as described in Table 1. The Genebank Accession Number AC091925 was used as reference *GR* sequence to which sequence variations were compared. For the exons 3 to 9-alpha, the primers were located in intronic DNA directly flanking these exons. Since exon 2 was too large to be amplified in a single PCR, five overlapping PCRs were developed to cover exon 2 (Table 1A). The PCR reaction was performed in 100 µl 1 x PCR buffer II (Applied Biosystems, Foster City, CA, USA), 3 mM $MgCl_2$ (except 2-4: 1.5 mM), 250 nM of dNTPs (10 µmol/µl, Amersham Biosciences, Freiburg, Germany), 3 Units of AmpliTaq Gold (5U/µl, Applied Biosystems, Foster City, CA, USA), 100 ng DNA and 400 nM of the forward and reverse primers. Prior to amplification, DNA was denatured and AmpliTaq Gold activated for 9 minutes at 95^{0}C, followed by 40 cycles of denaturation at 95^{0}C for 30 sec, annealing at 55^{0}C (except 2-4: 59^{0}C) for 30 sec, extension at 72^{0}C for 60 sec and a final period of extension at 72^{0}C for 10 min.

SSCP analysis. Ten µl of PCR product was mixed with 45 µl denaturating loading buffer. For optimal resolution, two loading buffers were used separately: buffer I contained 50 mM of NaOH, 1 mM of EDTA and 0.05% bromophenol blue, buffer II contained 96% of formamide, 20 mM EDTA and 0.05% bromophenol blue. DNA was denatured in buffers I and II by heating for 10 minutes at 50^{0}C and 95^{0}C respectively followed by direct cooling on ice. Six µl was loaded on a 12.5% polyacrylamide gel (Genegel excel, Amersham Biosciences, Freiburg, Germany). The products were separated on a Genephor electrophoresis unit (Amersham Biosciences, Freiburg, Germany) with accompanying bufferstrips (Amersham Biosciences, Freiburg, Germany), using 2 different temperatures for optimal resolution (5^{0}C and 18^{0}C). The DNA fragments were separated for 2 hours at 600V, 25mA and 15W. Gels were stained using the PlusOne™ DNA silver staining kit (Amersham Biosciences, Freiburg, Germany).

Sequencing. PCR products displaying an abnormal migration pattern on SSCP gels were sequenced. PCR products were purified using the QIAquick PCR purification kit. (Qiagen, Leusden, the Netherlands) The BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) was used according to the protocol as provided by the manufacturer. Sequencing primers used were the same as used in the PCR reaction (Table 1A), although for some reactions separate sequencing primers were developed (Table 1B). The products of the sequencing reaction were analyzed using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Taqman analysis of the Bcl1 polymorphism. Since the *Bcl1* polymorphism is

Table 1.
A. Primers used for PCR / SSCP analysis*

Exon 2-1	fw	5′ GAT TCG GAG TTA ACT AAA AG 3′	§
	rev	5′ ATC CCA GGT CAT TTC CCA TC 3′	
Exon 2-2	fw	5′ CCA AGC AGC GAA GAC TTT TG 3′	§
	rev	5′ TAC CTG GGG ACC CAG AAG AA 3′	§
Exon 2-3	fw	5′ CCA CAG AGA AGG AGT TTC CA 3′	§
	rev	5′ TTG CCT GAC AGT AAA CTG TG 3′	§
Exon 2-4	fw	5′ CCA GTA ATG TAA CAC TGC CCC 3′	§
	rev	5′ TTC GAC CAG GGA AGT TCA GA 3′	§
Exon 2-5	fw	5′ AGT ACC TCT GGA GGA CAG AT 3′	
	rev	5′ GTC CAT TCT TAA GAA ACA GG 3′	
Exon 3	fw	5′ AGT TCA CTG TGA GCA TTC TG 3′	§
	rev	5′ CGT GAG AAA TAA AAC CAA GT 3′	§
Exon 4	fw	5′ CAC CGG AAA CAA AGA CA 3′	§
	rev	5′ TTT TAT TGG GCA GTA ACA TT 3′	§
Exon 5	fw	5′ GAA TAA ACT GTG TAG CGC AG 3′	
	rev	5′ TAG TCC CCA GAA CTA AGA GA 3′	
Exon 6	fw	5′ GAT CTT CTG AAG AGT GTT GC 3′	§
	rev	5′ GGG AAA ATG ACA CAC ATA CA 3′	§
Exon 7	fw	5′ GAA AGT TCT CCA AAA TTC TG 3′	§
	rev	5′ TTG GTG TCA CTT ACT GTG CC 3′	§
Exon 8	fw	5′ GAC ACA GTG AGA CCC TAT CT 3′	§
	rev	5′ CAC CAA CAT CCA CAA ACT GG 3′	§
Exon 9 alpha	fw	5′ GGA ATT CCA GTG AGA TTG GT 3′	§
	rev	5′ TAT AAA CCA CAT GTA GTG CG 3′	§

B. Primers used for the sequencing reaction which are different from the primers in Table 1A

Exon 2-1	fw	5′ GAT TCG GAG TTA ACT AAA AG 3′ (conform 1A)
	rev	5′ TAC TGA GCC TTT TGG AAA AT 3′
Exon 2-5	fw	5′ GGA GGA CAG ATG TAC CAC TA 3′
	rev	5′ AAA AGC ACA TGA ATC TTT AGA G 3′
Exon 5	fw	5′ CTC CCA TCT TAA TAG TTT TAG AA 3′
	rev	5′ TGG GCT CAC GAT GAT ATA A 3′

C. Primers and probe used for Taqman analysis*

Bcl1	fw	5′ GCT CAC AGG GTT CTT GCC ATA 3′
	rev	5′ TTG CAC CAT GTT GAC ACC AAT 3′
	probe	5′ -FAM-TCT GCT GAT CAA TCT 3′
		5′ -VIC-TCT GCT GAT GAA TCT 3′

§ Primers used for both the PCR reaction and the sequencing reaction.
* Some of the primers and the probe have been described before[22].

located in intron 2 and is not covered by exonic PCR amplification reactions, a separate analysis was performed as described previously.[12] Briefly, an allelic discrimination using Taqman-chemistry on an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) was used. Primers and probe as depicted in Table 1C were used at concentrations of 400 nmol/l and 50 nmol/l respectively.

MTT assay. In vitro drug cytotoxicity was assessed with the MTT assay as described earlier.[2,5,30] Briefly, patient blasts were cultured with or without prednisolone disodiumphosphate in a concentration range between 0.06 and 250 µg/ml. At day 4 MTT is added, which can only be reduced into formazan by viable cells. The reduced product was measured spectrophotometrically at 562 nm. The leukemic cell survival is calculated by: (OD drug treated well / OD control wells without drug) x 100%. The LC50 value represents the concentration of the drug at which 50% of the cells are killed and is used as measure of *in vitro* drug cytotoxicity. *In vitro* prednisolone sensitivity was determined as sensitive (LC50 ≤ 0.1 µg/ml), intermediate (LC50 0.1 – 150 µg/ml) or resistant (LC50 ≥ 150 µg/ml), as has previously been described to be of prognostic value.[5, 31]

Statistics. The Chi-square test for trend and the Mann-Whitney test with correction for tied ranks were used to test for a difference in genotype distribution for the polymorphisms between the study population and a healthy population. A logistic regression analysis, the Chi-square test for trend and the Mann-Whitney test with correction for tied ranks were used to test for a relationship between the different genotypes (wildtype, heterozygous or homozygous) and *in vivo* or *in vitro* GC resistance. A power analysis yields that, with a p-value < 0.05, a standardized difference of 0.85 in such a study (42 *in vivo* prednisone sensitive and 15 resistant patients) is detected with 0.8 power. This allows to draw conclusions on large differences between sensitive and resistant patients, which is what we expect when GC resistance is determined by genetic variations. The probability of event-free survival (pEFS) was calculated using the Kaplan-Meier method and a correlation between different genotypes and pEFS was analyzed using the log-rank test.

RESULTS

In this study, 57 children with ALL were included for which *in vitro* and/or *in vivo* response towards GCs was known. Patient characteristics are given in Table 2. To

Table 2. Patient characteristics of the study population.

		N	(%)
Gender	Male	34	(60)
	Female	23	(40)
Age	0-1 years	5	(9)
	1-10 years	40	(70)
	≥ 10 years	12	(21)
WBC	< 50000 /µl	34	(60)
	≥ 50000 /µl	23	(40)
Immunophenotype	Common / pre-B ALL	45	(79)
	T-ALL	11	(19)
	pro-B ALL	1	(2)
***In vivo* prednisone response ***	Prednisone good response	42	(74)
	Prednisone poor response	15	(26)
In vitro* prednisolone response *	Sensitive	16	(40)
	Intermediate	16	(40)
	Resistant	8	(20)

* The *in vivo* prednisone response is determined on day 8 after 7 days of monotherapy of prednisone and one intrathecal dose of MTX: Prednisone good response (PGR) is defined as less than 1000 leukemic blasts/µl peripheral blood and prednisone poor response (PPR) as ≥ 1000 blasts/µl.[29]
** *In vitro* prednisolone response was determined using the MTT assay as sensitive (LC50 ≤ 0.1 µg/ml), intermediate (LC50 0.1 – 150 µg/ml) or resistant (LC50 ≥ 150 g/ml).[5, 31]

study whether *in vitro* or *in vivo* resistance correlated with the presence of genetic variations within the *GR* gene, we developed a PCR/SSCP strategy. The sensitivity of the SSCP technique varies between 80-95% using a single SSCP condition.[32] In the current study, we used 2 denaturating conditions and 2 temperature conditions to further improve the level of detection of genetic variations in the *GR*. PCR products showing an abnormal migration pattern on SSCP gel were sequenced. To check for the sensitivity and specificity of the assay, control samples with known genotype (wildtype, heterozygote and homozygote) for the polymorphisms N363S, the intron mutation upstream of exon 5 and N766N, obtained from a previous study of J.W. Koper and co-workers[22], were analyzed and successfully distinguished (data not shown). Secondly, in all PCR products with an aberrant SSCP pattern, a nucleotide variation was found after DNA sequencing. Thirdly, 50 PCR products with a normal SSCP pattern were sequenced, showing only wildtype alleles.

Each genetic variation had its specific denaturating buffer and temperature at which the resolution was most optimal (for example, N363S was detected with both buffer I and II at 18°C, whereas the intron mutation 16 basepairs upstream of exon 5 only was detected with buffer II at 5°C). Figure 2A shows an example of the SSCP pattern for the PCR products of exon 5 for 13 patients. The results

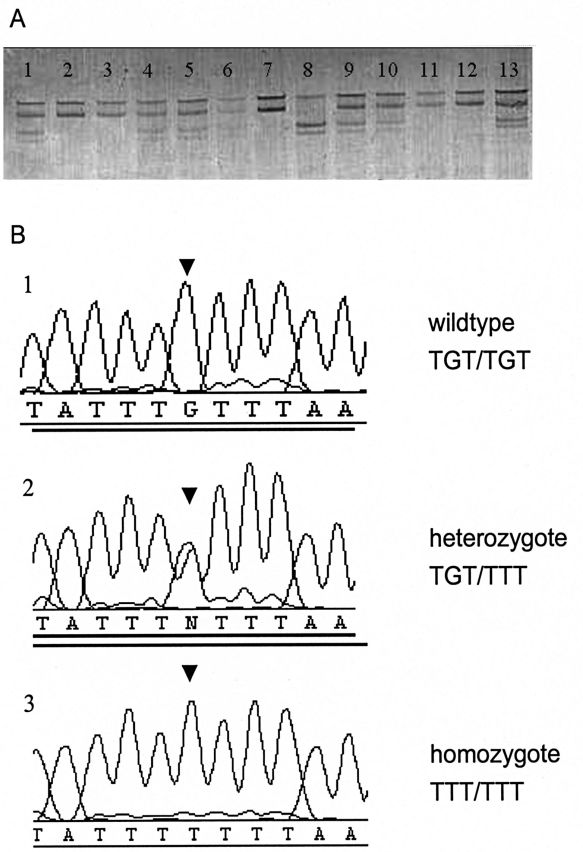


Figure 2. Example of the detection of mutations in the GR by PCR-SSCP and sequence analysis
A. SSCP pattern of exon 5 of 12 patient samples, representing an intron mutation 16 basepairs upstream of exon 5: wildtype in lanes 2, 3, 7, 11, 12, heterozygous in lanes 1, 4, 5, 6, 9, 10, 13 and a homozygous mutation in lane 8.
B. Sequence analysis of 3 patients for the site of the intron mutation 16 basepairs upstream of exon 5. 1- represents a wildtype patient, 2- represents a heterozygous and 3- a homozygous patient.

of the sequencing procedure for 3 of these patients (wildtype, heterozygote and homozygote) are shown in Figure 2B.

SSCP analysis of the coding exons 2-9 of the *GR* gene in 57 patients and subsequent DNA sequencing of aberrant SSCP patterns resulted into the detection of 6 different genetic variations. These 6 genetic variations were all identified as previously known polymorphisms, namely ER22/23EK, N363S, *Bcl1*, intron mutation 16 basepairs upstream of exon 5, H588H and N766N (Table 3). No previously unknown mutations were detected in these patients. In 55% of the patients with one or more *GR* gene sequence alterations in the ALL blasts, normal

Table 3. Genetic variations in the *GR* gene, incidence and relation to *in vivo* prednisone and *in vitro* prednisolone sensitivity.

Polymorphism		Number of patients tested	Incidence	*In vivo* Prednisone response		Number of patients tested	*In vitro* Prednisolone sensitivity			
				Good	poor		intermediate	resistant	sensitive	Log OR
ER22/23EK	GAGAGG/GAGAGG*	57	55 (96%)	41	14	40	16	8	15	
	GAGAGG/GA*AAA*G		2 (4%)	1	1		0	0	1	
NCBI SNP database:	GA*AAA*G/GA*AAA*G		0	0	0		0	0	0	
rs89/6190	GAG/AAG + AAG/AAG		2 (4)	1	1		0	0	1	ND
N363S	ATT/ATT	57	50 (88%)	37	13	40	14	7	14	
	ATT/*G*TT		7 (12%)	5	2		2	1	2	
NCBI SNP database:	*G*TT/*G*TT		0	0	0		0	0	0	
rs6195	A/G + G/G		7 (12%)	5	2		2	1	2	-0.011 (p=0.99)
Bcl1	TCA/TCA	53	24 (45%)	18	6	38	7	3	8	
	TCA/T*G*A		21 (40%)	14	7		6	2	5	
	T*G*A/T*G*A		8 (15%)	6	2		2	2	3	
	C/G + G/G		29 (55%)	20	9		8	4	8	0.689 (p=0.32)
Upstream exon 5**	G/G	57	34 (60%)	26	8	40	9	5	10	
	G/*T*		20 (35%)	13	7		7	2	4	
NCBI SNP database:	*T*/*T*		3 (5%)	3	0		0	1	2	
rs6188	G/T + T/T		23 (40)	16	7		7	3	6	0.613 (p=0.39)
H588H	ACC/ACC	57	56 (98%)	41	15	40	16	8	15	
	ACC/A*T*C		1 (2%)	1	0		0	0	1	
	A*T*C/A*T*C		0	0	0		0	0	0	
	C/T + T/T		1 (2%)	1	0		0	0	1	ND
N766N	AAT/AAT	56	44 (79%)	31	13	40	13	5	12	
	AAT/AA*C*		10 (18%)	8	2		3	2	3	
	AA*C*/AA*C*		2 (3%)	2	0		0	1	1	
	T/C + C/C		12 (21%)	10	2		3	3	4	-1.602 (p=0.11)

No statistically differences were found between the *in vivo* prednisone response and *in vitro* prednisolone sensitivity in relation to the occurrence of the different genetic variations. The Log Odds Ratio (analyzing wildtype versus homozygote and heterozygote genotypes) was determined using logistic regression analysis. ND means not done, since the numbers of the minor allele carriers are too small to test for statistical significance.
The incidentally described polymorphisms (see Figure 1) were not detected, besides H588H.
* The italic letter indicates the genetic alteration.
** intron mutation, 16 basepairs upstream of exon 5.

mononuclear cells at the time of complete remission were available for further analysis. In all cases, the sequence alterations such as found in the leukemic blasts were also present in these non-malignant cells, indicating that these alterations were indeed polymorphisms. The genotype distribution was consistent with a Hardy-Weinberg equilibrium.

In 38 out of 57 patients (67%), at least one minor allele of the polymorphisms was found. In 2 patients we found 4 minor alleles, in 11 patients 3 minor alleles, in 9 patients 2 minor alleles and in 16 patients 1 minor allele. In 19 patients no abnormalities were detected. (Note that only 53 patients were screened for the *Bcl1* polymorphism and only 56 patients were screened for the N766N polymorphism.)

The genotype distribution of the polymorphisms in our cohort was comparable to the incidence as reported in literature, except for the N363S and ER22/23EK polymorphisms. (Figure 1C) The minor allele of the N363S polymorphism was observed in a higher percentage of ALL patients (12%) and the minor allele of ER22/23EK polymorphism was found in only 4% of the patients as compared to 6% and 7.4% respectively in the normal, healthy population from the same topographical region. The test to compare the incidence in the study population and the incidence as reported in the literature yielded borderline significance (p=0.06 and p=0.1).

The relationship between the polymorphisms and *in vivo* and *in vitro* GC resistance is shown in Table 3. We analyzed a possible relationship between GC resistance versus wildtype, heterozygote and homozygote as well as a possible relationship between GC resistance versus wildtype and one minor allele (i.e. heterozygote or homozygote). None of the polymorphisms appeared to be correlated with *in vivo* prednisone response (N=57) nor with *in vitro* prednisolone resistance (N=40) (Table 3). The presence of 2 minor alleles in one patient (e.g. N363S and *Bcl1*) did not correlate with *in vivo* or *in vitro* GC resistance as well.

In this small cohort of 57 patients, we did not find an association between the presence of polymorphisms and event-free survival (data not shown).

DISCUSSION

Glucocorticoids are important drugs in the multi-drug treatment protocols for children with ALL. The *in vivo* prednisone response as used in the BFM studies has been found to be one of the strongest prognostic factors for therapy outcome

in childhood ALL.[1, 29, 33] The *in vitro* prednisolone sensitivity of untreated leukemic cells, as determined by the MTT assay, is a strong risk factor for outcome as well, and can predict the occurrence of a relapse even within the group of patients with a good *in vivo* prednisone response.[2, 4, 5, 31]

To induce apoptosis in GC sensitive cells, GCs has to bind the intracellular GR. This results in transactivation and/or transrepression of GC responding genes, which eventually results in apoptosis in GC sensitive patients. Although GC resistance is an important cause of treatment failure, little is known about possible mechanisms of GC resistance in childhood ALL.[34] In this study we tested the hypothesis that GC resistance is the result of genetic variations within the *GR* gene which might alter the GC-binding capacity or transactivation or transrepression of GC responding genes.

Analysis of the coding region of the *GR* gene for sequence alterations in 57 patients revealed six different genetic variations. These genetic variations were identical to previously reported polymorphisms (i.e. ER22/23EK, N363S, *Bcl1*, intron mutation 16 basepairs upstream of exon 5, H588H and N766N). (Figure 1) No somatic mutations, which might result in altered GC sensitivity, were found in these 57 ALL cases. In 2 studies somatic mutations were found in cell lines from ALL patients. A L753F somatic mutation was shown to be present in CCRF-CEM cells and original ALL cells from which the cell line was established.[27] As the L753F somatic mutation was found in both the GC sensitive and resistant clones of the CCRF-CEM cell line, it probably has no correlation with GC sensitivity.[25, 26] The C643R somatic mutation was found in a cell line established from a Japanese patient with ALL (P30/OHK). It is not clear whether this mutation was also present in the original cells or that it was acquired during tissue culture. Transfection studies showed that the C643R allele had no transcriptional activity but no evidence was given whether this mutation was related to GC resistance in ALL cells.[28] All together these data suggest that somatic mutations do not occur frequently and can not explain resistance to GCs in pediatric ALL.

In contrast to somatic mutations, we detected several polymorphisms of the GR gene in children with ALL. Confirmation of the same genetic variations in normal peripheral blood mononuclear cells obtained during clinical remission confirmed that the variations indeed were polymorphisms. Other polymorphisms of the *GR* gene as reported in the literature with a low incidence were not detected in our population, nor did we detect any so far unreported genetic variations. Although we can not exclude that these polymorphisms with low incidence were missed by

our PCR/SSCP, we had optimized our strategy to achieve a high detection limit, so it is reasonable to assume that these low incidence polymorphisms were absent in our population.

The genotype distribution of the polymorphisms *Bcl1*, N766N and the intron mutation upstream of exon 5 was the same as reported in the literature. However, the minor allele of the ER22/23EK polymorphism was found in a lower percentage than in a healthy population of the same ethnicity (4% versus 7.4% respectively), whereas the minor allele of N363S was found in a higher percentage as compared to the healthy population of the same ethnicity (12% versus 6% respectively).[13] Since the number of patients in our study is small and no statistical significance was reached (p=0.1 and p=0.06 respectively), the altered frequency of both polymorphisms warrants further evaluation in a larger cohort of patients. The minor allele of the H588H polymorphism was found in one of our patients. It was reported before in cell lines that originate from four Japanese individuals.[24] In a previous Dutch study including a large healthy population, this polymorphism was not reported.[22]

In the literature, the clinically most relevant polymorphism of the *GR* gene is the *Bcl1* polymorphism.[12] Previously, it was detected with a restriction fragment length polymorphism-based technique, but recently the exact mutation was found to be a C/G single nucleotide polymorphism in intron 2, 646 basepairs downstream from exon 2, for which a specific real-time PCR approach has been developed.[12, 35] It is associated with increased response to a dexamethasone suppression test and with increased (abdominal) obesity, body mass index and blood pressure.[20, 36, 37] However, in our population the *Bcl1* polymorphism was not correlated with *in vivo* or *in vitro* GC sensitivity. Also for the other five polymorphisms no correlation with *in vivo* or *in vitro* GC response was found.

In conclusion, polymorphisms but not somatic mutations in the coding region of the *GR* gene occur in leukemic blasts of children with ALL. Our data suggest that genetic variations in the GR gene are not a major contributor for differences in cellular response to GCs in childhood ALL

Acknowledgement

We would like to thank Dr H.B.Beverloo, PhD from the department of Clinical Genetics, Erasmus MC, Rotterdam for supplying DNA from leukemic blasts and Prof. Dr. J.J.M. van Dongen, MD, PhD from the department of Immunology, Erasmus MC, Rotterdam for providing us DNA from patients in continuous complete remission.

In addition, we would like to thank R.X. Menezes, PhD, for statistical support.

References

1. Lauten M, Stanulla M, Zimmermann M, Welte K, Riehm H, Schrappe M. Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter. Klin Padiatr 2001;213:169-74.
2. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood 1998;92:259-66.
3. Pieters R, den Boer ML, Durian M*, et al.* Relation between age, immunophenotype and in vitro drug resistance in 395 children with acute lymphoblastic leukemia--implications for treatment of infants. Leukemia 1998;12:1344-8.
4. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. *In vitro* drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. Blood 1997;89:2959-65.
5. Den Boer ML, Harms DO, Pieters R*, et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol 2003;21:3262-8.
6. Karl M, Lamberts SWJ, Detera-Wadleigh SD*, et al.* Familial glucocorticoid resistance caused by a splice site deletion in the human glucocorticoid receptor gene. journal of clinical endocrinology and metabolism 1993;76:683-9.
7. Karl M, Lamberts SW, Koper JW*, et al.* Cushing's disease preceded by generalized glucocorticoid resistance: clinical consequences of a novel, dominant-negative glucocorticoid receptor mutation. Proc Assoc Am Physicians 1996;108:296-307.
8. Malchoff DM, Brufsky A, Reardon G*, et al.* A mutation of the glucocorticoid receptor in primary cortisol resistance. J Clin Invest 1993;91:1918-25.
9. Hurley DM, Accili D, Stratakis CA*, et al.* Point mutation causing a single amino acid substitution in the hormone binding domain of the glucocorticoid receptor in familial glucocorticoid resistance. J Clin Invest 1991;87:680-6.
10. Ruiz M, Lind U, Gafvels M*, et al.* Characterization of two novel mutations in the glucocorticoid receptor gene in patients with primary cortisol resistance. Clin Endocrinol 2001;55:363-71.
11. van Rossum EF, Koper JW, Huizenga NA*, et al.* A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. Diabetes 2002;51:3128-34.
12. Van Rossum EF, Koper JW, Van Den Beld AW*, et al.* Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. Clin Endocrinol (Oxf) 2003;59:585-92.
13. Huizenga NA, Koper JW, De Lange P*, et al.* A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab 1998;83:144-51.
14. Van Rossum EF, Lamberts SW. Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. Recent Prog Horm Res 2004;59:333-57.
15. Rosmond R, Bouchard C, Bjorntorp P. Tsp509I polymorphism in exon 2 of the glucocorticoid receptor gene in relation to obesity and cortisol secretion: cohort study. Bmj 2001;322:652-3.
16. Dobson MG, Redfern CP, Unwin N, Weaver JU. The N363S polymorphism of the glucocorticoid receptor: potential contribution to central obesity in men and lack of

association with other risk factors for coronary heart disease and diabetes mellitus. J Clin Endocrinol Metab 2001;86:2270-4.
17. Di Blasio AM, van Rossum EF, Maestrini S*, et al.* The relation between two polymorphisms in the glucocorticoid receptor gene and body mass index, blood pressure and cholesterol in obese patients. Clin Endocrinol (Oxf) 2003;59:68-74.
18. Lin RCY, Wang WYS, Morris BJ. High penetrance, overweight, and glucocorticoid receptor variant: case-controll study. BMJ 1999;319:1337-8.
19. Lin RC, Wang WY, Morris BJ. Association and linkage analyses of glucocorticoid receptor gene markers in essential hypertension. Hypertension 1999;34:1186-92.
20. Rosmond R, Chagnon YC, Holm G*, et al.* A glucocorticoid receptor gene marker is associated with abdominal obesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis. Obes Res 2000;8:211-8.
21. Clement K, Philippi A, Jury C*, et al.* Candidate gene approach of familial morbid obesity: linkage analysis of the glucocorticoid receptor gene. Int J Obes Relat Metab Disord 1996;20:507-12.
22. Koper JW, Stolk RP, de Lange P*, et al.* Lack of association between five polymorphisms in the human glucocorticoid receptor gene and glucocorticoid resistance. Hum Genet 1997;99:663-8.
23. Feng J, Zheng J, Bennett WP*, et al.* Five missense variants in the amino-terminal domain of the glucocorticoid receptor: No association with puerperal psychosis or schizophrenia. Am J Med Genet 2000;96:412-7.
24. Nagano M, Nakamura T, Ozawa S, Maekawa K, Saito Y, Sawada J. Allele-specific long-range PCR/sequencing method for allelic assignment of multiple single nucleotide polymorphisms. J Biochem Biophys Methods 2003;55:1-9.
25. Strasser-Wozak EMC, Hattmannstorfer R, Hala M*, et al.* splice site mutation in the glucocorticoid receptor gene causes resistance to glucocorticoid-induced apoptosis in a human acute leukemic cell line. Cancer Res 1995;55:348-53.
26. Powers JH, Hillmann AG, Tang DC, Harmon JM. Cloning and expression of mutant glucocorticoid receptors from glucocorticoid-sensitive and -resistant human leukemic cells. Cancer Res 1993;53:4059-65.
27. Hillmann AG, Ramdas J, Multanen K, Norman MR, Harmon JM. Glucocorticoid receptor gene mutations in leukemic cells acquired in vitro and in vivo. Cancer Res 2000;60:2056-62.
28. Nagano M, Nakamura T, Niimi S*, et al.* Substitution of arginine for cysteine 643 of the glucocorticoid receptor reduces its steroid-binding affinity and transcriptional activity. Cancer Lett 2002;181:109-14.
29. Riehm H, Reiter A, Schrappe M*, et al.* Die corticosteroid-abhangige dezemierung der leukamiezellzahl im blut als prognose-faktor bei der akuten lymphoblastischen leukemie im kindesalter (therapiestudie ALL-BFM 83). Klin Padiatr 1986;199:151-60.
30. Pieters R, Loonen AH, Huismans DR*, et al.* In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 1990;76:2327-36.
31. Pieters R, Huismans DR, Loonen AH*, et al.* Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet 1991;338:399-403.
32. Vidal-Puig A, Moller DE. Comparative sensitivity of alternative single-strand conformation polymorphism (SCP) methods [Technical Brief]. Biotechniques 1994;17:490-6.
33. Schrappe M, Reiter A, Zimmermann M*, et al.* Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Munster. Leukemia 2000;14:2205-22.
34. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid

sensitivity and resistance in acute lymphoblastic leukemia. Leukemia 2003;17:17-25.
35. Fleury I, Beaulieu P, Primeau M, Labuda D, Sinnett D, Krajinovic M. Characterization of the BclI Polymorphism in the Glucocorticoid Receptor Gene. Clin Chem 2003;49:1528-31.
36. Buemann B, Vohl MC, Chagnon M*, et al.* Abdominal visceral fat is associated with a BclI restriction fragment length polymorphism at the glucocorticoid receptor gene locus. Obes Res 1997;5:186-92.
37. Ukkola O, Rosmond R, Tremblay A, Bouchard C. Glucocorticoid receptor Bcl I variant is associated with an increased atherogenic profile in response to long-term overfeeding. Atherosclerosis 2001;157:221-4.

Chapter 4

The expression of the glucocorticoid receptor, but not the differential expression of specific isoforms, is associated with glucocorticoid resistance in childhood ALL

Published in adapted form in: *Haematologica*, 2005; **90**: 1279-81

Wim J.E. Tissing[1], Melchior Lauten[2], Jules P.P. Meijerink[1], Monique L. den Boer[1], Jan Willem Koper[3], Pieter Sonneveld[4], Rob Pieters[1]

[1] Dept of Pediatric Oncology/Haematology, Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center, Rotterdam, The Netherlands
[2] Dept of Pediatric Haematology and Oncology, Hannover Medical School, Hannover, Germany
[3] Dept of Endocrinology, Erasmus MC Rotterdam, The Netherlands
[4] Dept of Haematology, Erasmus MC Rotterdam, The Netherlands

ABSTRACT

Background and objectives. Resistance to glucocorticoid (GC) induced apoptosis is a major prognostic factor in the treatment of acute lymphoblastic leukaemia (ALL). We analyzed whether expression levels of the functional glucocorticoid receptor splice variant *GR-alpha* or the *GR-beta* and *GR-P* splice variants (not able to bind GC) are related to GC resistance in childhood ALL.
Design and methods. 54 patients were analyzed. Within this group, each of 21 *in vitro* prednisolone sensitive cases was matched (for age, immunophenotype and leukocyte count at diagnosis) to a resistant case. Expression levels were determined by quantitative real-time RT-PCR.
Results. GR-alpha represented 71% of total GR expression, *GR-beta* 0.1% and *GR-P* 29%. The mRNA expression level of the functional *GR-alpha* splice variant was significantly lower in resistant compared to sensitive patients. The expression of GR-beta and GR-P as percentage of total GR expression did not differ. Validation on a cell line panel showed that *GR-alpha* mRNA expression levels closely matched GR-protein levels.
Interpretation and conclusions. A low *GR-alpha* expression level is associated with *in vitro* GC resistance. *GR-beta* and *GR-P* expression are not related to *in vitro* GC resistance. The low expression level of *GR-beta* makes it questionable whether *GR-beta* has any biological relevance.

INTRODUCTION

Glucocorticoids (GC) are widely used in the treatment of childhood acute lymphoblastic leukemia (ALL). In the majority of children newly diagnosed with ALL, GCs such as prednisone and dexamethasone have a significant antileukemic effect.[1] In the BFM (Berlin-Frankfurt-Münster) treatment protocols, the *in vivo* response to prednisone was consistently found to be one of the strongest prognostic factors for treatment outcome.[2, 3] Like the *in vivo* response to prednisone, the *in vitro* response to prednisolone is correlated with outcome as well, even for patients who demonstrated a good *in vivo* prednisone response.[4-6] Although GCs have been used in the treatment of ALL for decades, little is known about the mechanisms of GC resistance.

GCs exert their function by binding to the glucocorticoid receptor (GR). The importance of the number of receptors in relation to resistance to GCs in childhood

ALL has been subject of many dexamethasone binding studies, which have suggested a correlation between low GR numbers and a higher rate of induction failure or relapse. Paradoxically, a high GR expression was not necessarily associated with a good clinical response, indicating that other factors may also underlie GC resistance.[7] In contrast, two recent studies concluded that GR protein expression levels were not related to *in vivo* and *in vitro* GC resistance in childhood ALL. [8, 9]

Besides encoding for the functional GR-alpha splice variant, the human GR gene also encodes for various splice variants which are unable to bind GCs.[10] (Figure 1A) Whereas some studies reported a dominant negative effect of the GR-beta product on GR-alpha function, others did not find any effect for this splice variant.[11-14] Functional analysis of the GR-P splice variant in cell lines has suggested that the GR-P product enhances GR-alpha function.[15, 16] In the only study so far relating GR splice variants to GC resistance in childhood ALL, GR-alpha (protein and mRNA) and *GR-beta* (mRNA) expression levels were not related to *in vitro* GC resistance (13 sensitive and 9 resistant, non-matched patients).[9]

There have been different hypotheses on how GR splice variants may induce GC resistance, as these splice variants might interfere with the transactivation or transrepression function of GR-alpha as recently reviewed.[17] Upon GC binding, the liganded GR-alpha is transported into the nucleus. As a homodimer, it may regulate the transcription of various genes by binding to glucocorticoid responsive element (GRE) sequences located in the promoter region of GC responsive genes.[18] GR splice variants other than GR-alpha may impair GR-mediated transactivation by forming heterodimers with a GR-alpha, thereby inhibiting GRE binding and transactivation.[11] Apart from binding GRE, GR-alpha can interact with transcription factors such as NF-κB or AP-1, thereby inducing transrepression of the NF-κB or AP-1 responsive genes.[19] One might speculate that a competition between GR-beta, GR-P, and GR-alpha for this binding may lower transrepressive activity.[11]

In the present study we tested the hypothesis that the mRNA expression of *GR-alpha* or the splice variants *GR-beta* or *GR-P* are correlated to *in vitro* GC resistance in primary patient samples of children with ALL.

MATERIALS / METHODS

Patients. The total group comprised 54 children with ALL at initial diagnosis. Within this group 21 patients with leukemic cells *in vitro* sensitive to prednisolone

were matched each to 21 *in vitro* resistant leukemic patients (matched group). Matching criteria were age category, immunophenotype and WBC (white blood cell count) category at diagnosis. Age categories were defined as 1-9 years and ≥10 years, and WBC categories were determined as $<50 \times 10^3/\mu l$ and $\geq 50 \times 10^3/\mu l$.

Patient material was obtained at diagnosis, after written informed consent was obtained from the patients or their parents. The mononuclear cell fraction was collected by Lymphoprep density gradient centrifugation (density 1.077g/m^3, Nycomed Pharma, Oslo, Norway), followed by immunodepletion of non-leukemic cells using immunomagnetic beads for samples with blast-counts below 90 percent.[20] Cells were cryopreserved in RPMI 1640 medium (Gibco, Paisly, UK) supplemented with 10% dimethyl sulfoxide (Merck, Darmstadt, Germany) and 20% fetal calf serum (Gibco, Paisly, UK) and stored in liquid nitrogen. No differences have been observed in *GR-alpha*, *GR-beta* and *GR-P* expression levels between fresh and cryopreserved patient material (data not shown).

Isolation of mRNA and cDNA synthesis. Total RNA was extracted using the Trizol method (Gibco BRL, Life Technologies), with minor modifications to the protocol supplied by the manufacturer to improve RNA quality.[21] RNA pellets were dissolved in 20 µl TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0), and quantified spectrophotometrically. cDNA was synthesized following standard procedures.[22]

Quantitative real-time RT-PCR. The mRNA levels of *GR-alpha*, *GR-beta*, *GR-P* and one endogenous reference gene, i.e. *GAPDH*, were measured by quantitative real-time RT-PCR based on Taqman-chemistry using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). PCR products were detected by a dual-fluorescent non-extendable probe containing a 6-carboxyfluorescein (FAM) reporter-group and a 6-carboxytetramethylrhodamine (TAMRA) quencher-group for all reactions. Primer and probe combinations were designed using Oligo 6.0 primer analysis software (Molecular Biology Insights, Cascade, CO, USA), and purchased from Eurogentec (Belgium). (Figure 1A) GenBank accession codes

Table 1. Primer and probe combinations used for the quantitative real time RT-PCR.

Gene	Forward primer	Probe	Reverse primer
GR-alpha	5′-TGT TTT GCT CCT GAT CTG A-3′	5′ FAM-TGA CTC TAC CCT GCA TGT ACG AC-TAMRA 3′	5′-TCG GGG AAT TCA ATA CTC A-3′
GR-beta	Idem	Idem	5′-TGA GCG CCA AGA TTG T-3′
GR-P	Idem	Idem	5′-CCT TTG TTT CTA GGC CTT C-3′
GAPDH	5′-GTC GGA GTC AAC GGA TT-3′	5′ FAM-TCA ACT ACA TGG TTT ACA TGT TCC AA-TAMRA 3′	5′-AAGCTT CCC GTT CTC AG –3′

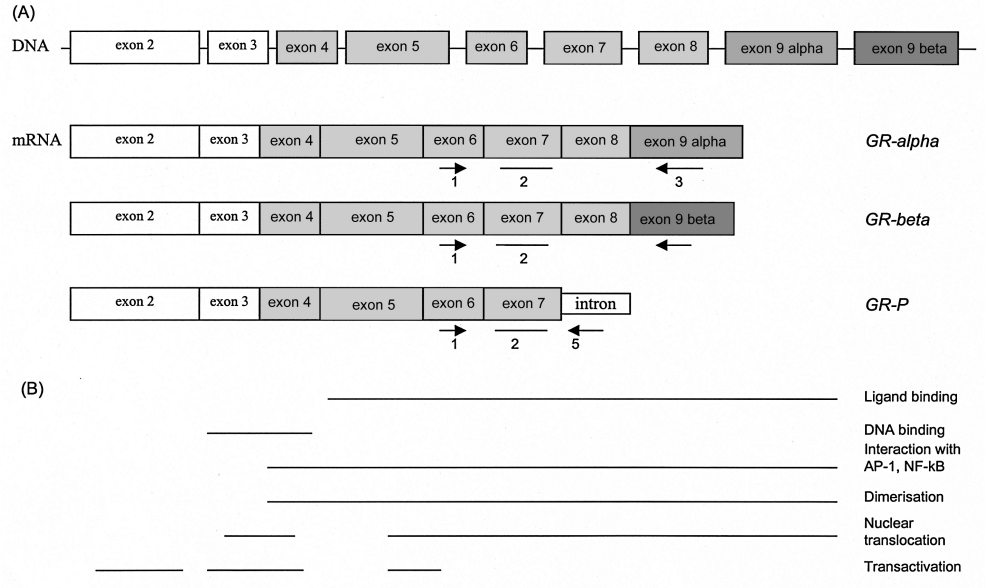


Figure 1. Schematic overview of the glucocorticoid receptor gene and *GR-alpha*, *GR-beta* and *GR-P* splice variants

(A) The location of the primers and probes are indicated. 1: forward primer for *GR-alpha*, *GR-beta* and *GR-P*, 2: probe for *GR-alpha*, *GR-beta* and *GR-P* , 3: *GR-alpha* reverse primer, 4: *GR-beta* reverse primer, 5: *GR-P* reverse primer.

(B) The functional domains of the GR.

used for the human *GR* gene were AC091925 and AC0126634, and ACJ04038 for the *GAPDH* gene (Table 1). All primers and probes had melting temperatures of 65±1°C and 75±1°C respectively (nearest neighbor method at a salt concentration of 303 mM of Na^+ equivalent and 300 nM of primer concentration). The PCR reactions were very specific, showing only one single PCR product on gel. (Figure 2) Amplification efficiency was over 95% for all reactions (data not shown). One hundred nanograms of genomic DNA served as a negative control and did not show any amplification for any of these reactions, confirming the specificity of these reactions for mRNA detection. Forty nanograms of patient sample cDNA was amplified in duplo in the presence of 300 nM forward and reverse primers, 50 nM of probe, 200 µM dNTPs, 4 mM $MgCl_2$ and 1.25 U of Ampli*Taq*™ gold DNA polymerase in Taqman buffer A (Applied Biosystems) in a total volume of 50 µl. Samples were heated for 10 min at 95°C, and amplified for 40 cycles of 15 sec at 95°C and 60 sec at 60°C. A cDNA serial dilution derived from a mRNA mixture from several leukemic cell lines was amplified on each plate, and served as a

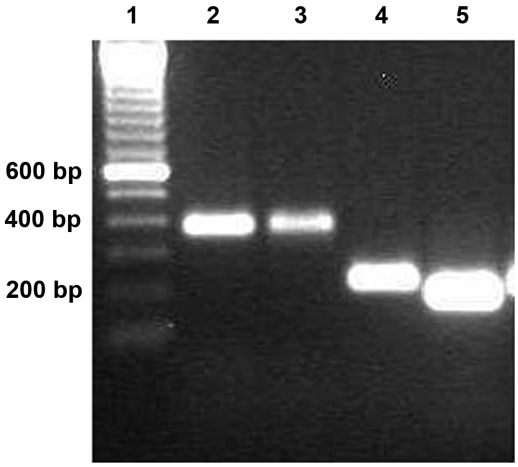


Figure 2. Verification of the real-time PCR products
The specificity of the real-time PCR and site of the amplification products was verified by loading equal amounts of each PCR reaction on a 1% agarose gel. Shown is the EtBr staining profile for *GR-alpha* (lane 2), *GR-beta* (lane 3), *GR-P* (lane 4), *GAPDH* (lane 5) and the 100 bp ladder as a marker (lane 1).

positive control to verify the amplification efficiency within each experiment.[21] For each patient sample, the average Ct-value from two independent experiments was used to calculate the expression levels of *GR-alpha*, *GR-beta* and *GR-P* mRNA relative to the *GAPDH* expression level using the comparative Ct method[23] using the equation:

relative expression = $2^{-[Ct(target)-Ct(GAPDH)]} \times 10^3$ (arbitrary units, AU)

The multiplication factor 10^3 was chosen to get whole figures.

Correlation mRNA and protein levels. 9 cell lines were used to control for a possible correlation between GR mRNA and protein expression levels. (K562, MUV2, Molt, RS4-11, HL60, SEMK4, Jurkat, TOM-1 and REH) The mRNA levels were measured as described above. Protein extraction and western blot was performed using standard procedures. The polyclonal anti-human GR antibody PA1-511A (Affinity Bioreagents, Golden, CO, USA) was used as primary antibody in a 1:500 dilution. As secondary antibody we used a peroxidase-conjugated goat-anti-rabbit antibody (DAKO, Glostrup, Denmark). The blot was developed by SuperSignal West Femto Maximum chemiluminescent substrate (Peirce, Rockford, IL, USA), and captured using the ChemiGenius bio imaging system (Syngene, Cambridge, UK) and GeneSnap imaging capture software. Band intensities were quantified using Genetools analysis software. Like all commercially available anti-

GR-antibodies, the PA1-511A antibody recognizes all GR splice variants. GR-alpha protein (94 kD) represents the major quantity of total GR expression and was determined by evaluating the band at 94 kD on Western blot (normalized to Beta-actin); GR-P protein was determined at 66 kD and normalized to Beta-actin. GR-beta protein (97 kD) could not be visualized on Western Blot.

MTT-assay. In vitro drug cytotoxicity was assessed with the MTT assay.[4, 6, 24] Briefly, patient leukemic blasts were cultured with or without prednisolone disodiumphosphate in a concentration range between 0.06 and 250 µg/ml. At day four, MTT is added which can only be reduced into formazan by viable cells. The reduced product was quantified spectrophotometrically at 562 nm. The leukemic cell survival is calculated by:

(OD drug treated well / OD control wells without drug) x 100%.

The LC50 value represents the concentration of the drug at which 50% of the cells are killed; it is used as measure of *in vitro* drug cytotoxicity. Leukemic cells having LC50 values lower than 0.1 µg/ml were assigned *in vitro* glucocorticoid sensitive, samples having LC50 values exceeding 150 µg/ml were considered *in vitro* resistant, such as previously defined.[6, 25]

Statistical methods. The non-parametric Mann-Whitney U test was used for analyzing the unmatched patients. To analyze the results from the matched patient samples, the Wilcoxon's rank test for matched samples was used. The Spearman's rank-correlation test was used to test the correlation between mRNA and protein expression. A *p*-value ≤ 0.05 was considered statistically significant (two-tailed tested).

RESULTS

In this study it was tested whether *in vitro* prednisolone resistance in childhood ALL was due to variations in the expression of the functional *GR* (*GR-alpha*). Furthermore, the expression levels of two alternatively spliced variants *GR-beta* and *GR-P* were also investigated. Patient characteristics are depicted in table 2.

First, we analyzed the *GR* mRNA expression levels in the total group (N=54). *GR-alpha* had the highest mRNA expression level with a median expression of 21.8 AU relative to *GAPDH* (P_{25}-P_{75}: 11.5-33.8 AU), comprising 71% of the total *GR* expression (sum of the 3 splice variants *GR-alpha*, *GR-beta* and *GR-P*). *GR-P* mRNA expression was much lower at 29% of total *GR* expression and a median expression relative to *GAPDH* of 8.6 AU (P_{25}-P_{75}: 3.6-14.0 AU). At 0.1%, the *GR-*

Table 2. Patient characteristics of the total group and the matched group.

		Total group N=54	Matched group N=42	
			In vitro sensitive	*In vitro* resistant
Gender	Male	28	10	10
	Female	26	11	11
Age at diagnosis	1 - 9 years	36	15	15
	> 10 years	26	11	11
Initial WBC	< 50000 /µl	32	14	14
	≥ 50000 /µl	22	7	7
Immunophenotype	pre-B / common ALL	40	15	15
	T-ALL	14	6	6
***In vitro* prednisolone sensitivity**	Sensitive	29		
	resistant	25		

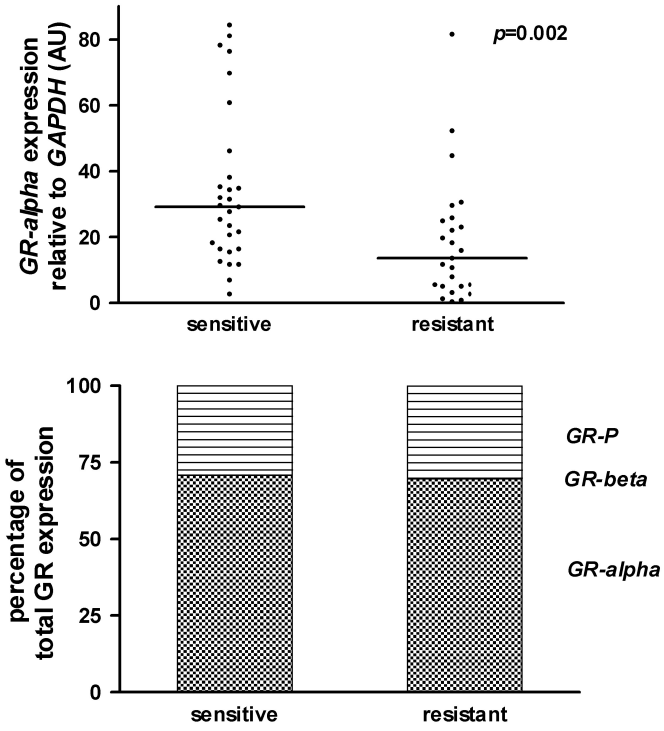


Figure 3. Expression of the splice variants *GR-alpha*, *GR-beta* and *GR-P* in relation to GC resistance in the total group

(A) Distribution of *GR-alpha* mRNA expression relative to *GAPDH* in sensitive (N=29) and resistant (N=25) patients. There is a significant difference between the sensitive and resistant patients (p=0.002). Horizontal lines indicate the median value. • indicates individual patients.

(B) *GR-alpha*, *GR-beta* and *GR-P* expression as percentage of the total GR expression in relation to GC resistance. There is no significant difference between *in vitro* sensitive and resistant patients. Note that the relative expression of *GR-beta* is very low at approximately 0.1%.

beta mRNA expression level was very low, near the border of the detection limit by quantitative real-time RT-PCR.

In the total study group, the expression of the functional *GR-alpha* splice variant was significantly lower in the resistant patients than in the sensitive patients (1.95 fold, p=0.002, Figure 3A). The mRNA expression level of *GR-beta* and *GR-P* was significantly lower as well (p=0.022 and 0.001 respectively). As a percentage of total GR expression, the mRNA expression levels of *GR-alpha*, *GR-beta* and *GR-P* did not differ between sensitive and resistant patients. (Figure 3B)

Using a matched pair analysis comparing 21 *in vitro* prednisolone sensitive patients with 21 *in vitro* resistant patients, matched for age, immunophenotype and WBC, *GR-alpha* mRNA expression levels were again significantly lower in the resistant patients than in the sensitive patients (1.6 fold, p=0.039, Figure 4). Again, *GR-beta* and *GR-P* had a significantly lower expression level in the *in vitro* resistant patients. (p=0.05 and 0.011 respectively) As in the total group, the percentages of *GR-alpha*, *GR-beta* and *GR-P* were not different between the matched sensitive and resistant patients (data not shown).

To analyze whether the mRNA expression of *GR-alpha* correlated with the protein level, we measured mRNA levels in combination with protein levels of cell lysates (obtained at the same time) of nine hematological cell lines. As shown in Figure 5, *GR-alpha* mRNA levels correlated strongly with GR-alpha protein level (rho 0.950, p<0.01). A similar trend was observed for GR-P, but due to the low expression level, this did not reach statistical significance. The expression level of GR-beta is so low, that it could not be visualized on Western Blot.

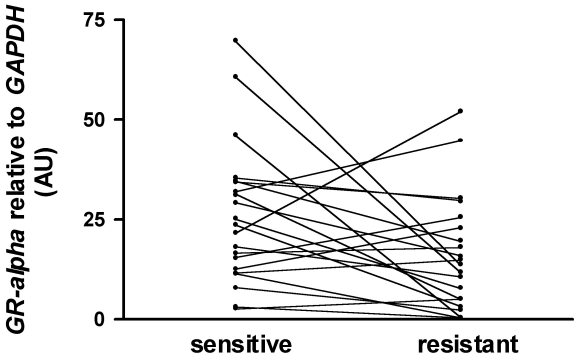


Figure 4. Expression of *GR-alpha* in relation to GC resistance in the matched group

GR-alpha mRNA expression relative to *GAPDH* for 42 matched patients. There is a significant difference between the *in vitro* sensitive and resistant patients (p=0.04). Lines connect the matched patients. • indicates individual patients.

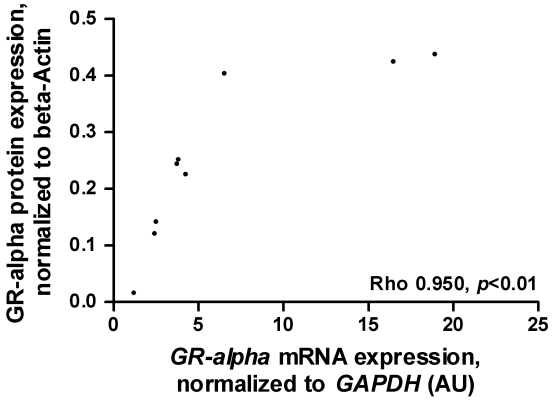


Figure 5. The correlation between GR-alpha protein and mRNA levels

DISCUSSION

As GC resistance is a poor prognostic factor in the treatment of childhood ALL[1, 4-6, 25, 26], it is important to increase our understanding of the mechanisms involved in GR resistance.

In the present study, we analyzed whether the expression level of the functional *GR-alpha* and the expression levels of *GR-beta* and *GR-P* are related to GC resistance in primary ALL patient cells. With *GR-alpha* being the predominant splice variant at 71% of total GR expression, *GR-beta* being expressed at only 0.1% and *GR-P* at 29%, the expression levels are comparable with those reported in the literature.[9, 16, 27, 28] Since the mRNA expression of *GR-beta* was very low, it is questionable whether this splice variant has any biological relevance in leukemia at all.

GR-alpha mRNA expression levels were almost twice as low in the *in vitro* resistant patients as in the sensitive patients, both in the total group (1.95 fold, N=54) and in the matched group (1.6 fold, N=42). This is in concordance with earlier studies, in which a lower dexamethasone capacity was correlated with a poor response to single-agent glucocorticoid therapy.[7] In our study we proved for the first time that this correlation is due to a lower expression of the functional GR-alpha, instead of increased expression of GR-beta and GR-P, competing with the functional GR-alpha for GRE or transcription factor binding, as proposed in the literature.[11-16] In contrast to our findings, one recent study showed that a low level of *GR-alpha* (the functional splice variant) is not related to GC resistance.[9]

However, this study included only a small number of patients, which may explain the negative results.

Although the expression of *GR-beta* and *GR-P* was significantly lower in the *in vitro* resistant versus the sensitive patients, the expression as percentage of total GR expression was not different between these patients. So, GR-beta and GR-P are not likely to play a clinically relevant role in GC resistance in childhood ALL. This is in agreement with the fact that these splice variants have a truncated ligand-binding domain and are not able to bind GC. (Figure 1B) However, it contrasts earlier hypotheses, that these truncated splice variants interfere with the function of GR-alpha either by forming heterodimers with this splice variant, disabling its function, or by competing with GR-alpha to bind NF-κB and AP-1.[11-14]

Because our data represented mRNA level measurements only, we also analyzed the correlation between mRNA and GR protein expression. Since not enough patient material for mRNA and protein measurements was available from the same patients, we used nine different cell lines instead, covering a wide dynamic range of GR expression levels. We showed a strong and significant correlation between *GR-alpha* mRNA and protein expression in 9 cell lines. This is in concordance with the results from other studies in a variety of human cells and in ALL patients.[9, 13] A general GR antibody was used to quantify the protein level of GR-alpha and GR-P using Western blotting at 94 and 66 kD respectively. Although there are commercially available antibodies specific for GR-alpha and GR-beta, we doubt the specificity of these antibodies, because another splice variant, GR-gamma, which has a three basepair insertion between exon 3 and 4 will always be included in the quantification of GR-alpha, GR-beta and GR-P as well (both on mRNA and protein level).[8, 29] In a pilot study there were suggestions that the expression of GR-gamma might be related to GC resistance in childhood ALL.[30] In another more recent study, a significantly higher GR-gamma expression in 8 *in vitro* resistant patients was found as compared to 7 *in vitro* sensitive patients.[9] As the median expression level of GR-gamma was found to be only 2.8% of total GR expression, it may be hard to envision how this splice variant might influence GC sensitivity. The relevance of this splice variant for GC sensitivity needs further research.

In conclusion, low *GR-alpha* expression levels are related to *in vitro* prednisolone resistance in childhood ALL. Expression levels of *GR-beta* and *GR-P* do not contribute to *in vitro* prednisolone resistance in childhood ALL.

References

1. Reiter A, Schrappe M, Ludwig WD*, et al.* Chemotherapy in 998 unselected childhood acute lymphoblastic leukemia patients. Results and conclusions of the multicenter trial ALL-BFM 86*.* Blood 1994;84:3122-33.
2. Lauten M, Stanulla M, Zimmermann M, Welte K, Riehm H, Schrappe M. Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter*.* Klin Padiatr 2001;213:169-74.
3. Schrappe M, Reiter A, Zimmermann M*, et al.* Long-term results of four consecutive trials in childhood ALL performed by the ALL-BFM study group from 1981 to 1995. Berlin-Frankfurt-Munster*.* Leukemia 2000;14:2205-22.
4. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs*.* Blood 1998;92:259-66.
5. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. *In vitro* drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia*.* Blood 1997;89:2959-65.
6. Den Boer ML, Harms DO, Pieters R*, et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia*.* J Clin Oncol 2003;21:3262-8.
7. Kaspers GJ, Pieters R, Veerman AP. Glucocorticoid resistance in childhood leukemia*.* International Journal of Pediatric Hematology/Oncology 1997;4:583-96.
8. Lauten M, Cario G, Asgedom G, Welte K, Schrappe M. Protein expression of the glucocorticoid receptor in childhood acute lymphoblastic leukemia*.* Haematologica 2003;88:1253-8.
9. Haarman EG, Kaspers GJL, Pieters R, Rottier MMA, Veerman AJP. Glucocorticoid receptor alpha, beta and gamma expression vs *in vitro* glucocorticod resistance in childhood leukemia*.* Leukemia 2004;18:530-7.
10. Hollenberg SM, Weinberger C, Ong ES*, et al.* Primary structure and expression of a functional human glucocorticoid receptor cDNA*.* Nature 1985;318:635-41.
11. Vottero A, Chrousos GP. Glucocorticoid receptor beta: View I*.* Trends Endocrinol Metab 1999;10:333-8.
12. Carlstedt-Duke J. Glucocorticoid Receptor beta: View II*.* Trends Endocrinol Metab 1999;10:339-42.
13. Pujols L, Mullol J, Perez M*, et al.* Expression of the human glucocorticoid receptor alpha and beta isoforms in human respiratory epithelial cells and their regulation by dexamethasone*.* Am J Respir Cell Mol Biol 2001;24:49-57.
14. Leung DYM, Chrousos GP. Is there a role for glucocorticoid receptor beta in glucocorticoid-dependent asthmatics? Am J Respir Crit Care Med 2000;162:1-3.
15. Moalli PA, Pillay S, Krett NL, Rosen ST. Alternatively spliced glucocorticoid receptor messenger RNAs in glucocorticoid-resistant human multiple myeloma cells*.* Cancer Res 1993;53:3877-9.
16. de Lange P, Segeren CM, Koper JW*, et al.* Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells*.* Cancer Res 2001;61:3937-41.
17. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia*.* Leukemia 2003;17:17-25.
18. Tsai SY, Carlstedt-Duke J, Weigel NL*, et al.* Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation*.* Cell 1988;55:361-9.
19. Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA, Baldwin AS. Characterization

of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. Mol Cell Biol 1995;15:943-53.
20. Kaspers GJ, Veerman AJ, Pieters R*, et al.* Mononuclear cells contaminating acute lymphoblastic leukaemic samples tested for cellular drug resistance using the methyl-thiazol- tetrazolium assay. Br J Cancer 1994;70:1047-52.
21. Stam RW, den Boer ML, Meijerink JP*, et al.* Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood 2003;101:1270-6.
22. Stams WA, den Boer ML, Beverloo HB*, et al.* Sensitivity to L-asparaginase is not associated with expression levels of asparagine synthetase in t(12;21)+ pediatric ALL. Blood 2003;101:2743-7.
23. Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn 2001;3:55-61.
24. Pieters R, Loonen AH, Huismans DR*, et al.* In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 1990;76:2327-36.
25. Pieters R, Huismans DR, Loonen AH*, et al.* Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet 1991;338:399-403.
26. Riehm H, Reiter A, Schrappe M*, et al.* Die corticosteroid-abhangige dezemierung der leukamiezellzahl im blut als prognose-faktor bei der akuten lymphoblastischen leukemie im kindesalter (therapiestudie ALL-BFM 83). Klin Padiatr 1986;199:151-60.
27. Oakley RH, Sar M, Cidlowski JA. The human glucocorticoid receptor beta isoform. Expression, biochemical properties, and putative function. J Biol Chem 1996;271:9550-9.
28. Krett NL, Pillay S, Moalli PA, Greipp PR, Rosen ST. A variant glucocorticoid receptor messenger RNA is expressed in multiple myeloma patients. Cancer Res 1995;55:2727-9.
29. Rivers C, Levy A, Hancock J, Lightman S, Norman M. Insertion of an amino acid in the DNA-binding domain of the glucocorticoid receptor as a result of alternative splicing. J Clin Endocrinol Metab 1999;84:4283-6.
30. Beger C, Gerdes K, Lauten M*, et al.* Expression and structural analysis of glucocorticoid receptor isoform gamma in human leukaemia cells using an isoform-specific real-time polymerase chain reaction approach. Br J Haematol 2003;122:245-52.

Chapter 5

Glucocorticoid-induced glucocorticoid receptor expression and promoter-usage is not linked to glucocorticoid resistance in childhood ALL

Manuscript submitted

Wim J.E. Tissing[1,2,] Jules P.P. Meijerink[1], Bas Brinkhof[1], Mathilde J.C. Broekhuis[1], Renee X. Menezes[1,3,] Monique L. den Boer[1], Rob Pieters[1]

1- Dept of Pediatric Oncology / Haematology, Erasmus MC-Sophia Children's Hospital, Erasmus University Medical Center Rotterdam, The Netherlands
2- Dept of Pediatric Oncology / Haematology, Beatrix Children's Hospital, University of Groningen and University Medical Center Groningen, Groningen, the Netherlands
3- Dept of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands

ABSTRACT

Glucocorticoid (GC) resistance is an adverse prognostic factor in childhood ALL, but little is known about causes of GC resistance. Upregulation of the glucocorticoid receptor (GR) has been suggested as an essential step to the induction of apoptosis in leukemic cells. In this study we investigated whether base-line mRNA expression levels of the five different *GR* promoters transcripts (1A1, 1A2, 1A3, 1B and 1C) or differences in the degree of regulation of the *GR* or *GR* promoter transcripts upon GC exposure are related to GC resistance.

Material & Methods. mRNA levels of the five *GR* promoter transcripts and of the *GR* were measured by quantitative real-time RT-PCR (Taqman) technology in primary ALL cells prior to and after 3, 8 and 24 hours of prednisolone exposure.

Results. The base-line and GC-induced expression levels of the five different *GR* promoter transcripts did not correlate with GC resistance. GC exposure induced an upregulation of the *GR* in ALL cells, which is opposite to what is found in tissues in which GC exposure does not induce apoptosis. However, in GC resistant ALL samples no defective upregulation of the *GR* was detected.

Conclusion. *GR* expression is induced upon GC exposure in primary ALL patient samples. However, GC resistance in childhood ALL can not be attributed to differences in *GR* promoter usage (at base-line and upon GC exposure), nor to an inability of resistant cells to upregulate the expression of the *GR* upon GC exposure.

INTRODUCTION

Glucocorticoids (GC) like prednisolone and dexamethasone have been used in the treatment of childhood acute lymphoblastic leukemia (ALL) for many years. Although poor early prednisone response as determined in the BFM (Berlin-Frankfurt-Munster) treatment protocols and *in vitro* cellular resistance to prednisolone are important adverse riskfactors in the treatment of childhood ALL[1-3], little is known about causes underlying GC resistance.[4, 5]

To induce apoptosis, GCs have to bind to the intracellular GC receptor (GR). Most cell types demonstrate a down regulation of the amount of GR upon GC exposure[6-8], probably as part of a physiological feed-back mechanism.[9] In contrast, an upregulation of GR mRNA and protein levels has been demonstrated in various cell lines of lymphoid leukemias[10-12] and has been described to be essential for GC

induced apoptosis.[13] Hence, resistance towards GCs in patient cells may be caused by lack of GR upregulation upon GC exposure.[13, 14]

Besides the functional GR-alpha transcript, several other 3' splice variations of the GR have been described that are unable to bind GCs (GR-beta and GR-P). These variants retain a normal DNA binding motif and may compete with GR-alpha for binding to glucocorticoid responsive elements (GREs) of targeted genes or with transcription factors interacting with the GR, thereby interfering with GR-alpha function and causing resistance.[15, 16] We have shown before that leukemic cells from ALL patients resistant to GCs express less of the functional GR-alpha variant[17], and that base-line mRNA expression levels of the GR transcripts GR-beta and GR-P are not relevant for GC resistance.[17, 18]

The *GR* gene has three different promoters, i.e. 1A, 1B and 1C which results into transcripts that include the corresponding exon 1A, 1B and 1C respectively. Since exon 1A can be alternatively spliced in 3 variants (1A1, 1A2 and 1A3), in total five different 5' *GR* transcript variants exist. (Figure 1) These *GR* promoter transcripts are expressed at various levels in different cancer cell lines.[19-22] Differential usage of these *GR* promoter transcripts might be responsible for differences in GC cytotoxicity in hematological malignancies.[19] This is supported by the fact that the 1A promoter, but not 1B and 1C, contains a GRE that may enhance GR-1A transcript production upon GC exposure.[21]

In the present study we determined whether resistance to GCs in leukemic cells of children with ALL can be explained by altered base-line and / or GC-induced

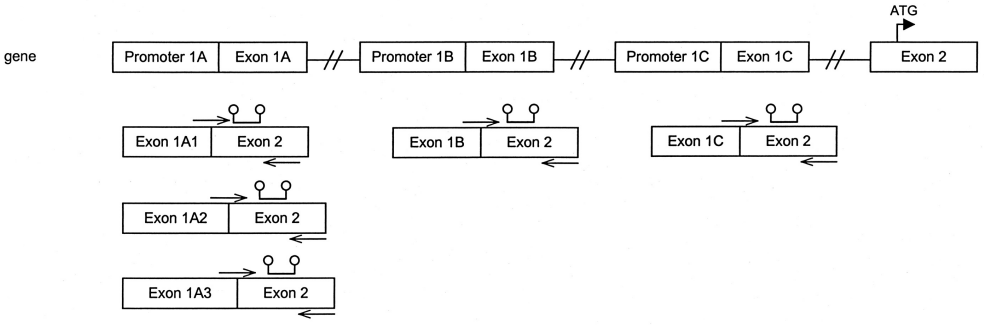


Figure 1. Schematic overview of the different 5' glucocorticoid receptor transcripts derived from the use of 3 different promoters

The *GR* promoter transcripts 1A, 1B and 1C are alternatively spliced to exon 2. The locations of the forward primer (⟶), the reverse primer (⟵) and the probe (⚲⚲) are indicated.

expression of 5' *GR* promoter transcripts (1A1, 1A2, 1A3, 1B and 1C) and of the 3' *GR* splice variants (*GR-alpha*, *GR-beta* and *GR-P*).

MATERIAL & METHODS

Patient samples. Two study populations were included. For the base-line *GR* promoter study, 24 pediatric ALL patients with leukemic blasts *in vitro* sensitive to prednisolone were matched each to a patient with leukemic blasts *in vitro* resistant to prednisolone. Patients were matched according to age (1-9 years of age and ≥ 10 years of age), immunophenotype (precursor B-ALL and T-ALL) and white blood cell (WBC) count (<50 x 10^9 / l and ≥ 50 x 10^9 / l). None of the patients had poor prognostic cytogenetic abnormalities like the t(9,22) and 11q23 (MLL) rearrangements. For the GR regulation study, 22 unmatched patients were included. Patient material was obtained prior to initial therapy after written informed consent was obtained from the patient and / or their parents according to regulations and approval by the local ethical committee.
Lymphoprep density gradient centrifugation (density 1.077 g/ml, Nucomed Pharma, Oslo, Norway) was used to separate the mononuclear cell fraction, and, when necessary, immunomagnetic beads were used to deplete non-leukemic cells from the samples. All samples therefore contained more than 90% leukemic blasts.

MTT-assay. In vitro drug cytotoxicity was assessed using the MTT assay as described earlier.[23, 24] Briefly, patient blasts were cultured with or without prednisolone disodiumphosphate in a concentration range of 0.06 to 250 µg/mL. At day 4 MTT was added which can be reduced into formazan by viable cells only. The reduced product was quantified spectrophotometrically at 562 nm. The leukemic cell survival was calculated by: (OD drug treated well / OD control well without drug) x 100%. The value of the concentration of the drug at which 50% of the cells are killed represents the LC50, which was used as measure of *in vitro* drug cytotoxicity. Leukemic cells having LC50 values lower than 0.1 µg/ml were assigned *in vitro* prednisolone sensitive, samples having LC50 values exceeding 150 µg/ml were considered *in vitro* resistant, as previously described to be of prognostic value.[23, 25]

Prednisolone exposure. To study the effect of prednisolone exposure on GR mRNA levels in childhood leukemia, leukemic blasts were incubated in culture medium as used in the MTT assay, supplemented with 250 µg/ml prednisolone

for 3, 8 and 24 hours. In each case, leukemic blasts were incubated as well in culture medium without prednisolone as control to discard variations due to other mechanisms than prednisolone exposure.

Isolation of RNA and cDNA synthesis. Total RNA was extracted using the Trizol method (Gibco BRL, Life Technologies, Breda, the Netherlands) according to the protocol provided by the manufacturer with minor modifications to improve RNA quality.[26] The RNA pellets were dissolved in 20 µl TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH= 8.0) and quantified spectrophotometrically. cDNA was synthesized as described before.[26]

Quantitative real-time RT-PCR. Levels of mRNA expression of the 3' *GR* transcripts *GR-alpha*, *GR-beta* and *GR-P*, the 5' *GR* promoter transcripts *GR-1A1*, *GR-1A2*, *GR-1A3*, *GR-1B* and *GR-1C* (i.e. target PCRs) and two endogenous reference genes (i.e. *GAPDH* and *RNaseP*) were measured by quantitative real-time RT-PCR based on Taqman-chemistry using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Specific primers and probes were designed using GenBank accession codes AC091925 for the human *GR* gene, AC012634 and ACJ04038 for the *GAPDH* gene and X15624 for *RNaseP* (table 1). All primers and probes had melting temperatures of 65±1°C and 75±1°C respectively (nearest neighbor method[27]). The PCR reactions were very specific, showing only a single PCR product on gel (data not shown). One hundred nanograms of genomic DNA served as a negative control and did not result in product amplification for any of these reactions, confirming the specificity of these reactions for RNA detection. Forty nanograms of patient sample cDNA was amplified in duplo in the presence of 300 nM forward and reverse primers, 50 nM probe, 200 µM dNTPs, 4 mM $MgCl_2$ and 1.25 U of Ampli*Taq*™ gold DNA polymerase in Taqman buffer A (Applied Biosystems) in a total volume of 50 µl. Samples were heated for 10 min at 95°C and amplified in 40 cycles of 15 sec at 95°C and 60 sec at 60°C. A positive control was amplified on each plate to verify the amplification efficiency within each experiment.[26] The average Ct-value was used to calculate mRNA expression levels of the PCR targets relative to the expression level of the two reference genes using the comparative Ct method[27] using the equation:

relative expression = $2^{-[Ct(target)-Ct(reference\ gene)]} \times 100$.

Statistical methods. The non-parametric Wilcoxon signed rank test for matched samples was used to compare *GR* promoter transcript expression of matched sensitive and resistant cases, as well as for the analysis of the *GR* promoter transcript regulation upon prednisolone exposure. To test whether the *GR* was

Table 1. Primers and probes used to discriminate the different 5' *GR*-promoter and 3' *GR* transcripts (*GR-alpha*, *GR-beta* and *GR-P*) expression in pediatric ALL.
Primers and probes were designed using AC091925 and AC012634 for the *GR* gene, ACJ04038 for *GAPDH* and X15624 for *RNaseP*

GR-1A1	fw	5'-CAC TGG ACC TTA GAA GTT GAT A-3'
	rev	5'-ATA CAG TCC CAT TGA GAG TGA-3'
	probe	5'-(FAM)-CCC TAA GAG GAG GAG CTA CTG AA-(TAMRA)- 3'
GR-1A2	fw	5'-GAA TAG AAA CAG AAA GAG GTT GAT A-3'
	rev	5'-ATA CAG TCC CAT TGA GAG TGA-3'
	probe	5'-(FAM)-CCC TAA GAG GAG GAG CTA CTG AA-(TAMRA)-3'
GR-1A3	fw	5'-AGT GTC TGA GAA GGA AGT TGA TA-3'
	rev	5'-ATA CAG TCC CAT TGA GAG TGA-3'
	probe	5'-(FAM)-CCC TAA GAG GAG GAG CTA CTG AA-(TAMRA)-3'
GR-1B	fw	5' –GGC CCA AAT TGA TAT TCA-3'
	rev	5'-ATA CAG TCC CAT TGA GAG TGA-3'
	probe	5'-(FAM)-CCC TAA GAG GAG GAG CTA CTG AA-(TAMRA)-3'
GR-1C	fw	5'-CTG CTC CTT CTG CGT TC-3'
	rev	5'-ATA CAG TCC CAT TGA GAG TGA-3'
	probe	5'-(FAM)-CCC TAA GAG GAG GAG CTA CTG AA-(TAMRA)-3'
GR-alpha	fw	5'-TGT TTT GCT CCT GAT CTG A-3'
	Rev	5'-TCG GGG AAT TCA ATA CTC A-3'
	Probe	5'-(FAM)-TGA CTC TAC CCT GCA TGT ACG AC-(TAMRA)-3'
GR-beta	fw	5'-TGT TTT GCT CCT GAT CTG A-3'
	Rev	5'-TGA GCG CCA AGA TTG T-3'
	Probe	5'-(FAM)-TGA CTC TAC CCT GCA TGT ACG AC-(TAMRA)-3'
GR-P	fw	5'-TGT TTT GCT CCT GAT CTG A-3'
	Rev	5'-CCT TTG TTT CTA GGC CTT C-3'
	Probe	5'-(FAM)-TGA CTC TAC CCT GCA TGT ACG AC-(TAMRA)-3'
GAPDH	Fw	5'-GTC GGA GTC AAC GGA TT-3'
	Rev	5'-AAGCTT CCC GTT CTC AG -3'
	Probe	5'-(FAM)-TCA ACT ACA TGG TTT ACA TGT TCC AA-(TAMRA)-3'
RNaseP	Fw	5'-TTG GGA AGG TCT GAG ACT A-3'
	Rev	5'-TCA GCC ATT GAA CTC ACT T-3'
	Probe	5'-(FAM)-AGG TCA GAC TGG GCA GGA GAT-(TAMRA)-3'

upregulated upon GC exposure and whether the degree of upregulation was related to GC resistance, a linear mixed-effects model was fitted to the log-2 *GR* expressions, using as explanatory variables exposure time, GC sensitivity, prednisolone exposed or control sample, and the individual patient, the last one having a random effect, the others with fixed effects. Log-2 transformed *GR* expression levels were used to correct for the non-normal distribution of the data. By associating a random effect to each patient in the study, the model not

only allows for samples corresponding to the same patient to be more correlated than otherwise, but also patients are taken as representing a larger population of similar patients. A p-value ≤ 0.05 was considered statistically significant (two-tailed tested).

RESULTS

Base-line GR promoter usage. We explored whether GC resistant ALL cells utilize other promoter sites at the GR than GC sensitive cells. For this, expression levels from the five different 5′ *GR* promoter transcripts, i.e. transcripts 1A1, 1A2, 1A3 derived from promoter 1A, transcript 1B from promoter 1B and transcript 1C, from promoter 1C, were measured by quantitative real-time RT-PCR according to the strategy as outlined in Figure 1. This study included 24 ALL patients *in vitro* sensitive to prednisolone, who were matched each to an *in vitro* resistant patient (matching according to age, immunophenotype and WBC count). The patient characteristics are depicted in table 2. For each patient, *GR* 1A1, 1A2, 1A3, 1B and 1C transcript expression levels were calculated as percent of the total expression level of the five transcripts combined. The highest expression was found for promoters 1B and 1C, i.e. median 15.9% and 80.8%, respectively. The *GR* transcripts derived from the 1A promoter were lower expressed at 0.02%, 0.08% and 2.8% for the 1A1, 1A2 and 1A3 transcripts, respectively. In a matched pair analysis, we did not observe a relationship between the base-line expression levels of the five different

Table 2. Patient characteristics.

		Base-line expression of 5′ *GR* promoter transcripts	Effect of GCs on 5′ *GR* promoter and 3′ *GR* transcript levels
		N=48	N=22
Gender	Male	28	12
	Female	20	10
Age	1-9 yr	36	11
	≥ 10 yr	12	11
Immunophenotype	Precursor B-ALL	36	9
	T-ALL	12	13
WBC	< 50000 / µl	18	3
	≥ 50000 / µl	30	19
In vitro prednisolone toxicity	Sensitive	24	12
	Resistant	24	10

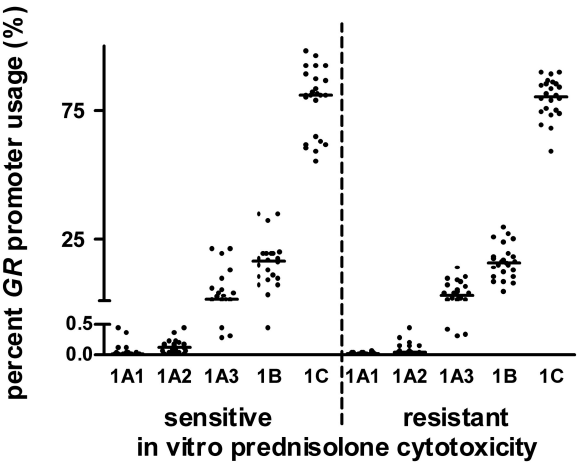


Figure 2. 5′ GR promoter transcript expression for *in vitro* prednisolone sensitive and resistant children with ALL
The mRNA expression of the 5′ *GR* promoter transcript *1A1*, *1A2*, *1A3*, *1B* and *1C* as percentage of total *GR* promoter expression for 48 patients is depicted. The results are depicted as median and P_{25} and P_{75} values. Comparisons in expression levels of these five transcripts between GC sensitive and resistant patient samples was not significant.

GR promoter transcripts and *in vitro* prednisolone toxicity (Figure 2), even when analyzing precursor B-ALL and T-ALL samples separately.

GC-induced GR promoter usage. We next investigated whether specific promoter transcripts of the *GR* gene were upregulated upon eight hours of prednisolone exposure. Patient characteristics are depicted in table 2. Similar to what was found for the base-line expression values, no significant changes for the percent expression of the five 5′ *GR* promoter transcripts (1A1-3, 1B and 1C) were found between *in vitro* prednisolone sensitive and resistant cases. (Figure 3)

GC-induced GR expression. The effect of GC exposure on *GR* mRNA expression levels was analyzed by incubating leukemic cells for 3, 8 and 24 hours in culture medium with or without prednisolone. Characteristics of the included patients are depicted in table 2. For each time point, mRNA expression levels of the 3′ *GR* splice variants *GR-alpha*, *GR-beta* and *GR-P* were determined relative to the reference genes *GAPDH* and *RNaseP*. Both reference genes revealed the same data and, therefore, in the remaining part of this paper we describe the results obtained with *GAPDH* only. Total *GR* mRNA expression levels were defined as the sum of *GR-alpha*, *GR-beta* and *GR-P* and are depicted in Figure 4.
Using a linear mixed-effects model, we found the *GR* to be upregulated upon GC

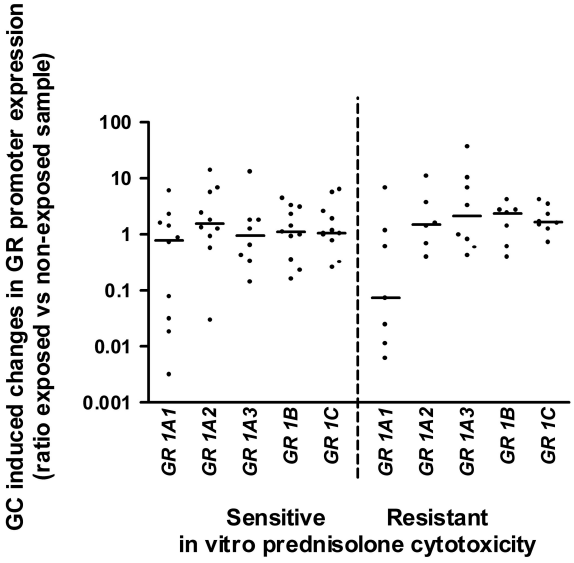


Figure 3. Glucocorticoid sensitivity is not related to differential use of *GR* promoters after 8 hours prednisolone exposure in ALL

The ratio for the 5′ *GR* promoter transcripts between the prednisolone exposed and control samples was calculated. The results are depicted as median and P_{25} and P_{75} values. Comparisons of the ratio's of these five transcripts between GC sensitive and resistant patient samples was not significant.

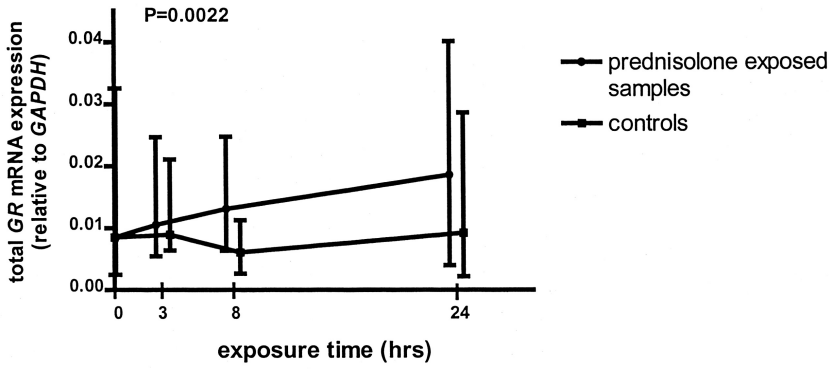


Figure 4. Regulation of the glucocorticoid receptor upon prednisolone exposure in ALL

Leukemic cells were incubated in culture medium with or without 250 µg/ml prednisolone for three, eight and twenty-four hours. The total *GR* mRNA expression as sum of *GR-alpha*, *GR-beta* and *GR-P*, for both the prednisolone incubated and the control samples is depicted. The results are depicted as median and P_{25} and P_{75} values.

exposure for 3, 8 and 24 hours as compared to the control samples, incubated without prednisolone (p=0.002). However, the degree of upregulation was not related to GC sensitivity (p=0.12). The relative distribution between *GR-alpha*, *GR-beta* and *GR-P* expression levels did not change over time (with median expression levels of 68.8%, 0.03% and 31.1%, respectively), in both the GC sensitive and resistant patient samples.
No difference was found within T-ALL or precursor B-ALL samples when analyzed separately as well. In addition, the three 3' *GR* transcripts (*GR-alpha*, *GR-beta* and *GR-P*) were also co-ordinately regulated in both sensitive and resistant patients.

DISCUSSION

The ability to upregulate GR expression upon GC exposure may be related to GC sensitivity in ALL as suggested in the literature after several cell line studies.[13, 14] It is currently unknown whether GR upregulation upon GC exposure occurs in pediatric ALL cells directly obtained from patients and whether an inability to upregulate the GR is related to GC resistance. Also no studies have been performed to address whether differential expression of the five different 5' *GR* promoter transcripts (1A1, 1A2, 1A3, 1B and 1C) is linked to GC resistance.

Our study shows that the base-line relative expression of the five different 5' *GR* promoter transcripts in pediatric ALL samples was the same as described in cell lines[19, 22]: 1B (15.9%) and 1C (80.8%) are the most abundantly expressed, followed by 1A3 (2.8%), 1A2 (0.08%) and 1A1 (0.02%). No correlation between the base-line expression levels of the different 5' *GR* promoter transcripts and GC sensitivity was found. This is in contrast with a recent study in which mouse T-lymphocytes showing a relative high expression of the *GR-1A* promoter were more susceptible to GC induced cell death than those showing a relative low expression of *GR-1A*.[28] Since these studies were done using T-lymphocytes only, we analyzed samples of patients with a precursor B-ALL and T-ALL separately. Again no relation between *GR* promoter expression and *in vitro* prednisolone toxicity was found.

Since the GR-1A promoter contains a weak GRE sequence in contrast to the GR-promoters 1B and 1C (in which no GRE can be recognized), the regulation of the three GR promoters upon GC exposure has been hypothesized to be different.[21] Differential regulation of the 5 different 5' *GR* transcripts has been shown in two separate studies with the CEM-C7 T lymphoblast cell line as model system, in which GC treatment specifically led to the upregulation of 1A3 promoter transcripts.[19, 22]

However, in the present study we did not detect a significant difference in the GC induced expression level of any of the five different 5' *GR* promoter transcripts, neither in the total study population, nor when analyzing the prednisolone sensitive versus resistant patient samples. Since all 5' *GR* promoter transcripts (1A1-3, 1B and 1C) were activated at the same level, the GRE sequence such as present in promoter 1A but not 1B and 1C may not be functional in ALL cells. *In vitro* GC resistance was not caused by a differential regulation of the different *GR* promoter transcripts.

Besides promoter usage, the inability of leukemic cells to upregulate the GR upon GC exposure could be an explanation for GC resistance. In correspondence with leukemic cell line studies[10-12], we also found an upregulation of the *GR* upon prednisolone exposure in leukemic blasts from children with ALL. In contrast, other human cell types, in which GCs do not induce apoptosis, are characterized by a downregulation of the GR upon GC exposure.[6-8] Thus, upregulation of the GR may be an important hallmark of ALL cells that normally undergo apoptosis upon GC exposure. We found that the three 3' *GR* splice variants *GR-alpha*, *GR-beta* and *GR-P* were co-ordinately regulated over time. The percentage of these three splice variants of total *GR* expression was the same as reported in the literature in non-GC-exposed ALL samples[17] and did not change after 3, 8 and 24 hours prednisolone exposure.

Since relatively large differences in the degree of regulation of the GR were observed between sensitive and resistant cell lines, it has been hypothesized that this difference underlies GC resistance in childhood ALL.[13, 14] However, in our study we did not find differences in the degree of regulation of *GR* mRNA between *in vitro* sensitive and resistant leukemic samples. Since the studies relating GC resistance to the level of GR upregulation have been done using T-cell leukemia cell lines, we also analyzed the precursor B-ALL and T-ALL samples separately. Again no relation between the level of *GR* regulation and GC resistance could be found. The 3' *GR* splice variants *GR-alpha*, *GR-beta* and *GR-P* were found to be co-ordinately regulated upon GC exposure in both sensitive and resistant ALL samples, ruling out the possibility that GC resistance is determined by a preferential regulation of GR-beta and GR-P that may inhibit GR-alpha function.[15, 16] These results are in correspondence with one previous cell line study, in which *GR-alpha* and *GR-beta* mRNA expression levels were co-ordinately upregulated upon GC exposure.[22]

As an alternative explanation for GC resistance, it could be that instead of mRNA levels, protein levels of the GR upon GC exposure are correlated with

GC resistance (i.e. post-transcriptional regulation instead of transcriptional regulation). However, it has been demonstrated before in leukemic cell lines that the GC mediated upregulation of GR expression is a transcriptional response.[10, 14, 29] Unfortunately, we did not have sufficient material to confirm these cell line data in patient material.

In conclusion, nor the base-line expression of the five 5' *GR* promoter transcripts, nor the expression of these after prednisolone exposition is related to GC resistance in pediatric ALL. Exposure to prednisolone did not induce a decrease of the *GR* as seen in other body tissues, but an upregulation in ALL cells. However, GC resistance in ALL is not related to a defective upregulation.

References

1. Lauten M, Stanulla M, Zimmermann M, Welte K, Riehm H, Schrappe M. Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter. Klin Padiatr 2001;213:169-74.
2. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood 1998;92:259-66.
3. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. *In vitro* drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. Blood 1997;89:2959-65.
4. Schmidt S, Rainer J, Ploner C, Presul E, Riml S, Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ 2004;11 Suppl 1:S45-55.
5. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia 2003;17:17-25.
6. Silva CM, Powell-Oliver FE, Jewell CM, Sar M, Allgood VE, Cidlowski JA. Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid. Steroids 1994;59:436-42.
7. Andreae J, Tripmacher R, Weltrich R, *et al.* Effect of glucocorticoid therapy on glucocorticoid receptors in children with autoimmune diseases. Pediatr Res 2001;49:130-5.
8. Schmidt TJ, Meyer AS. Autoregulation of corticosteroid receptors. How, when, where, and why? Receptor 1994;4:229-57.
9. Webster JC, Cidlowski JA. Downregulation of the glucocorticoid receptor. A mechanism for physiological adaptation to hormones. Ann N Y Acad Sci 1994;746:216-20.
10. Eisen LP, Elsasser MS, Harmon JM. Positive regulation of the glucocorticoid receptor in human T-cells sensitive to the cytolytic effects of glucocorticoids. J Biol Chem 1988;263:12044-8.
11. Obexer P, Certa U, Kofler R, Helmberg A. Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA-, protein- and nucleotide synthesis. Oncogene 2001;20:4324-36.
12. Tonko M, Ausserlechner MJ, Bernhard D, Helmberg A, Kofler R. Gene expression profiles of proliferating vs. G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. Faseb J 2001;15:693-9.
13. Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res 1999;59:1378-

85.
14. Riml S, Schmidt S, Ausserlechner MJ, Geley S, Kofler R. Glucocorticoid receptor heterozygosity combined with lack of receptor auto-induction causes glucocorticoid resistance in Jurkat acute lymphoblastic leukemia cells. Cell Death Differ 2004.
15. Vottero A, Chrousos GP. Glucocorticoid receptor beta: View I. Trends Endocrinol Metab 1999;10:333-8.
16. de Lange P, Segeren CM, Koper JW, *et al.* Expression in hematological malignancies of a glucocorticoid receptor splice variant that augments glucocorticoid receptor-mediated effects in transfected cells. Cancer Res 2001;61:3937-41.
17. Tissing WJE, Lauten M, Meijerink JPP, *et al.* The expression of the glucocorticoid receptor, but not the differential expression of specific isoforms, is associated with glucocorticoid resistance in childhood ALL. Haematologica 2005;90:1279-81.
18. Haarman EG, Kaspers GJL, Pieters R, Rottier MMA, Veerman AJP. Glucocorticoid receptor alpha, beta and gamma expression vs *in vitro* glucocorticod resistance in childhood leukemia. Leukemia 2004;18:530-7.
19. Breslin MB, Geng CD, Vedeckis WV. Multiple promoters exist in the human gr gene, one of which is activated by glucocorticoids. Mol Endocrinol 2001;15:1381-95.
20. Nunez BS, Vedeckis WV. Characterization of promoter 1B in the human glucocorticoid receptor gene. Mol Cell Endocrinol 2002;189:191-9.
21. Geng CD, Vedeckis WV. Steroid-responsive sequences in the human glucocorticoid receptor gene 1A promoter. Mol Endocrinol 2004;18:912-24.
22. Pedersen KB, Vedeckis WV. Quantification and glucocorticoid regulation of glucocorticoid receptor transcripts in two human leukemic cell lines. Biochemistry 2003;42:10978-90.
23. Den Boer ML, Harms DO, Pieters R, *et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol 2003;21:3262-8.
24. Pieters R, Loonen AH, Huismans DR, *et al.* In vitro drug sensitivity of cells from children with leukemia using the MTT assay with improved culture conditions. Blood 1990;76:2327-36.
25. Pieters R, Huismans DR, Loonen AH, *et al.* Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet 1991;338:399-403.
26. Stam RW, den Boer ML, Meijerink JP, *et al.* Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood 2003;101:1270-6.
27. Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn 2001;3:55-61.
28. Purton JF, Monk JA, Liddicoat DR, *et al.* Expression of the glucocorticoid receptor from the 1A promoter correlates with T lymphocyte sensitivity to glucocorticoid-induced cell death. J Immunol 2004;173:3816-24.
29. Denton RR, Eisen LP, Elsasser MS, Harmon JM. Differential autoregulation of glucocorticoid receptor expression in human T- and B-cell lines. Endocrinology 1993;133:248-56.

Chapter 6

mRNA expression levels of (co)chaperone molecules of the glucocorticoid receptor are not involved in glucocorticoid resistance in pediatric ALL

Leukemia, 2005; **19**: 727-33

Wim J.E. Tissing, Jules P.P. Meijerink, Monique L. den Boer, Bas Brinkhof, and Rob Pieters[1]

Division of Pediatric Oncology/Haematology, Erasmus MC-Sophia Childrens Hospital, University Medical Center Rotterdam, The Netherlands

ABSTRACT

Resistance to glucocorticoids (GC) is an important adverse risk factor in the treatment of acute lymphoblastic leukemia (ALL). To induce apoptosis, GCs bind to the GC receptor (GR), which is regulated by various (co)chaperone proteins like HSP-70, HSP-40, HIP, BAG-1, HOP, HSP-90, P-23, FKBP-51, FKBP-52 and CYP-40. In this study, we tested the hypothesis that mRNA expression levels of these molecules are determinants of GC resistance in childhood ALL.

Methods. 20 children with ALL cells *in vitro* sensitive to prednisolone (LC50<0.1 µg/ml) were compared each with a resistant patient (LC50>150 µg/ml), matched for immunophenotype, age and white blood cell count. mRNA expression levels of the (co)chaperone molecules were measured by quantitative real-time RT-PCR and normalized to *GAPDH* and *RNaseP* levels. *In vitro* resistance to prednisolone was measured by MTT assay.

Results. HSP-90 mRNA expression levels were 2000-fold higher as compared to *HSP-70*. Using matched pair analysis, mRNA expression levels of the various (co)chaperone molecules were not significantly different between *in vitro* sensitive and resistant patients.

Conclusion. Glucocorticoid resistance in childhood ALL can not be attributed to different mRNA expression levels of the investigated (co)chaperone molecules, involved in GC binding and transport to the nucleus.

INTRODUCTION

The glucocorticoids (GC) prednisone and dexamethasone have been used in the treatment of childhood acute lymphoblastic leukemia (ALL) since many decades. *In vivo* and *in vitro* response to GCs are strong prognostic factors for clinical outcome[1-3], but mechanisms underlying GC resistance in childhood ALL remain largely unknown.[4] After entering the cell by passive diffusion, GCs bind to the glucocorticoid receptor (GR). Binding depends on the assembly of various (co)chaperone proteins within a GR-chaperone complex, which directs the GR to adopt a GC-binding conformation. The first step in this process is the binding of heat shock protein 70 (HSP-70) and HSP-40 to the GR in an energy-dependent process whereby BAG-1 (BCL-2 associated gene product-1) and HIP (HSP-70 interacting protein) can act as negative and positive regulators respectively.[5] Secondly, HSP-90 and HOP (HSP-70 / HSP-90 organizing protein) bind to this

complex leading to opening of the GC-binding cleft of the GR in an ATP dependent way.[5] This complex is stabilized by P-23 (protein 23) and several immunophillins like FKBP-51 (FK506 binding protein-51), FKBP-52 and CYP-40 (cyclophilin D).[5, 6] (Figure 1) Upon binding, the GC – GR complex is transported to the nucleus where it transactivates GC responsive genes and/or transrepresses other transcription factors like AP-1 and NF-κB. For the transport of the GC-GR complex into the nucleus, (co)chaperone molecules like HSP-90 and FKBP-52 are suggested to

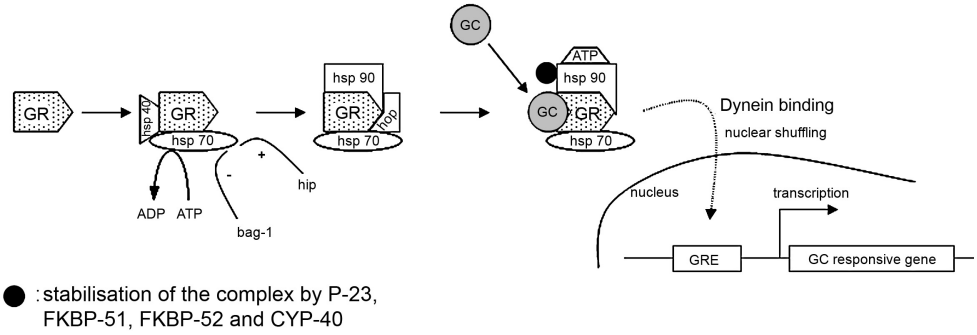


Figure 1. (Co)chaperone protein interactions with the glucocorticoid receptor (GR)

The stepwise assembly of the glucocorticoid receptor – (co)chaperone complex, leading to opening of the GC binding cleft and enabling GC binding. In a first, ATP dependent step, HSP-70 and HSP-40 bind to the GR. This step is inhibited by BAG-1 and activated by HIP. Hereafter HOP and HSP-90 bind to the GR, which opens, in an ATP dependent step, the glucocorticoid (GC) binding cleft, enabling the GR to bind GC. This complex is stabilized by P-23 and the immunophilins FKBP-51, FKBP-52 and CYP-40. One of the models hypothesized in the literature is that FKBP-52 promotes binding of the GC-GR complex to Dyneins for transportation into the nucleus, whereas FKBP-51 promotes retainment of the complex in the cytoplasm.

be necessary for binding of the GC-GR complex to Dynein, which is part of the microtubule based movement machinery.[5, 7] FKBP-51 has an opposite effect, keeping the complex in the cytoplasm.[8]

Sofar, only two (co)chaperone proteins have been studied in relation to GC resistance in ALL. Kojika *et al.* reported aberrant HSP-90 protein expression (80 and 160 kD instead of 90 and 180 kD) and extremely low HSP-70 protein expression in 2 out of 9 resistant human leukemic cell lines.[9] In the only study using patient material, no relation was found between HSP-90 protein and mRNA expression, and *in vivo* GC sensitivity in children with ALL.[10]

In the present study we tested whether GC resistance in childhood ALL is associated with altered mRNA expression of the GR (co)chaperone molecules *HSP-70*, *HSP-40*, *HIP*, *BAG-1*, *HOP*, *HSP-90*, *P-23*, *FKBP-51*, *FKBP-52* and *CYP-40*.

MATERIAL AND METHODS

Patients. In this case control study, 20 patients *in vitro* sensitive to prednisolone (LC_{50}<0.1 µg/ml) were matched each to a patient *in vitro* resistant to prednisolone (LC_{50}>150 µg/ml). Patients were matched for age (1-10 years or ≥10 years), immunophenotype (common- / pre-B-ALL or T-ALL) and white blood cell count (WBC < 50000/µl or ≥50000/µl) at diagnosis. Patient material was taken at diagnosis with informed consent from the patients or their parents.
The mononuclear cell fraction was separated by Lymphoprep density gradient centrifugation (density 1.077 g/m^3, Nucomed Pharma, Oslo, Norway). When necessary, non-leukemic cells were depleted by immunomagnetic beads to purify the samples to more than 90% of blasts.

Isolation of RNA and cDNA synthesis. Total RNA was extracted using the Trizol method (Gibco BRL, Life Technologies, Breda, the Netherlands) according to the protocol provided by the manufacturer with minor modifications.[11] RNA pellets were dissolved in 20 µl TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH= 8.0), and quantified spectrophotometrically. cDNA was synthesized as described before[11], diluted to a final concentration of 8 ng/µl, and stored at -80°C.

Quantitative Real-time RT-PCR. mRNA levels of *HSP-70*, *HSP-40*, *HIP*, *BAG-1*, *HOP*, *HSP-90*, *P-23*, *FKBP-51*, *FKBP-52, CYP-40* and *GR-alpha* (i.e. target PCRs) and two endogenous reference genes (i.e. *GAPDH* and *RNaseP*) were measured by quantitative real-time RT-PCR based on Taqman-chemistry using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). PCR products were detected by a double labeled, non-extendible probe containing a FAM reporter group and a TAMRA quencher-group (table 1).[11] All primers and probes had melting temperatures of 65±1°C and 75±1°C respectively (nearest neighbor method[12]). One hundred nanograms of genomic DNA served as a negative control and did not result in detectable amplification for any of these reactions, confirming the specificity of these reactions for RNA detection. Forty nanograms of patient sample cDNA was amplified in duplo in the presence of 300 nM forward and reverse primers, 50 nM probe, 200 µM dNTPs, 4 mM $MgCl_2$ and 1.25 U of Ampli*Taq*™ gold DNA polymerase in Taqman buffer A (Applied Biosystems) in a total volume of 50 µl. Samples were heated for 10 min at 95°C and amplified in 40 cycles of 15 sec at 95°C and 60 sec at 60°C. A serial diluted positive control was amplified on each plate to verify the amplification efficiency within each experiment.[11] The average Ct-value was used to calculate the mRNA expression levels of the PCR

Table 1. Primer and probe combinations used for the quantitative real-time RT-PCR. GenBank accession codes used for designing the primers and probes are depicted.

Gene	Sequence	
Hsp-90	Fw	5′ GAT AAA CCC TGA CCA TTC C 3′
NM005348	Rev	5′ TTT-ATG-AAA-CTG-CGC-TCC-TGT-CTT 3′
	Probe	5′ FAM - TTT-ATG-AAA-CTG-CGC-TCC-TGT-CTT - TAMRA 3′
Hsp-70	Fw	5′ GGA GGC GGA GAA GTA CA 3′
NM005345	Rev	5′ GCT GAT GAT GGG GTT ACA 3′
NM005346	Probe	5′ FAM - AGA TCA GCG AGG CGG ACA A - TAMRA 3′
Hsp-40	Fw	5′ CGC CGA GGA GAA GTT C 3′
NM006145	Rev	5′ CAT CAA TGT CCA TGC CTT 3′
	Probe	5′ FAM - TGA GTT CTT CGG TGG CAG AAA T - TAMRA 3′
P-23	Fw	5′ AAC GGA CAG ATC AAT TTT ATG 3′
NM006601	Rev	5′ TTG TGA ATC ATC ATC TGC TC 3′
	Probe	5′ FAM - TGA TGA ACA ACA TGG GTG GTG A - TAMRA 3′
HOP	Fw	5′ ATG ACC ACT CTC AGC GTC 3′
NM006819	Rev	5′ CTC CTT GGC TTT GTC GTA 3′
	Probe	5′ FAM - CAG TAT GGA TGA GCA GGA AGA GAT TG - TAMRA 3′
FKBP-51	Fw	5′ GAA TGG TGA GGA AAC GC 3′
NM004117	Rev	5′ ATG CCT CCA TCT TCA AAT AA 3′
	Probe	5′ FAM - TAT GGC TCG GCT GGC AGT C - TAMRA 3′
FKBP-52	*F*w	5′ GCG GGG CAC TGT GTA 3′
NM002014	Rev	5′ AGC CAG CCT CTC AAA CA 3′
	Probe	5′ FAM - CAA CAA AGC CGA CAA GAC C - TAMRA 3′
CYP-40	*F*w	5′ GCC-CTT-TTC-ATC-GAA-TTA-T 3′
NM005038	Rev	5′ ATA-TTC-CCC-CGT-CAT-CTC 3′
	Probe	5′ FAM - CAA GCA TGA TCG GGA GGG TT - TAMRA 3′
BAG-1	Fw	5′ GGG GTT CCA CAG TCT TTT 3′
NM004323	Rev	5′ CTG AAC CTT TTT TAC CAA GC 3′
	Probe	5′ FAM - AAT CTC TGA AGG AAA TGG AAA CAC C - TAMRA 3′
HIP	Fw	5′ CCG CAA AGT GAA CGA G 3′
NM003932	Rev	5′ TGA TGG TTC GTC TGC C 3′
	Probe	5′ FAM - CTG AGG GAG TGG GTG GAG AG - TAMRA 3′
GR-alpha	Fw	5′ TGT TTT GCT CCT GAT CTG A 3′
AC012634	Rev	5′ TCG GGG AAT TCA ATA CTC A 3′
	Probe	5′ FAM - TGA CTC TAC CCT GCA TGT ACG AC - TAMRA 3′
GAPDH	Fw	5′ GTC GGA GTC AAC GGA TT 3′
ACJ04038	Rev	5′ AAGCTT CCC GTT CTC AG 3′
	Probe	5′ FAM - TCA ACT ACA TGG TTT ACA TGT TCC AA - TAMRA 3′
RNaseP	Fw	5′ TTG GGA AGG TCT GAG ACT A 3′
X15624	Rev	5′ TCA GCC ATT GAA CTC ACT T 3′
	Probe	5′ FAM - AGG TCA GAC TGG GCA GGA GAT - TAMRA 3′

targets relative to the expression level of the two housekeeping genes using the comparative Ct method[12] using the equation:
relative expression = $2^{-[Ct(target)-Ct(reference\ gene)]} \times 100$.

MTT-assay. In-vitro drug cytotoxicity was assessed using the MTT assay as described earlier.[2, 13] Briefly, patient blasts were cultured with or without prednisolone disodiumphosphate in a concentration range between 0.06 and 250 µg/mL. At day 4, MTT is added which can only be reduced into formazan by viable cells. The reduced product was quantified spectrophotometrically at 562 nm. The leukemic cell survival was calculated by: (OD drug treated well / OD control wells without drug) x 100%. The LC50 value represents the concentration of the drug at which 50% of the cells are killed and is used as measure of *in-vitro* drug cytotoxicity. Leukemic cells having LC50 values lower than 0.1 µg/ml were assigned *in vitro* glucocorticoid sensitive, samples having LC50 values exceeding 150 µg/ml were considered *in vitro* resistant, as previously described to be of prognostic value.[13, 14]

Statistical methods. The non-parametric Wilcoxon signed rank test for matched samples and the non-parametric Mann-Whitney U test were used to analyze the results from the patient samples. A p-value ≤ 0.05 was considered statistically significant (two-tailed tested).

RESULTS

Forty patients were included in this case control study. For each *in vitro* sensitive patient, an *in vitro* resistant control, matched for age, immunophenotype and WBC, was selected. Patient characteristics are depicted in table 2.
The expression levels of the different (co)chaperone molecules were measured by quantitative real-time RT-PCR. The PCR reactions were very specific, showing

Table 2. Patient characteristics.

		N	(%)
Gender	Male	22	(55)
	Female	18	(45)
Age at diagnosis	1 – 9 yr	30	(75)
	≥ 10 yr	10	(25)
WBC at diagnosis	< 50000/ml	22	(55)
	≥ 50000/ml	18	(45)
Immunophenotype	Common- / pre-B-ALL	28	(70)
	T-ALL	12	(30)

only one single PCR product of predicted size on gel. (Figure 2) Amplification efficiency was 95% or higher for all reactions (data not shown), which allows direct normalization of expression levels of each target PCR to the expression levels of the endogenous house-keeping genes *GAPDH* and *RNaseP*. Figure 3 shows the mRNA expression levels of the various (co)chaperone molecules relative to *GAPDH* for the whole study population. Compared to the other (co)chaperone molecules, the mRNA expression level of *HSP-90* was relatively high, whereas the expression level of *HSP-70* was relatively low, approximately 2000-fold lower than *HSP-90*. The median mRNA expression levels of the different (co)chaperone molecules and the GR for the *in vitro* sensitive and resistant patients are depicted in table 3.

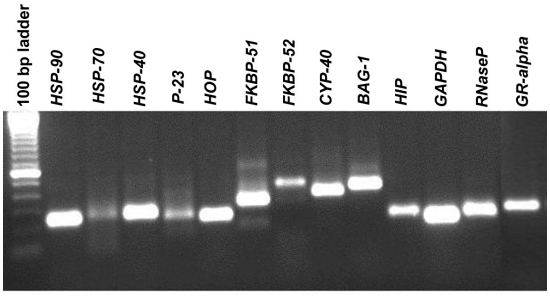


Figure 2. PCR products of the (co)chaperone molecules
Example of real-time RT-PCR products resolved on agarose gel for the various target genes as tested in this study, to show that the PCR reactions are very specific, resulting in only one PCR product per reaction.

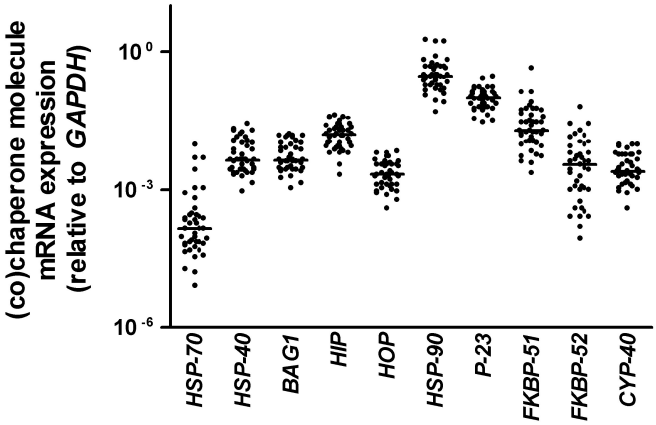


Figure 3. Expression levels of (co)chaperone molecules in pediatric ALL cells
mRNA expression levels, relative to *GAPDH*, of all study patients are depicted. Lines indicate the median values.

Since we analyzed every GC sensitive patient in relation to its matched control, the ratios of the mRNA expression levels of the various (co)chaperone molecules for each prednisolone sensitive case relative to its matched prednisolone resistant control case were calculated and shown in Figure 4. The median ratio was about 1 for each of the (co)chaperone molecules. Using a matched pair analysis, no

Table 3. mRNA expression levels of the different (co)chaperone molecules and the glucocorticoid receptor relative to GAPDH for the in vitro sensitive and resistant patients.

	Sensitive patients Median (p_{25} – p_{75})	Resistant patients Median (p_{25} – p_{75})
HSP-70	0.0001 (0.0001-0.0002)	0.0002 (0.0001-0.0008)
HSP-40	0.0045 (0.0027-0.0108)	0.0039 (0.0028-0.0100)
BAG-1	0.0055 (0.0038-0.0106)	0.0038 (0.0025-0.0083)
HIP	0.0156 (0.0101-0.0260)	0.0148 (0.0083-0.0203)
HOP	0.0033 (0.0018-0.0042)	0.0018 (0.0012-0.0024)
HSP-90	0.3148 (0.2232-0.4686)	0.2595 (0.1900-0.4662)
P-23	0.1156 (0.0751-0.1390)	0.0924 (0.0598-0.1217)
FKBP-51	0.0295 (0.0152-0.0537)	0.0170 (0.0100-0.0302)
FKBP-52	0.0044 (0.0010-0.0084)	0.0027 (0.0010-0.0094)
CYP-40	0.0029 (0.0020-0.0059)	0.0024 (0.0013-0.0049)
GR	0.0257 (0.0178-0.0326)	0.0158 (0.0092-0.0249)

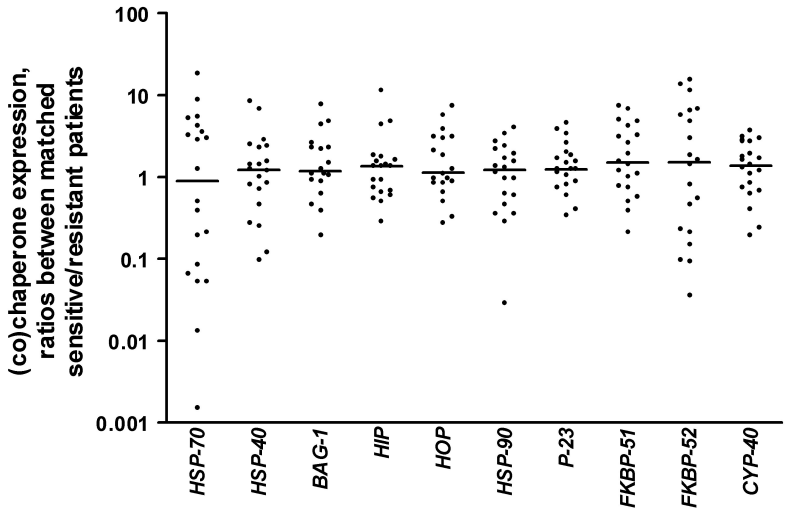


Figure 4. Ratio of the (co)chaperone mRNA expression levels of the matched prednisolone sensitive and resistant ALL patients
Distribution of the ratio of the (co)chaperone molecules for the matched sensitive and resistant patients. mRNA expression levels, relative to *GAPDH*, were used for calculation of the ratio. Lines indicate the median values.

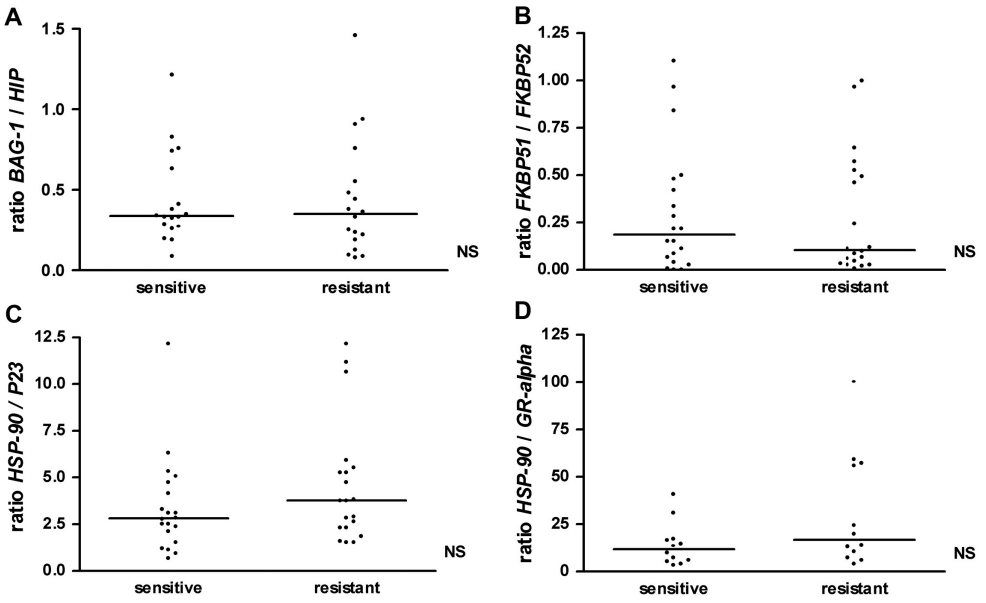


Figure 5. Ratio of the mRNA expression levels of various related (co)chaperone molecules

(a) The ratio of *BAG-1* (a negative regulator of HSP-70) and *HIP* (a positive regulator of HSP-70). (b) The ratio of *FKBP-51* and *FKBP-52*, which have been reported to compete with eachother for binding the GC-GR complex. Binding to *FKBP-52* promotes shuttling to the nucleus and thereby promotes the anticipated effect of the GC. (c) The ratio of *HSP-90 and P-23*, reported to stabilize the binding of the GR to the promoter of GR responsive genes and to destabilize this binding respectively. (d) The ratio of *HSP-90* and *GR-alpha* (the functional GR), which has been speculated to be related to GC sensitivity. Lines indicate the median values, NS means not significant.

differences in expression for any of these molecules were found between *in vitro* sensitive and resistant patients. Normalization to *RNaseP* instead of *GAPDH* led to the same conclusions (data not shown).

With *BAG-1* being an inhibitor of *HSP-70* and *HIP* being a positive regulator, the ratio of these 2 cochaperone molecules was determined and related to GC sensitivity. No difference was found between the matched *in vitro* sensitive and resistant patients with respect to the ratio of the mRNA expression levels of *BAG-1* and *HIP*. (Figure 5A) As *FKBP-51* and *FKBP-52* might compete for binding to the GR complex, thereby affecting the nuclear shuffling of the GC-GR complex, the ratio of these molecules was calculated and related to GC sensitivity. No difference for the *FKBP-51* / *FKBP-52* mRNA ratio was found between the matched *in vitro* prednisolone sensitive and resistant patients. (Figure 5B) Since HSP-90 has been reported to be involved in the stabilization of the GC-GR complex binding to the

promoter of GR responsive genes while P-23 has the opposite effect, the ratio of the mRNA expression levels of these molecules was studied in relation to GC resistance. However, we did not find any difference between sensitive and resistant patients. (Figure 5C) The ratio of HSP-90 and the functional glucocorticoid receptor (GR-alpha) mRNA levels might be related to GC resistance as well. We were able to calculate a HSP-90 / GR-alpha ratio in 12 matched patient samples. No relation was found between this ratio and GC resistance. (Figure 5D)

DISCUSSION

GCs enter the cell by passive diffusion to bind the GR. To enable binding, the GR undergoes a stepwise maturation process in which it is bound by various (co)chaperone proteins, eventually leading to the opening of the GC binding pocket.[5] (Figure 1) Which of the proteins are absolutely necessary for this process remains unclear. Some reports state that only HSP-90 and HSP-70 are minimally required[15], but most studies report the necessity of at least 5 proteins (HSP-90, HSP-70, HSP-40, HOP and P-23).[16, 17]

In the present case control study we analyzed the mRNA expression levels of the (co)chaperone proteins of the GR in relation to GC resistance in childhood ALL. The chaperone molecule *HSP-70* had a relatively low mRNA expression level, while the mRNA expression level of *HSP-90* was relatively high (2000 fold higher than *HSP-70* expression), which is in concordance with earlier studies reporting protein and mRNA levels of HSP-90 and HSP-70 in acute leukemias.[10, 18, 19] As HSP-70 is part of the more immature GR-(co)chaperone complex, its low expression level might determine a rate limiting step in the initial maturation process of the GR. For none of the (co)chaperone molecules as tested in this study, a relation between mRNA expression level and *in vitro* GC resistance was found.

We tested different other hypotheses on a possible relation between GR (co)chaperone expression and GC resistance. The ratio of BAG-1, which is a negative regulator of HSP-70[20] and HIP, which is a positive regulator might be correlated to GC resistance. [21] However, such a correlation was not found in our study population. BAG-1 is also reported to be an inhibitor of DNA binding and subsequent transcriptional activation by the GR[22], but we did not find a relation between *BAG-1* mRNA expression levels and GC resistance either.

For transportation of the GC-GR (co)chaperone complex to the nucleus, FKBP-51 is replaced by FKBP-52 in this complex. Some New World primate genera are

very resistant to GC. In these animals it was shown that an elevated protein expression of FKBP-51 contributed to GC resistance.[23] Studies analyzing protein expression levels showed that FKBP-52 potentiates reporter gene activation, which is attenuated by FKBP-51.[8, 24] We hypothesized that the ratio of *FKBP-51* and *FKBP-52* mRNA levels is related to GC resistance. As we could not show such a correlation in our study population, and we did not show a relation between *FKBP-51* mRNA expression and GC resistance either, the relevance of *FKBP-51* for GC resistance as found in some new world primates can not be extrapolated to GC resistance in childhood ALL.

HSP-90 stabilizes the binding of the GC – GR complex to the promoter of GR responsive genes, while P-23 induces removal of this complex from the promoter.[25] Longer GR residence time at these promoter sites is associated with greater transcriptional output, so expression of HSP-90 and P-23 might be related to GC resistance. By studying the ratio of *HSP-90* and *P-23* mRNA expression levels and GC resistance, we conclude that this ratio is not correlated to GC resistance in childhood ALL. Another study reported a negative effect of HSP-90 protein levels on GR function when the ratio HSP-90/GR increased.[26] No such negative effect of mRNA expression levels was found in our patient cohort.

The conclusion of an earlier study, that *HSP-90* expression levels are not related to GC resistance[10], are confirmed in this (larger) study and we show that the mRNA expression levels of other (co)chaperone molecules of the GR are not related to GC resistance as well. It might be, that not mRNA levels, but protein levels of the different (co)chaperone molecules are correlated with GC resistance (i.e. post-transcriptional regulation instead of transcriptional regulation). However, a correlation between mRNA and protein levels has been shown previously for HSP-90, HSP-70, BAG-1, FKBP-51 and FKBP-52 in different cell lines.[27-31] Due to the limitation of patient material, it was not possible to confirm these data in our population. Another hypothesis relating (co)chaperone molecules to GC resistance might be that not the expression level, but rather the functional capacity of the (co)chaperone molecules to form a complex with the GR instead of the expression level of the (co)chaperone molecules is related to GC resistance. This complex formation process might for example be influenced by mutations in one of the genes encoding for the (co)chaperone proteins. When one step of the maturation process of the GR is disturbed, GC binding might decrease, resulting in reduced transactivation or transrepression of GC responsive genes. Using dexamethasone binding assays, it was shown that decreased binding of dexamethasone was related

to GC resistance in childhood ALL. Although this finding was regarded to be due to a lower expression level of the GR[32], it may alternatively be related to a reduced rate in complex maturation due to defects in one of these molecules other than just the expression level of these molecules. However, to study the formation of the GR – (co)chaperone complex will be very difficult when using patient material, as the different stages of this complex will pass quickly. A last hypothesis on the relevance of the (co)chaperone expression for GC sensitivity might be, that not the expression level before start of therapy, but the (co)chaperone expression level upon GC exposure is related to GC sensitivity and resistance. In this sense it is of interest that, using micro-array studies, one of the genes found to be upregulated upon GC exposure is FKBP-51.[33] However, till date there are no data on differences in gene expression upon GC exposure between GC sensitive and resistant ALL cells.

In conclusion, childhood ALL cells have a relatively high mRNA expression level of *HSP-90* and a low expression level of *HSP-70*. The expression levels of the different (co)chaperone molecules of the GR are not related to GC resistance.

References

1. Lauten M, Stanulla M, Zimmermann M, Welte K, Riehm H, Schrappe M. Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter. Klin Padiatr 2001;213:169-74.
2. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood 1998;92:259-66.
3. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. *In vitro* drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. Blood 1997;89:2959-65.
4. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia 2003;17:17-25.
5. Pratt WB, Toft DO. Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med 2003;228:111-33.
6. Ratajczak T, Ward BK, Minchin RF. Immunophilin chaperones in steroid receptor signalling. Curr Top Med Chem 2003;3:1348-57.
7. Galigniana MD, Harrell JM, Murphy PJ, *et al.* Binding of hsp90-associated immunophilins to cytoplasmic dynein: direct binding and in vivo evidence that the peptidylprolyl isomerase domain is a dynein interaction domain. Biochemistry 2002;41:13602-10.
8. Davies TH, Ning YM, Sanchez ER. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. J Biol Chem 2002;277:4597-600.
9. Kojika S, Sugita K, Inukai T, *et al.* Mechanisms of glucocorticoid resistance in human leukemic cells: implication of abnormal 90 and 70 kDa heat shock proteins. Leukemia 1996;10:994-9.
10. Lauten M, Beger C, Gerdes K, *et al.* Expression of heat-shock protein 90 in

glucocorticoid-sensitive and -resistant childhood acute lymphoblastic leukaemia. Leukemia 2003;17:1551-6.
11. Stam RW, den Boer ML, Meijerink JP*, et al.* Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood 2003;101:1270-6.
12. Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn 2001;3:55-61.
13. Den Boer ML, Harms DO, Pieters R*, et al.* Patient stratification based on prednisolone-vincristine-asparaginase resistance profiles in children with acute lymphoblastic leukemia. J Clin Oncol 2003;21:3262-8.
14. Pieters R, Huismans DR, Loonen AH*, et al.* Relation of cellular drug resistance to long-term clinical outcome in childhood acute lymphoblastic leukaemia. Lancet 1991;338:399-403.
15. Rajapandi T, Greene LE, Eisenberg E. The molecular chaperones Hsp90 and Hsp70 are both necessary and sufficient to activate hormone binding by glucocorticoid receptor. J Biol Chem 2000;275:22597-604.
16. Morishima Y, Murphy PJ, Li DP, Sanchez ER, Pratt WB. Stepwise assembly of a glucocorticoid receptor.hsp90 heterocomplex resolves two sequential ATP-dependent events involving first hsp70 and then hsp90 in opening of the steroid binding pocket. J Biol Chem 2000;275:18054-60.
17. Kanelakis KC, Pratt WB. Regulation of glucocorticoid receptor ligand-binding activity by the hsp90/hsp70-based chaperone machinery. Methods Enzymol 2003;364:159-73.
18. Yufu Y, Nishimura J, Nawata H. High constitutive expression of heat shock protein 90 alpha in human acute leukemia cells. Leuk Res 1992;16:597-605.
19. Xiao K, Liu W, Qu S, Sun H, Tang J. Study of heat shock protein HSP90 alpha, HSP70, HSP27 mRNA expression in human acute leukemia cells. J Tongji Med Univ 1996;16:212-6.
20. Kanelakis KC, Morishima Y, Dittmar KD*, et al.* Differential effects of the hsp70-binding protein BAG-1 on glucocorticoid receptor folding by the hsp90-based chaperone machinery. J Biol Chem 1999;274:34134-40.
21. Kanelakis KC, Murphy PJ, Galigniana MD*, et al.* hsp70 interacting protein Hip does not affect glucocorticoid receptor folding by the hsp90-based chaperone machinery except to oppose the effect of BAG-1. Biochemistry 2000;39:14314-21.
22. Kullmann M, Schneikert J, Moll J*, et al.* RAP46 is a negative regulator of glucocorticoid receptor action and hormone-induced apoptosis. J Biol Chem 1998;273:14620-5.
23. Scammell JG, Denny WB, Valentine DL, Smith DF. Overexpression of the FK506-binding immunophilin FKBP51 is the common cause of glucocorticoid resistance in three New World primates. Gen Comp Endocrinol 2001;124:152-65.
24. Riggs DL, Roberts PJ, Chirillo SC*, et al.* The Hsp90-binding peptidylprolyl isomerase FKBP52 potentiates glucocorticoid signaling in vivo. Embo J 2003;22:1158-67.
25. Stavreva DA, Muller WG, Hager GL, Smith CL, McNally JG. Rapid glucocorticoid receptor exchange at a promoter is coupled to transcription and regulated by chaperones and proteasomes. Mol Cell Biol 2004;24:2682-97.
26. Kang KI, Meng X, Devin-Leclerc J*, et al.* The molecular chaperone Hsp90 can negatively regulate the activity of a glucocorticosteroid-dependent promoter. Proc Natl Acad Sci U S A 1999;96:1439-44.
27. Yang WL, Nair DG, Makizumi R*, et al.* Heat shock protein 70 is induced in mouse human colon tumor xenografts after sublethal radiofrequency ablation. Ann Surg Oncol 2004;11:399-406.
28. Yang X, Chernenko G, Hao Y*, et al.* Human BAG-1/RAP46 protein is generated as

four isoforms by alternative translation initiation and overexpressed in cancer cells. Oncogene 1998;17:981-9.
29. Chen LJ, Su XW, Qiu PX, Huang YJ, Yan GM. Thermal preconditioning protected cerebellar granule neurons of rats by modulating HSP70 expression. Acta Pharmacol Sin 2004;25:458-61.
30. Ward BK, Mark PJ, Ingram DM, Minchin RF, Ratajczak T. Expression of the estrogen receptor-associated immunophilins, cyclophilin 40 and FKBP52, in breast cancer. Breast Cancer Res Treat 1999;58:267-80.
31. Giraudier S, Chagraoui H, Komura E*, et al.* Overexpression of FKBP51 in idiopathic myelofibrosis regulates the growth factor independence of megakaryocyte progenitors. Blood 2002;100:2932-40.
32. Kaspers GJ, Pieters R, Veerman AP. Glucocorticoid resistance in childhood leukemia. International Journal of Pediatric Hematology/Oncology 1997;4:583-96.
33. Schmidt S, Rainer J, Ploner C, Presul E, Riml S, Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ 2004;11 Suppl 1:S45-55.

Chapter 7

Genome-wide identification of prednisolone-responsive genes in acute lymphoblastic leukemia cells

Manuscript submitted

Wim J.E. Tissing[1,2], Monique L. den Boer[1], Jules P.P. Meijerink[1], Renee X. Menezes[1,3], Sigrid Swagemakers[4], Peter J. van der Spek[4], Stephen E. Sallan[5], Scott A. Armstrong[5], Rob Pieters[1]

1- Dept of Pediatric Oncology / Hematology, Erasmus MC / Sophia Children's Hospital, Rotterdam, the Netherlands
2- Dept of Pediatric Oncology / Hematology, Beatrix Children's Hospital, University of Groningen and University Medical Center Groningen, Groningen, the Netherlands
3- Dept of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands
4- Dept of Bioinformatics, Erasmus MC, Rotterdam, the Netherlands
5- Dept of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA, USA

ABSTRACT

Glucocorticoids are keystone drugs in the treatment of childhood acute lymphoblastic leukemia (ALL) and therefore it is important to get more insight in signal transduction pathways involved in glucocorticoid induced apoptosis in ALL. Affymetrix U133A GeneChips were used to identify genes that are transcriptionally regulated upon 3 and 8 hours of prednisolone exposure in leukemic cells of 13 children newly diagnosed with ALL. Following 3 hours of exposure no significant changes in gene expression could be identified as compared to exposure to culture medium. Following 8 hours of exposure, 57 probesets (51 unique genes) were differentially expressed ($p < 0.0005$ and false discovery rate < 10%) with 44 probesets (39 genes) being upregulated (median 2.4-fold) and 13 probesets (12 genes) downregulated (median 1.7-fold). Twenty-one of those genes have not been identified before to be transcriptionally regulated by prednisolone. Two of the three most highly upregulated genes were tumor suppressor genes, i.e. *Thioredoxin interacting protein* (*TXNIP,* 3.7-fold) and *Zinc finger and BTB domain containing 16* (*ZBTB16*, 8.8-fold). About 50% of the differentially expressed genes were functionally categorized in three major routes, namely MAPK pathways (9 genes), NF-κB signaling (11 genes) and carbohydrate metabolism (5 genes). Biological characterization of these genes and pathways might elucidate the action of glucocorticoids in ALL cells, which may point to causes of glucocorticoid resistance, ways to circumvent glucocorticoid resistance and new potential targets for therapy.

INTRODUCTION

Glucocorticoids like prednisone and dexamethasone have been used extensively in the treatment of childhood acute lymphoblastic leukemia (ALL) since many years. The *in vivo* and *in vitro* prednisone response as determined with a tetrazolium-based (MTT) toxicity assay have been shown to correlate with each other and long-term clinical outcome in children with ALL.[1-3]

The classically proposed way of glucocorticoid action is that glucocorticoids bind to the intracellular glucocorticoid receptor (GR). The glucocorticoid-GR complex then translocates to the nucleus, where it binds to glucocorticoid responsive elements (GRE), resulting in the transcriptional activation of glucocorticoid-responsive genes.[4] Alternatively, the glucocorticoid-GR complex can directly bind

to transcription factors like Nuclear Factor-κB (NF-κB) or Activator Protein-1 (AP-1), resulting in so-called transrepression complexes. These complexes disrupt the transcriptional regulation of genes that are normally affected by these transcription factors.[5] Depending on the cell type, transcriptional activation and repression result in immunosuppression, stress response or induction of apoptosis.

Interestingly, glucocorticoids only induce apoptosis in lymphoid cells like ALL, multiple myelomas, malignant lymphomas and thymocytes, not in other tissues. Nevertheless, despite the major impact of glucocorticoid resistance on clinical outcome, knowledge about the signal transduction pathways leading to glucocorticoid induced apoptosis in ALL cells is limited.[6,7] Several microarray studies have been performed to determine glucocorticoid-regulated genes in leukemia. [8-12] However, the cell lines (i.e. immortalized cells) as used in these studies do not represent an ideal model to study mechanisms involved in survival and apoptosis of primary ALL cells. Recently the first microarray study was published in which, besides cell lines, *in vivo* prednisone-exposed patient ALL samples were analysed.[13] In the present study, we used freshly obtained leukemic cells of pediatric patients at initial diagnosis of ALL to identify which genes are transcriptionally regulated upon in *vitro* prednisolone exposure.

MATERIAL AND METHODS

Patients. The study population consisted of 13 patients diagnosed with precursor-B or T-ALL. Pretreatment bone marrow or peripheral blood was obtained after written informed consent from the patients and/or their legal guardians. The mononuclear cell fraction was separated by Lymphoprep density gradient centrifugation (density 1.077 g/ml, Nycomed Pharma, Oslo, Norway). When necessary, non-leukemic cells were depleted by immunomagnetic beads to purify the samples to more than 90% of leukemic cells.

Prednisolone exposure. Leukemic cells were incubated in RPMI 1640 medium (Dutch modification without L-glutamine) supplemented with 2 mM L-glutamine, 5 µg/ml insulin, 5µg/ml transferrin, 5 ng/ml sodium selenite, 20% heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 0.125 µg/ml fungizone, 200 µg/ml gentamycin with and without 250 µg/ml prednisolone. After 3 and 8 hours of incubation, 20 x 10^6 cells were removed from the culture for RNA isolation.

RNA extraction, labeling and hybridization. Total RNA was extracted using the

Trizol method (Gibco BRL, Life Technologies, Breda, the Netherlands) according to the protocol provided by the manufacturer with minor modifications.[14] RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). RNA (5-15 µg) was used for subsequent production of biotinylated antisense cRNA, as described before.[15] Samples with less than 10 µg labeled cRNA were excluded. Labeled cRNA was hybridized to the U133A GeneChip oligonucleotide microarray (Affymetrix) according to the protocol provided by the manufacturer.

Statistics. Raw gene expression values were calculated using Affymetrix Microarray Suite version 5.0.[16] Data were normalized using the variance stabilization procedure (VSN) as proposed by Huber et al.[17] Data distribution of unexposed controls suggested a gamma- or log-normal distribution. Therefore a generalized linear model with gamma error distribution and identity link function was used to describe the effect of exposure (prednisolone exposed or control) on gene expression levels. This model was fitted to each gene separately and the effect of exposure was evaluated via the corresponding ANOVA *p*-values. Differentially expressed genes with respect to exposure were selected by controlling the false discovery rate (FDR), using Benjamini & Hochberg's procedure.[18] A *p*-value < 0.0005 and a FDR (adapted for multiple testing) < 10% was considered statistically significant. Normalization and subsequent analysis were run using R 1.9.1[19], also making use of the Bioconductor packages VSN and Multtest. (www.Bioconductor.org, see URL) The fold up- or down-regulation was calculated using the formula: $e^{(\text{vsn value pred sample} - \text{vsn value control sample})}$.

The TELiS database (www.telis.ucla.edu/index.htm)[20] was used to search for transcription factor binding motifs (TFBMs) which were over- or underrepresented in the genes differentially expressed upon prednisolone exposure, using a promoter size of –1000 to + 200 and a stringency of 0.9.

RESULTS

Diagnostic samples of thirteen patients with ALL were exposed to prednisolone for three and eight hours. Prednisolone exposed and control (culture medium only) leukemic cells of the same patient were analyzed pairwise per patient to correct for the effect of culture in time. Paired samples could be successfully analyzed for 9/13 patients at three hours and for 10/13 patients at eight hours of exposure time.

After 3 hours of prednisolone exposure, no differentially expressed probesets

Table 1. Prednisolone induced changes in gene expression in pediatric ALL.
After 8 hours of prednisolone exposure, 57 probesets (51 unique genes and 2 ESTs) were differentially expressed at $p<0.0005$ and FDR<10%. Aliases between brackets.

probe ID	Accession No	Gene Name#	Description	Fold change*	*p*-value
			Upregulated genes		
204560_at	NM_004117	*FKBP5*	FK506-binding protein 5	35.4	2.7e-07
205883_at	NM_006006	*ZBTB16 (PLZF)*	zinc finger and BTB domain containing 16	8.8	6.0e-05
201008_s_at	AI439556	*TXNIP (VDUP)*	thioredoxin interacting protein	4.4	8.7e-06
201009_s_at	AA812232	*TXNIP (VDUP)*	thioredoxin interacting protein	3.0	0.0002
221756_at	AL540260	*LIMK2*	LIM domain kinase 2	4.1	6.8e-06
212158_at	AL577322	*SDC2*	syndecan 2	4.0	1.6e-05
204698_at	U88964	*ISG20*	interferon stimulated exonuclease gene 20kDa	3.7	3.3e-05
33304_at	NM_002201	*ISG20*	interferon stimulated exonuclease gene 20kDa	3.4	1.2e-05
201369_s_at	U07802	*ZFP36L2 (ERF2)*	zinc finger protein 36, C3H type-like 2	3.6	6.1e-06
201368_at	NM_006887	*ZFP36L2 (ERF2)*	zinc finger protein 36, C3H type-like 2	2.6	2.2e-07
208078_s_at	NM_030751 / NP_775490	*TCF8 / SNF1LK*	transcription factor 8 (represses interleukin 2 expression) / SNF1-like kinase	3.5	6.3e-05
208763_s_at	AL110191	*DSIPI (TSC22D3, GILZ)*	delta sleep inducing peptide, immunoreactor	3.3	5.5e-05
202670_at	AI571419	*MAP2K1 (MEK1)*	mitogen-activated protein kinase kinase 1	3.1	1.1e-05
203542_s_at	NM_001206	*KLF9 (BTEB1)*	kruppel-like factor 9	3.1	0.0002
203543_s_at	AI690205	*KLF9 (BTEB1)*	kruppel-like factor 9	3.1	5.5e-05
203574_at	NM_005384	*NFIL3*	nuclear factor, interleukin 3 regulated	3.0	7.5e-06
209185_s_at	AF073310	*IRS2*	insulin receptor substrate 2	2.8	7.7e-06
215890_at	X61094	*GM2A*	GM2 ganglioside activator	2.6	2.0e-05
203973_s_at	NM_005195	*CEBPD*	CCAAT/enhancer binding protein (C/EBP), delta	2.5	3.4e-05
213792_s_at	AA485908	*INSR*	insulin receptor	2.5	0.0001
212242_at	AL565074	*TUBA1*	tubulin, alpha 1 (testis specific)	2.4	0.0001
201041_s_at	NM_004417	*DUSP1 (MKP1)*	dual specificity phosphatase 1	2.4	5.9e-05
212188_at	AI718937	*KCTD12*	potassium channel tetramerisation domain containing 12	2.3	4.9e-06
212192_at	AA551075	*KCTD12*	potassium channel tetramerisation domain containing 12	2.3	3.5e-05
218638_s_at	NM_012445	*SPON2*	spondin 2	2.3	4.6e-05
207996_s_at	NM_004338	*C18orf1*	chromosome 18 open reading frame 1	2.2	9.6e-06
204618_s_at	NM_005254	*GABPB2*	GA binding protein transcription factor, beta subunit 2	2.2	4.3e-05
210001_s_at	AB005043	*SOCS1*	suppressor of cytokine signaling 1	2.2	0.0002
200921_s_at	NM_001731	*BTG1*	B-cell translocation gene 1, anti-proliferative	2.2	6.1e-05
202643_s_at	AI738896	*TNFAIP3 (A20)*	tumor necrosis factor, alpha-induced protein 3	2.1	0.0001
201037_at	NM_002627	*PFKP*	phosphofructokinase, platelet	2.0	0.0002
207945_s_at	NM_001893	*CSNK1D*	casein kinase 1, delta	1.8	6.1e-05
201739_at	NM_005627	*SGK*	serum/glucocorticoid regulated kinase	1.8	0.0002
203819_s_at	AU160004	*IMP-3*	IGF-II mRNA-binding protein 3	1.7	2.1e-05
221563_at	N36770	*DUSP10 (MKP5)*	dual specificity phosphatase 10	1.5	6.9e-05
215977_x_at	X68285	*GK*	glycerol kinase	1.5	6.4e-05
213310_at	AI613483	*EIF2C2*	eukaryotic translation initiation factor 2C, 2	1.4	0.0001
215046_at	AL133053	*FLJ23861*	EST	1.4	0.0002
217356_s_at	S81916	*PGK1*	phosphoglycerate kinase 1	1.4	0.0001
211926_s_at	AI827941	*MYH9*	myosin, heavy polypeptide 9, non-muscle	1.3	2.3e-05
218761_at	NM_017610	*RNF111 (ARK)*	ring finger protein 111	1.3	6.8e-05
217795_s_at	W74580	*THEM43*	transmembrane protein 43	1.3	8.1e-05
201859_at	NM_002727	*PRG1*	proteoglycan 1	1.3	0.0001
218528_s_at	NM_022781	*RNF38*	ring finger protein 38	1.3	0.0001
			Down regulated genes		
205749_at	NM_000499	*CYP1A1*	cytochrome P450, family 1, subfamily A, polypeptide 1	- 2.0	8.6e-06
209969_s_at	BC002704	*STAT1*	signal transducer and activator of transcription 1	- 2.0	0.0001
219066_at	NM_021823	*MDS018*	EST	- 2.0	0.0001
205013_s_at	NM_000675	*ADORA2A*	adenosine A2a receptor	- 2.0	0.0001
205006_s_at	NM_004808	*NMT2*	N-myristoyltransferase 2	- 1.7	6.4e-05
203612_at	NM_004053	*BYSL*	bystin-like	- 1.7	0.0002
219665_at	NM_024815	*NUDT18*	nudix-type motif 18	- 1.7	3.5e-05
221933_at	AI338338	*NLGN4X*	neuroligin 4, X-linked	- 1.7	9.8e-05
204070_at	NM_004585	*RARRES3*	retinoic acid receptor responder (tazarotene induced) 3	- 1.7	0.0002
211430_s_at	M87789	*IGHG1*	immunoglobulin heavy constant gamma 1	- 1.4	7.7e-05
218046_s_at	NM_016065	*MRPS16*	mitochondrial ribosomal protein S16	- 1.4	8.9e-06
219344_at	NM_018344	*SLC29A3 (ENT3)*	solute carrier family 29, member 3	- 1.4	2.7e-05
203814_s_at	NM_000904	*NQO2*	NAD(P)H dehydrogenase, quinone 2	- 1.4	4.8e-05

Human genome nomenclature

* The fold change reflects the change in expression of genes after 8 hours prednisolone exposure compared to culture medium exposed control cells

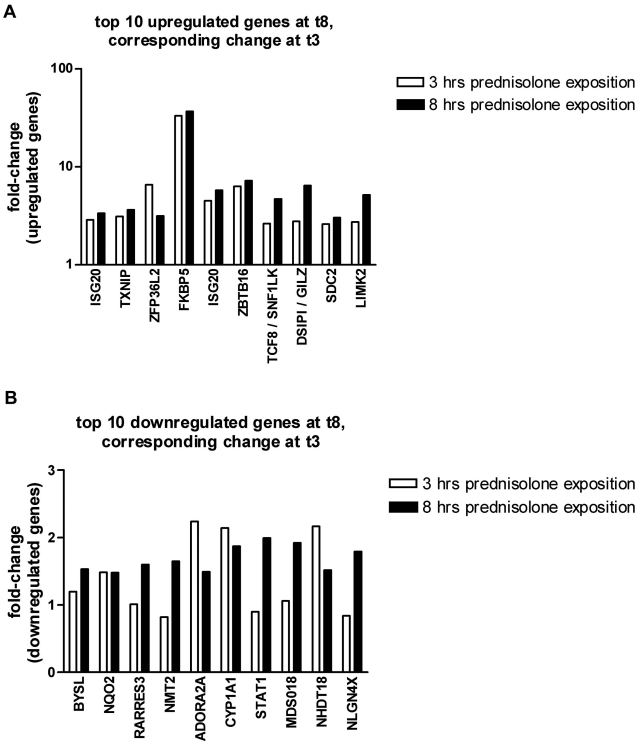


Figure 1. Comparison between three and eight hours on prednisolone-induced changes in gene expression in pediatric ALL
The fold up- (A) and down- (B) regulation of the top 10 probesets that were affected after 8 hours of prednisolone exposure are shown at both three (white bars) and eight (black bars) hours of exposure. The median fold change is given.

were identified at the $p < 0.0005$ and FDR < 10% level. However, 2 genes were differentially expressed with $p < 0.005$ and FDR < 20%, i.e. ZFP36L2 (*zinc finger protein 36, C3H type-like 2*) and DSIPI (*delta sleep inducing peptide, immunoreactor*, alias GILZ). Eight hours of prednisolone exposure revealed differential expression of 57 probesets (51 unique genes and 2 expressed sequence tags -ESTs) at $p < 0.0005$ and FDR < 10% level. As shown in Table 1, 44 probesets (39 genes) were upregulated (median 2.4-fold, 25th-75th percentile: 1.8 - 3.1 fold) and 13 probesets (12 genes) were downregulated (median 1.7-fold, 25th-75th percentile: 1.5 – 2 fold). Probeset 208078_s_at represented 2 different genes: *TCF8* and *SNF1LK*. Analysis at lower significance level ($p < 0.001$ and FDR < 20%) revealed that 144 probesets were differentially expressed.

As shown in Figure 1A the top 10 probesets that were found to be significantly upregulated at $p < 0.005$ and FDR < 10% level after eight hours of prednisolone

Table 2. Genes differentially expressed upon 8 hours prednisolone exposure in this study and previously reported in the literature.

gene	Child ALL (this study) Up- or down Regulation	Lymphoid cell lines (literature) Up- or down regulation	Cell line studied*	reference
ZFP36L2	Up	Up	697	11
FKBP5	Up	Up	697,Jurkat, CEM	9-11
LIMK2	Up	Up	CEM	38, 43
NFIL3	Up	Up	CEM	38, 43
ISG20	Up	Up	697	11
TXNIP	Up	Up	697, CEM	8, 10, 38, 43
C18ORF1	Up	Up	CEM	8, 43
BTEB1	Up	Up	697	10
DUSP1 (MKP-1)	Up	Down#	697	10
DSIPI (GILZ)	Up	Up	697, CEM	8, 10
BTG1	Up	Up	WEHI7.2, S49.A2, CEM, 697	11, 12, 38, 43
ZBTB16	Up	Up	CEM	8
TCF8 / SNF1LK	Up	Up	697	11
TUBA1	Up	Up	CEM	38, 43
DUSP10 (MKP-5)	Up	Down	697	10
PGK1	Up	Down	CEM	8
SGK	Up	Up	WEHI7.2, S49.A2	12
PRG1	Up	Up	CEM, 697	10, 38, 43
EIF2C2	Up	Down	CEM	38, 43
PFKP	Up	Down	WEHI7.2, S49.A2	12
BYSL	Down	Down	CEM	8, 43
SOCS1	Up	Up	CEM, 697	8, 11, 38, 43
	Child ALL (this study) Up- or down Regulation	Non-Leukemic cell lines (literature) Up- or down regulation		
MAP2K1	Up	Up	Human bone marrow stromal cells (TM5)	44
SDC2	Up	Up	Human glomerular epithelial cells	45
CEBPD	Up	Up	Different rat and rabbit cell types	46
GK	Up	Up	Rat adipocytes	47
STAT1	Down	Down	Human peripheral blood mononuclear cells	48
INSR	Up	Up	Human promonocytic cells	49
CYP1A1	Down	Up	Human aorta endothelial cells	50

*697: Human pre-B leukemia cell line
Jurkat: T-lineage leukemic cell line
CEM: T-lineage leukemic cell line
WEH17.2: T-cell lymphoma cell line
S49A2: T-cell lymphoma cell line
upregulation reported in non-leukemic cell lines[51]

exposure were also upregulated after three hours of prednisolone exposure albeit at a lower significance (p-value < 0.05). Seven of the ten most significantly downregulated genes at eight hours of prednisolone exposure were also downregulated after three hours of prednisolone exposure but a much lower significance ($p < 0.35$) (Figure 1B).

Table 2 shows a summary of literature on previously identified prednisolone-responsive genes in leukemic cell lines (23 genes) and non-leukemic cells (7 genes) that were also found in the present study to be affected after eight hours of prednisolone exposure in primary ALL cells. Five genes were found to be upregulated in our study but downregulated in array studies that included leukemic cell lines: *DUSP1*(although reported to be upregulated in mast cells and fibroblasts*), DUSP10*, *PGK1*, *EIF2C2* and *PFKP. CYP1A1* was found to be the most prominently downregulated gene in primary ALL cells. In contrast, this gene was found to be upregulated in human aorta endothelial cells.

Forty out of the 51 genes (57 probesets) differentially expressed upon prednisolone exposure had an annotation in the Gene Ontology database. The

Table 3. Biological function of prednisolone responsive genes in pediatric ALL.
The function of the different genes was studied in the literature (PUBMED search). Some genes have multiple functions, depending on cell type, stage in cell cycle.

Pro-apoptotic	Proliferation	Metabolism	Other	unknown
ADORA2A		***CYP1A1*** (drug metabolizing enzyme)	FKBP5	KCTD12
NFIL3		GM2A (glycosphingolipid metabolism)	LIMK2	C18orf1
ZBTB16		IRS2	MYH9	SDC2
STAT1		GK	*SLC29A3*	RNF38
ISG20	ZFP36L2	INSR (carbohydrate metabolism)	CEBPD	**BYSL**
TXNIP	MAP2K1	PGK1	KLF9	THEM43
BTG1	CEBPD	PFKP	**NQO2**	**NUDT18**
RARRES3	DUSP1 (MKP-1)	SGK	SPON2	
SOCS1	**NMT2**		CSNK1D	
DSIPI (GILZ)	RNF111		TCF8 / SNF1LK	
	DUSP10 (MKP-5)		**IGHG1**	
	TNFAIP3		TUBA1	
	SGK		**NLGN4X**	
	IMP-3		PRG1	
			EIF2C2	
			GABPB2	
			MRPS16	

Bold letters indicate genes that are downregulated upon glucocorticoid exposure

Table 4. Transcription factor binding motifs in prednisolone responsive genes.
Transcription factor binding motifs (TFBMs) over- / under-represented in 39 genes upregulated upon 8 hours prednisolone exposure.

Transcription factor	TBFM matrix	Fold difference*	*p*-value
Over-represented			
cAMP responsive element binding protein	V$CREB_Q2	2.8	0.0002
CRE-binding protein 1	V$CREBP1_Q2	2.8	0.0061
cAMP responsive element binding protein	V$CREB_02	2.5	7.8e-05
activator protein 2	V$AP2_Q6	2.5	6.9e-07
cAMP responsive element binding protein	V$CREB_01	2.5	0.0028
cAMP responsive element binding protein	V$CREB_Q4	2.2	0.0063
stimulating protein 1	V$SP1_Q6	2.1	5.5e-05
GC box elements	V$GC_01	1.9	0.0024
F1	V$MZF1_01	1.6	6.4e-05
stimulating protein 1	V$SP1_01	1.6	0.0037
activator protein 4	V$AP4_Q5	1.4	0.0055
Under-represented			
YY1 - yin and yang 1	V$YY1_01	- 2.5	0.0006
octamer factor 1	V$OCT1_03	- 2.0	0.0018
GATA binding factor 3	V$GATA3_01	- 1.4	0.0074
GATA binding factor 1	V$GATA1_01	- 1.3	0.0024
GATA binding factor 2	V$GATA2_01	- 1.3	0.0041
cap signal for transcription initiation	V$CAP_01	- 1.1	0.0009

* Fold difference in the frequency of specified TFBMs in upregulated genes compared to the frequency observed in genes that are not differentially expressed upon prednisolone exposure.

representation of these 40 probesets in each of the functional categories did not statistically differ from the total of U133A Gene Chip probesets that are annotated by Gene Ontology. Besides analysis through the Gene Ontology database, we also analyzed the literature for putative functions of the 51 genes. (Table 3) Among the 51 prednisolone responsive genes, two of the three most highly upregulated genes are the putative tumor suppressor genes *TXNIP* (*Thioredoxin interacting protein*, alias *VDUP1*, 3.7 fold) and *ZBTB1* (*Zinc finger and BTB domain containing 16*, alias *PLZF*, 8.8 fold). Besides these two cell-cycle involved tumor suppressor genes, ~50% of the prednisolone-responsive genes could be assigned to three major pathways, i.e. the MAPK pathways (9 genes), NF-κB signaling pathways of gene transcription (11 genes), and carbohydrate metabolism (5 genes).

Coordinated up- and downregulation of multiple genes upon prednisolone exposure may depend on the presence of specific transcription factor binding motifs (TFBMs) in the promoter regions of genes. Forty-five of the 51 genes were annotated in the TELiS database, of which 35 genes were upregulated and 10

genes were downregulated upon prednisolone exposure. Seventeen TFBMs were found to be over- or underrepresented in the upregulated genes compared to non-regulated genes (*p*-value < 0.01 and a FDR < 14%). Of these 17 TFBMs, 11 were over-represented and 6 were under-represented. (Table 4) cAMP responsive element binding protein (CREB) is the most often represented TFBM in the upregulated genes. The number of 12 downregulated genes was too small to allow for a meaningful analysis.

Although this study contained both *in vitro* prednisolone sensitive and resistant cases, the sample size in each subgroup was too small for a statistically relevant analysis.

DISCUSSION

Despite the clinical importance of glucocorticoids in the treatment of ALL, the genes which are transcriptionally regulated upon glucocorticoid exposure in pediatric ALL and the specific (in)activation of pathways leading to glucocorticoid induced apoptosis are unknown.

In the present study, 3 hours of prednisolone exposure did not sufficiently alter the level of gene expression to be able to (statistically) discriminate prednisolone responsive genes in pediatric ALL. In contrast to these primary cells, in leukemic cell lines significant changes in gene expression can be observed already after 3 hours of prednisolone exposure.[8, 9] The same phenomenon that leukemic cell lines respond faster to a drug than corresponding primary cells was found for L-asparaginase.[21] Besides the faster response, also different genes were found to be affected by these drugs in the leukemic cell lines. These studies emphasize the fact that leukemic cell lines behave differently to drugs than primary cells and hence may not be suitable models for pharmacodynamic studies.

Exposure of pediatric ALL cells for eight hours to prednisolone affected the expression of 57 probesets (51 genes); 44 probesets were upregulated and 13 probesets were downregulated. *FKBP5 (FK506 binding protein 5*) was the most significantly upregulated gene (35-fold). Its product, FKBP-51, functions as co-chaperone molecule of the glucocorticoid receptor that affects the transport of the glucocorticoid receptor into the nucleus. The expression of this gene has been reported before to be highly glucocorticoid-inducable in cell lines and primary patient's cells.[13, 22, 23] The most significantly downregulated gene was *CYP1A1* (2-fold) which is involved in drug metabolism and detoxification. Although we found

only 57 probesets significantly regulated upon 8 hours prednisolone exposition, more prednisolone-responsive genes can be identified if less stringent cut-off levels are used for the p-value and FDR. For example, at $p < 0.001$ and FDR < 20% 144 probesets are regulated upon 8 hours prednisolone exposure.

Interestingly, a gene expression profiling study has been published recently in which glucocorticoid-responsive genes were studied in both leukemic cell lines and primary cells of pediatric ALL.[13] In that study other statistical considerations were made than in our study, such as a minimal change in gene expression of 1.6-fold in at least 6 our of 13 studied patients. Despite differences in methodology, five out of 28 identified responsive genes found in patients that were *in vivo* treated with prednisone (*FKBP5*, *SOCS1*, *ZFP36L2*, *SNF1LK* and *ZBTB16*) were also found in our study using *in vitro* exposed leukemic cells of children with ALL.

Signal transduction pathways possibly involved in glucocorticoid induced apoptosis

TXNIP / ZBTB16. Both *TXNIP* and *ZBTB16* were found to be upregulated following 8 hours of prednisolone exposure in the present study. TXNIP has recently been described as a tumor suppressor protein that induces a cell cycle arrest upon formation of a transcriptional repressor complex with ZBTB16.[24] TXNIP prevents thioredoxin-mediated apoptosis signal-regulating kinase 1 (ASK1) ubiquitination and degradation[25, 26], and inhibits the thioredoxin radical scavenging function.[27] Thereby, TXNIP can act as a pro-apoptotic regulator. These data support a role for these genes in the induction of apoptosis upon prednisolone exposure in childhood ALL, as has been suggested recently by Wang et al..[28]

MAP kinase pathways. In the group of 51 prednisolone-regulated genes, 9 genes were associated with the three mitogen-activated protein (MAP) kinase pathways, i.e. ERK, JNK and p38 MAPK. These MAP kinase pathways are involved in cell survival (ERK and JNK) and cell death (p38 MAPK) and have been reported to play critical roles in the pathogenesis of various hematological malignancies.[29, 30] Miller et al. recently showed that pharmacological inhibition of ERK and JNK enhanced glucocorticoid-induced apoptosis, whereas inhibition of p38 MAPK activity opposed glucocorticoid-induced apoptosis in lymphoid cells.[31] Four genes (*DUSP1, DUSP10, DSIPI* (alias *GILZ*) and *SGK*) that we found to be induced upon prednisolone exposure are negative regulators of the MAP kinase pathways. In the literature, overexpression of *DSIPI* was shown to promote apoptosis in

thymocytes.[32] Overexpression of DUSP1 in a precursor-B ALL cell line did not alter glucocorticoid sensitivity[33], suggesting that DUSP1 may not be essential for mediating the toxic effect of glucocorticoid in ALL cells. *SGK* is related to the PI3K / AKT pathway (which is linked to the ERK pathway) and has been reported to be a survival molecule.[34]

Since we found both positive and negative regulators of the MAPK pathways among the prednisolone regulated genes, the net effect of these genes on cell survival needs to be addressed in functional studies in childhood ALL.

NF-κB. There are two classically proposed ways of an inhibitory effect of glucocorticoids on NF-κB function. First, the glucocorticoid-GR complex may interact with NF-κB directly, thereby opposing its function. Secondly, glucocorticoids upregulate IκBα (inhibitor of NF-κB α), which negatively regulates NF-κB. However, we did not find a significant upregulation of *IκBα* in our study (p = 0.002, FDR 30%). Other postulated mechanisms of glucocorticoid mediated inhibition of NF-κB are the glucocorticoid induced upregulation of DSIPI and TNFAIP3.[35, 36] Both genes were identified in our study as prednisolone-responsive genes (3.35 fold and 2.12 fold respectively, $p < 0.0005$ and FDR < 10%). This observation implies that the last two mechanisms might be more relevant for NF-κB inhibition (and hence induction of apoptosis) than the two classically proposed models in childhood ALL.

Carbohydrate metabolism. Five genes directly involved in carbohydrate metabolism are upregulated upon prednisolone exposure: *IRS2*, *INSR*, *PFKP*, *GK* and *PGK1*. Activity of these 5 genes results in higher ATP levels in the cell due to a higher glucose uptake of the cell (*IRS2* and *INSR*), a higher rate of glyconeogenesis (*GK*) and glycolysis (*PFKP* and *PGK1*). Interestingly, in an earlier study looking for the baseline expression of genes determining glucocorticoid resistance in primary, untreated ALL cells, we found *GLUT3* and *GAPDH*, two genes involved in carbohydrate metabolism to be overexpressed in prednisolone resistant ALL cells.[16] Moreover, the glycolytic rate of prednisolone resistant leukemic cell lines was higher as compared to sensitive cell lines and inhibition of glycolysis by 2-deoxy-D-glucose sensitized resistant cells to prednisolone whereas no effect on sensitive cells was found. (Holleman, manuscript in preparation) Taken together, these studies strongly suggest an important role for carbohydrate metabolism in glucocorticoid induced apoptosis.

Transcription factor binding motifs. The TELiS database[20] was used to study which TFBMs were over- or under-represented in the 39 upregulated genes. (Table 4)

Four TFBMs representing cAMP responsive element binding protein (CREB) were overrepresented in the promoter regions of the 35 upregulated genes as compared to non-regulated genes. CREB is a ubiquitous transcription factor involved in cell proliferation and survival. Interestingly, the interaction between glucocorticoids and CREB has been reported before.[37] Forskolin was shown to increase cellular cAMP levels and to promote the phosphorylation of CREB. In combination with dexamethasone, forskolin synergistically induced apoptosis in the glucocorticoid resistant CEM-C1 lymphoid cell line, suggesting a role for CREB in glucocorticoid response. Surprisingly, the glucocorticoid responsive element (GRE) sequence was not over-represented in the promoter regions of the 35 upregulated genes, which is in line with a previous report on glucocorticoid induced genes in three ALL cell lines.[38] However, at least three out of the 51 genes found to be regulated upon prednisolone exposure, contain GREs in the promoter regions, namely *FKBP5*[23, 39], *DSIPI*[40] and *SGK*.[41, 42] The fact that the GRE TFBMs were not overexpressed in the upregulated genes in our study, might be the result of the delicate positioning of GRE-like sequences in glucocorticoid responding genes, that are not recognized in the TELiS database. Another explanation might be that some genes are regulated "directly" by glucocorticoids binding to their GREs, whereas other genes are regulated more "indirectly" by other transcription factors, which in turn are regulated by glucocorticoids.

In conclusion, we found 51 prednisolone responsive genes in leukemic cells taken from children at initial diagnosis of ALL. Further functional research may identify which genes/pathways are essential for the glucocorticoid-responsiveness of cells. Out of the 51 identified glucocorticoid-responsive genes, 50% can be linked to 3 pathways, i.e. cell proliferation and survival, NF-κB signalling and glucose metabolism. Two of the upregulated genes are tumorsuppressor genes: *TXNIP* and *ZBTB16*, which is possibly related to the induction of apoptosis by glucocorticoids. Knowledge on the pathways leading to glucocorticoid induced apoptosis is essential to develop more targeted therapy and ways to modulate glucocorticoid resistance in pediatric ALL

References

1. Lauten M, Stanulla M, Zimmermann M, Welte K, Riehm H, Schrappe M. Clinical outcome of patients with childhood acute lymphoblastic leukaemia and an initial leukaemic blood blast count of less than 1000 per microliter. Klin Padiatr 2001;213:169-74.
2. Kaspers GJ, Pieters R, Van Zantwijk CH, Van Wering ER, Van Der Does-Van Den Berg A, Veerman AJ. Prednisolone resistance in childhood acute lymphoblastic leukemia: vitro-vivo correlations and cross-resistance to other drugs. Blood 1998;92:259-66.
3. Hongo T, Yajima S, Sakurai M, Horikoshi Y, Hanada R. *In vitro* drug sensitivity testing can predict induction failure and early relapse of childhood acute lymphoblastic leukemia. Blood 1997;89:2959-65.
4. Tsai SY, Carlstedt-Duke J, Weigel NL, et al. Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell 1988;55:361-9.
5. De Bosscher K, Vanden Berghe W, Haegeman G. The interplay between the glucocorticoid receptor and nuclear factor-kappaB or activator protein-1: molecular mechanisms for gene repression. Endocr Rev 2003;24:488-522.
6. Tissing WJ, Meijerink JP, den Boer ML, Pieters R. Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia 2003;17:17-25.
7. Schmidt S, Rainer J, Ploner C, Presul E, Riml S, Kofler R. Glucocorticoid-induced apoptosis and glucocorticoid resistance: molecular mechanisms and clinical relevance. Cell Death Differ 2004;11 Suppl 1:S45-55.
8. Tonko M, Ausserlechner MJ, Bernhard D, Helmberg A, Kofler R. Gene expression profiles of proliferating vs. G1/G0 arrested human leukemia cells suggest a mechanism for glucocorticoid-induced apoptosis. Faseb J 2001;15:693-9.
9. Obexer P, Certa U, Kofler R, Helmberg A. Expression profiling of glucocorticoid-treated T-ALL cell lines: rapid repression of multiple genes involved in RNA-, protein- and nucleotide synthesis. Oncogene 2001;20:4324-36.
10. Planey SL, Abrams MT, Robertson NM, Litwack G. Role of apical caspases and glucocorticoid-regulated genes in glucocorticoid-induced apoptosis of pre-B leukemic cells. Cancer Res 2003;63:172-8.
11. Yoshida NL, Miyashita T, U M, et al. Analysis of gene expression patterns during glucocorticoid-induced apoptosis using oligonucleotide arrays. Biochem Biophys Res Commun 2002;293:1254-61.
12. Wang Z, Malone MH, He H, McColl KS, Distelhorst CW. Microarray analysis uncovers the induction of the proapoptotic BH3-only protein Bim in multiple models of glucocorticoid-induced apoptosis. J Biol Chem 2003;278:23861-7.
13. Schmidt S, Rainer J, Riml S, et al. Identification of glucocorticoid-responsive genes in children with acute lymphoblastic leukemia. Blood 2005;epub ahead of print.
14. Stam RW, den Boer ML, Meijerink JP, et al. Differential mRNA expression of Ara-C-metabolizing enzymes explains Ara-C sensitivity in MLL gene-rearranged infant acute lymphoblastic leukemia. Blood 2003;101:1270-6.
15. Armstrong SA, Staunton JE, Silverman LB, et al. MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. Nat Genet 2002;30:41-7.
16. Holleman A, Cheok MH, den Boer ML, et al. Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med 2004;351:533-42.
17. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 2002;18 Suppl 1:S96-104.

18. Benjamini Y, Hochberg Y. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J Roy Stat Soc B 1995;57:289-300.
19. R development core team. R: a language and environment for statistical computing. R foundation for statistical computing, Vienna, Austria 2005: http:www.r-project.org.
20. Cole SW, Yan W, Galic Z, Arevalo J, Zack JA. Expression-based monitoring of transcription factor activity: the TELiS database. Bioinformatics 2005;21:803-10.
21. Fine BM, Kaspers GJ, Ho M, Loonen AH, Boxer LM. A genome-wide view of the *in vitro* response to L-asparaginase in acute lymphoblastic leukemia. Cancer Res 2005;65:291-9.
22. Vermeer H, Hendriks-Stegeman BI, van der Burg B, van Buul-Offers SC, Jansen M. Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. J Clin Endocrinol Metab 2003;88:277-84.
23. U M, Shen L, Oshida T, Miyauchi J, Yamada M, Miyashita T. Identification of novel direct transcriptional targets of glucocorticoid receptor. Leukemia 2004;18:1850-6.
24. Han SH, Jeon JH, Ju HR, et al. VDUP1 upregulated by TGF-beta1 and 1,25-dihydroxyvitamin D3 inhibits tumor cell growth by blocking cell-cycle progression. Oncogene 2003;22:4035-46.
25. Liu Y, Min W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. Circ Res 2002;90:1259-66.
26. Junn E, Han SH, Im JY, et al. Vitamin D3 up-regulated protein 1 mediates oxidative strss via suppressing the thioredoxin function. J. Immunol 2000;164:6287-95.
27. Schulze PC, Yoshioka J, Takahashi T, He Z, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. J Biol Chem 2004;279:30369-74.
28. Wang Z, Rong YP, Malone MH, Davis MC, Zhong F, Distelhorst CW. Thioredoxin-interacting protein (*txnip*) is a glucocorticoid-regulated primary response gene involved in mediating glucocorticoid-induced apoptosis. Oncogene (epub ahead of print) 2005.
29. Platanias LC. Map kinase signaling pathways and hematologic malignancies. Blood 2003;101:4667-79.
30. Ravandi F, Talpaz M, Kantarjian H, Estrov Z. Cellular signaling pathways: new targets in leukaemia therapy. Br J Haematol 2002;116:57-77.
31. Miller AL, Webb MS, Copik AJ, et al. p38 MAP Kinase is a key mediator in glucocorticoid-induced apoptosis of lymphoid cells: correlation between p38 MAPK activation and site-specific phosphorylation of the human glucocorticoid receptor at serine 211. Mol Endocrinol 2005;19:1569-83.
32. Delfino DV, Agostini M, Spinicelli S, Vito P, Riccardi C. Decrease of Bcl-xL and augmentation of thymoctye apoptosis in GILZ overexpressing transgenic mice. Blood 2004;104:4134-41.
33. Abrahams MT, Robertson NM, Litwack G, Wickstrom E. Evaluation of glucocorticoid sensitivity in 697 pre-B acute lymphoblastic leukemia cells after overexpression or silencing of MAP kinase phosphatase-1. J Cancer Res Clin Oncol 2005;131:347-54.
34. Mikosz CA, Brickley DR, Sharkey MS, Moran TW, Conzen SD. Glucocorticoid receptor-mediated protection from apoptosis is associted with induction of the serine/threonine survival kinase gene, sgk-1. J Biol Chem 2001;276:16649-54.
35. Ayroldi E, Migliorati G, Bruscoli S, et al. Modulation of T-cell activation by the glucocorticoid-induced leucine zipper factor via inhibition of nuclear factor kappaB. Blood 2001;98:743-53.
36. Chen F. Endogenous inhibitors of nuclear factor-kappaB, an opportunity for cancer control. Cancer Res 2004;64:8135-8.

37. Medh RD, Saeed MF, Johnson BH, Thompson EB. Resistance of human leukemic CEM-C1 cells is overcome by synergism between glucocorticoid and protein kinase A pathways: correlation with c-Myc suppression. Cancer Res 1998;58:3684-93.
38. Medh RD, Webb MS, Miller AL, et al. Gene expression profile of human lymphoid CEM cells sensitive and resistant to glucocorticoid-evoked apoptosis. Genomics 2003;81:543-55.
39. Hubler TR, Scammell JG. Intronic hormone response elements mediate regulation of FKBP5 by progestins and glucocorticoids. Cell Stress Chaperones 2004;9:243-52.
40. Asselin-Labat ML, David M, Biola-Vidamment A, et al. GILZ, a new target for the transcription factor FoxO3, protects T lymphocytes from interleukin-2 withdrawal-induced apoptosis. Blood 2004;104:215-23.
41. Naray-Fejes-Toth A, Fejes-Toth G, Volk KA, Stokes JB. SGK is a primary glucocorticoid-induced gene in the human. J Steroid Biochem Mol Biol 2000;75:51-6.
42. Leong ML, Maiyar AC, Kim B, O'Keeffe BA, Firestone GL. Expression of the serum- and glucocorticoid-inducible protein kinase, SGK, is a cell survival response to multiple types of environmental stress stimuli in mammary epithelial cells. J Biol Chem 2003;278:5871-82.
43. Webb MS, Miller AL, Johnson BH, et al. Gene networks in glucocorticoid-evoked apoptosis of leukemic cells. J Steroid Biochem Mol Biol 2003;85:183-93.
44. Jeon JW, Lee SJ, Kim JB, et al. Cellular proliferative effect of dexamethasone in immortalized trabecular meshwork cell (TM5) line. Yonsei Med J 2003;44:299-306.
45. Kasinath BS, Singh AK, Kanwar YS, Lewis EJ. Dexamethasone increases heparan sulfate proteoglycan core protein content of glomerular epithelial cells. J Lab Clin Med 1990;115:196-202.
46. Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 2002;365:561-75.
47. Taylor WM, Goldrick RB, Ishikawa T. Glycerokinase in rat and human adipose tissue: response to hormonal and dietary stimuli. Horm Metab Res 1979;11:280-4.
48. Hu X, Li WP, Meng C, Ivashkiv LB. Inhibition of IFN-gamma signaling by glucocorticoids. J Immunol 2003;170:4833-9.
49. Leal MA, Aller P, Calle C. Effect of dexamethasone on insulin receptor mRNA levels, RNA stability and isotype RNA pattern in U-937 human promonocytic cells. J Endocrinol Invest 1996;19:530-4.
50. Celander M, Weisbrod R, Stegeman JJ. Glucocorticoid potentiation of cytochrome p4501A1 induction by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in porcine and human endothelial cells in culture. Biochem Biophys Res Commun 1997;232:749-53.
51. Hermoso MA, Matsuguchi T, Smoak K, Cidlowski JA. Glucocorticoids and tumor necrosis factor alpha cooperatively regulate toll-like receptor 2 gene expression. Mol Cell Biol 2004;24:4743-56.

Chapter 8

Summary, discussion and perspectives

SUMMARY

Children who are diagnosed with acute lymphoblastic leukemia (ALL) have a relatively good prognosis: the event-free survival in most current treatment protocols is about 75-80%. Both an *in vivo* and *in vitro* poor response to glucocorticoids (GCs) like prednisone and dexamethasone (i.e. GC resistance) are important adverse risk factors related to a higher risk of relapse and unfavorable overall survival. However, little is known about mechanisms of GC resistance in childhood ALL. Knowledge of these mechanisms may lead to renewed insights how to overcome GC resistance. Secondly, knowledge about the underlying causes of GC resistance may indicate which genes may be targeted using new therapies, thereby possibly increasing survival and decreasing the many side effects of GC treatment. The aim of the present study was to investigate possible causes of GC resistance in children with ALL.

Chapter 2 reviews the current knowledge about mechanisms of GC resistance in childhood ALL. To induce apoptosis, GCs have to bind to the intracellular GC receptor (GR). One of the possible mechanisms for GC resistance is the presence of genetic mutations or polymorphisms in the *GR* gene that affect the function of the GR. Studies in a healthy population and in patients with a GC resistance syndrome have shown that specific polymorphisms in the *GR* gene (e.g. N363S, *Bcl1*) lead to increased sensitivity to GCs, whereas other polymorphisms and mutations in the *GR* gene (e.g. ER22/23EK) lead to decreased GC sensitivity.[1-3] No data are available on the relationship between genetic variants in the *GR* gene and resistance to GCs in childhood ALL. In **Chapter 3** we addressed this issue in primary cells of patients at initial diagnosis of ALL. The coding region of the *GR* gene was screened for nucleotide variations in leukemic cells of 57 children with ALL. Besides six known polymorphisms, no mutations or new polymorphism in the *GR* gene were found. None of the polymorphisms in the coding region of the *GR* gene was linked to an unfavorable *in vivo* or *in vitro* response to prednisone. We therefore conclude that polymorphisms or mutations in the *GR* gene are no major contributors to GC resistance in childhood ALL.

Another possible explanation for GC resistance in childhood ALL is an altered expression of the full length GR or the expression of specific 3' splice variants of the GR. Two decades ago, the ligand binding capacity of the GR was found to be related to GC resistance,[4, 5] although this could not always be confirmed.[6] In **chapter 4** we examined the relationship between the base-line expression levels

of the GR and the three 3′ splice variants GR-alpha, GR-beta and GR-P and *in vitro* prednisolone resistance in leukemic cells taken at diagnosis from 54 children with ALL. A lower absolute expression level of the GR, but not the relative expression levels of the GR-alpha, GR-beta and GR-P splice variants, was associated with *in vitro* prednisolone resistance. Although the relationship between a low total GR expression and *in vitro* prednisolone resistance was statistically significant, the interindividual differences in base-line expression of the GC receptor were rather small (~2-fold) compared to the > 1000-fold variation in level of resistance to prednisolone. Therefore, it seems likely that other mechanisms exist that may have a more pronounced contribution to GC resistance in pediatric ALL.

It is known that the expression of the GR in leukemic cell lines can be induced by exposure to GCs, which may be necessary to induce apoptosis in ALL cells.[7] Therefore, in addition to the base-line expression level of the GR, GC resistance may be caused by the inability of cells to upregulate the expression of the GR upon GC exposure. Since the *GR* gene contains three different promoter regions of which one seems to be controlled by the GR itself, we studied the relationship between the expression of the different promoter-encoded GR variants and GC resistance in **chapter 5**. The expression levels of the 5 different 5′ GR promoter transcripts (*1A1*, *1A2*, *1A3*, *1B* and *1C*) both at base-line and after eight hours of prednisolone exposure appeared not to be related to *in vitro* prednisolone resistance in leukemic samples of children with ALL. Next, we tested the hypothesis that *in vitro* prednisolone resistant pediatric ALL cells were defective in upregulating *GR* levels upon *in vitro* GC exposure in contrast to leukemic cells from *in vitro* sensitive patients. Opposite to what is found in tissues in which GC exposure does not induce apoptosis, we showed a significant upregulation of *GR* mRNA expression (and of the three 3′ splice variants *GR-alpha*, *GR-beta* and *GR-P*) after 8 hours of *in vitro* prednisolone exposure in pediatric ALL cells derived from children. However, the level of upregulation of total *GR* and the three 3′ *GR* splice variants did not differ between *in vitro* GC sensitive and resistant patients. Therefore we conclude that the degree of regulation of GR expression upon prednisone exposure does not contribute to GC resistance in childhood ALL.

The studies as mentioned above showed that resistance to GCs can not be explained by abnormalities in the GR itself. We next investigated the expression levels of the (co)chaperone molecules, which the GR needs to be in the correct configuration to bind GCs and for the subsequent nuclear transportation. (**chapter 6)** The mRNA expression levels of the (co)chaperone molecules in leukemic cells taken at initial

diagnosis from 40 children with ALL were measured using quantitative real-time RT-PCR. No correlation was found between the expression levels of *HSP-70*, *HSP-40*, *HIP*, *BAG-1*, *HOP*, *HSP-90*, *P-23*, *FKBP-51*, *FKBP-52* and *CYP-40* and *in vitro* prednisolone resistance. However, the underlying cause of GC resistance might be the inability of the GR and the different (co)chaperone molecules to form a complex, rather than the base-line mRNA expression levels of these molecules. It is difficult to study the formation of GR-(co)chaperone complexes since the binding and release of different components is a highly dynamic process. Besides base-line levels, also GC induced changes in the expression levels of co-chaperone molecules occur, which further complicates the study of functional GR complexes. It has previously been shown that the increase of FKBP-51 levels upon GC exposure was correlated to GC sensitivity in non-leukemic cells.[8] However in **chapter 7** we demonstrate that *FKBP-51* is upregulated upon GC exposure in both GC sensitive and resistant ALL cells.

Since genetic aberrations and expression levels of the GR or its (co)chaperone molecules can not explain GC resistance in childhood ALL, we explored the signal transduction pathways that may be activated or repressed by GCs in ALL cells. **Chapter 7** describes the results of a micro-array analysis of GC-responsive genes in childhood ALL. After exposing ALL cells of children *in vitro* to prednisolone for 3 hours, no genes were differentially expressed compared to cells exposed to culture medium without prednisolone. However, after 8 hours of prednisolone exposure, 51 genes were differentially expressed ($p<0.05$, FDR<10%) of which 39 genes were upregulated (median 2.4-fold) and 12 genes were downregulated (median 1.7-fold). Twenty-one genes were not previously described as GC regulated genes. Two tumor suppressor genes, *thioredoxin interacting protein* (*TXNIP*) and *zinc finger and BTB domain containing 16* (*ZBTB16*), were 3.7-fold and 8.8-fold upregulated respectively. Genes were functionally categorized in three major routes: i.e. MAPK pathways (9 genes), NF-κB signaling (11 genes) and carbohydrate metabolism (5 genes). This study points to pathways that are used by GCs to trigger apoptosis in ALL cells. Future research is needed to functionally explore these pathways and to study whether abnormalities occur in these pathways that explain GC resistance in childhood ALL.

DISCUSSION AND FUTURE PERSPECTIVES

N-terminal GR splice variants

Based on the studies described in the **chapters 3 to 6**, we concluded that abnormalities in the *GR* and associated proteins have only limited effect on the level of GC resistance in childhood ALL. However, more recently, new N-terminal splice variants of *GR-alpha* have been described that are produced via translational regulatory processes.[9] The level of expression of these splice variants differ significantly among tissues and since each splice variant regulates both a common and a unique set of genes in the same cell, it was proposed that N-terminal splice variants generate specificity in GC-responsive gene transcription in different tissues. It is of interest to determine whether these splice variants contribute to GC induced apoptosis in ALL and whether an aberrant expression is linked to GC resistance.

Posttranslational modification

In the **chapters 4 and 5**, we observed that GR mRNA and protein levels were correlated in leukemic cell lines but at present it is unknown whether this is also true for primary patient samples. Posttranslational modifications might be important for the actual GR protein levels in the leukemic cell. In COS-1 cells expressing mouse GR, ubiquitination and proteasomal degradation of the GR was shown.[10] A proteasome inhibitor effectively blocked GR protein down regulation. These cell line data suggest a role for the ubiquitin-proteasome pathway in regulating GR protein turnover. Another mechanism possibly regulating GR protein stability as well as its transcriptional activity is sumoylation. Sumoylation occurs through an interaction between GR and small ubiquitin-related modifier-1 (SUMO-1). This may lead to specific degradation of the GR protein, but may also stimulate its transactivation capacity. It is hypothesized that sumoylation of the GR protein may regulate GR activity.[11] It remains to be studied whether ubiquitination, proteasomal degradation and/or sumoylation are relevant for posttranscriptional regulation of the GR protein and whether GR protein levels are related to GC resistance in childhood ALL.

TXNIP / ZBTB16

In the study as described in **chapter 7**, we found *TXNIP* and *ZBTB16* to be upregulated upon GC exposure. These two tumor suppressor genes[12] are interesting

candidates for the way GCs trigger the apoptotic pathway in childhood ALL. TXNIP may induce apoptosis by the repression of thioredoxin (TXN). TXN is a scavenger of ROS (reactive oxygen species), thereby preventing ROS induced cell death.[13] Secondly, TXN represses ASK1 (apoptosis signal-regulating kinase 1, MAP3K5), thereby preventing apoptosis.[14] To address the contribution of these genes to GC-induced apoptosis, one may study the effect of GC exposure of leukemic cell lines in which TXNIP and / or ZBTB16 have been silenced.

MAPK pathways

In the study as described in **chapter 7**, we found nine GC-responsive genes that are involved in one or more of the mitogen activated protein kinase (MAPK) pathways. The proteins encoded by these genes are both positive and negative regulators of these MAPK pathways, but the net effect on proliferation is yet unclear. If the net effect is anti-proliferative, these genes may contribute to the induction of apoptosis in ALL. If the net effect is pro-proliferative, these genes contribute to cell survival and cell growth responses, which have been ascribed to these pathways as well.[15, 16] Since the MAPK pathways are complicated networks, it is difficult to study these pathways in detail. The best way to study the genes regulated upon GC exposure, is to silence these single genes by RNAi and analyze the functional effect. Alternatively, selective blocking agents (e.g. RO-318220 as a blocker of DUSP-1 / MKP-1[17]) may be used. If this decreases GC sensitivity, the blocked pathway might be relevant for GC induced apoptosis.

FLT3

Besides the top 51 of GC-responsive genes, also other genes are regulated upon GC exposure, but at a lower significance level than used to select the genes as described in **chapter 7**. One of these genes is FLT3 (92nd most significantly regulated gene, p=0.0004 and FDR=16%), which is upregulated in both sensitive and resistant leukemic cells. This gene is of special interest since the activity of FLT3 may be inhibited by tyrosine-kinase inhibitors such as PKC 412 and CEP-701. These tyrosine kinase inhibitors were shown to be effective in FLT3 mutated AML[18] and FLT3 overexpressing infant ALL.[19] If GCs upregulate FLT3 expression, GCs might be used to "sensitize" leukemic cells to FLT3-inhibitors.

Carbohydrate metabolism

Analysis of gene expression profiles before and after GC exposure identified five

genes that were linked to carbohydrate metabolism (**chapter 7**). Upregulation of these genes may lead to an increased ATP production in the cell. In other tissues, GCs were also shown to upregulate metabolic genes involved in stress responses that require rapid and intense physical exertions.[20] Therefore we conclude that these genes may not be involved in GC-mediated apoptosis. On the other hand, GC-resistance has been related to increased glycolysis.[21] Our group recently showed that GC resistance correlates with a higher glycolytic rate in ALL cell lines.[22] The glycolysis inhibitor 2-deoxy-D-glucose (2DG) selectively sensitized GC resistant, but not GC sensitive cell lines to GC induced apoptosis. If the degree of carbohydrate metabolism is indeed related to GC resistance in childhood ALL, strategies to circumvent this resistance may be developed. The increased carbohydrate metabolism might be antagonized with for example 2DG or TH-070.[23] If our hypothesis might prove to be valid, these two compounds are especially interesting since they are already being tested in phase I/II studies for other malignancies.

Selective GC agonists and antagonists

The last few years interesting compounds have become available which can be used in the search for the pathways of GC induced apoptosis and resistance mechanisms in childhood ALL. Some of these new compounds demonstrate equipotent anti-inflammatory effects as compared to GCs, but with reduced side effects. One of these selective GC agonists, ZK216348, preferentially induces transrepression with little or no transactivating activity in a murine model of skin inflammation.[24] This compound may be used to study which pathway of gene transcription (transrepression or transactivation) is mainly used in childhood ALL cells. Secondly, if these compounds induce apoptosis in ALL cells *in vitro*, this might warrant further clinical evaluation in children with ALL.

Besides GC **agonists**, novel GR-alpha selective non-steroidal **antagonists** (NSGCAs) have been developed. Some agents have already been shown to antagonize the induction of glucose and glutamine metabolism, whereas other genes (e.g. pro-inflammatory genes) were not affected.[25] If these compounds do not disturb GC induced apoptosis in ALL cells, they are of interest for two reasons. In the first place, these agents might decrease important side effects of GC treatment, like insulin resistance. Secondly, these agents might be used to sensitize resistant cells to GCs. Since we observed that GC-resistance is linked to an increased glycolytic rate in ALL cell lines[22], it might be worthwhile to combine

(De-)acetylation of promoter regions

Acetylation of lysine residues of histones by histone acetyl transferase (HAT) coactivators is one of the proposed mechanisms that result in an "open-state" of the chromatine, thereby facilitating access of transcription factors to bind to target genes. On the other hand, histone deacetylases (HDACs) silence gene transcription by preventing access to promoter regions.[26] Recently, SMAD6 was shown to silence GC-GR mediated transactivation together with HDAC3.[27] The promoter region of GC responsive genes becomes deacetylated which results in decreased activity and thus decreased transactivation upon GC exposure. It has been hypothesized that Smad6 functions as a regulator of GC action.[27] If SMAD6 suppresses GC induced apoptosis, this suggests an important role for the transactivating properties of GCs whereas in case of no effect the transrepression function via the inhibition of transcription factors like NF-κB and AP-1 might be more important for GC induced apoptosis in leukemic cells. Secondly, if increased SMAD6 expression will have a negative effect on GC induced apoptosis, it will be relevant to study the biological function of SMAD6 in ALL cells.

If the induction of apoptosis depends on transactivation of GC responsive genes, it is worthwhile to study the effect of HDAC inhibitors such as valproic acid.[28] Another HDAC inhibitor (depsipeptide / FK228) has been shown in our laboratory to have a cytotoxic effect in precursor B-ALL cells[29], but its effect on the induction of apoptosis by GCs awaits further studies.

Side effects of GC treatment

There is a high interindividual variability in the occurrence of side effects upon prednisone / dexamethasone treatment in children with ALL.[30] One of the possible reasons for this are polymorphisms in the GR as described in **chapter 3.** Using a dexamethasone suppression test, two polymorphisms (N363S and *Bcl1*) appeared to be related to increased sensitivity to GCs and one polymorphism (ER22/23EK) to decreased GC sensitivity in a healthy population.[1-3] We showed that these polymorphisms were not related to GC sensitivity of ALL cells. (**chapter 3**) However, these polymorphisms might affect the GC-responsiveness of normal tissues in the patient and may contribute to the occurrence of side-effects of GC treatment such as insulin resistance, increased body mass index, hypertension etcetera.[3, 31] To investigate the relationship between GR polymorphisms and side

effects of treatment we started a prospective national study in children who are treated with the current DCOG treatment protocol ALL-10.

One of the complications of GC treatment is the development of insulin resistance, sometimes leading to overt diabetes mellitus and the need of insulin therapy. Three insulin related genes are among the 51 significantly GC regulated genes as described in **chapter 7**. *INSR* (*insulin receptor*), *IRS2* (*insulin receptor substance-2*), but also *SOCS1* (*suppressor of cytokine signaling 1*) are upregulated. This last gene is reported to stimulate the ubiquitin-mediated degradation of IRS, leading to glucose intolerance.[32] We speculate that the balance between *SOCS1* and *IRS2*/*INSR* expression determines the level of glucose intolerance. Future studies should address this, clinically very relevant, side effect.

In conclusion, in this thesis we have shown that the main cause of GC resistance in childhood ALL is located downstream of GC binding to the GR. Although we have shown that the GR level is significantly 2-fold lower in GR resistant compared to GC sensitive cells, this does not explain the more than 1000-fold interindividual difference in resistance to prednisolone among children with ALL. Other possible causes of GC resistance related to the GR (polymorphisms within the GR gene, (co)chaperone molecules and GR regulation upon GC exposure) have been excluded. A gene expression array study revealed several novel GC-responsive genes. Further research needs to explore the functional pathways of GC induced apoptosis and GC resistance in childhood ALL which is necessary to find ways to circumvent GC resistance, that is a major adverse risk factor in childhood ALL. Secondly, the knowledge obtained by exploring GC mechanisms of action will lead to more specific, targeted GC therapy. This targeted therapy should have at least the same antileukemic efficacy, but reduced side effects than the currently used drugs like prednisone and dexamethasone.

References

1. van Rossum EF, Koper JW, Huizenga NA*, et al.* A polymorphism in the glucocorticoid receptor gene, which decreases sensitivity to glucocorticoids in vivo, is associated with low insulin and cholesterol levels. Diabetes 2002;51:3128-34.
2. Huizenga NA, Koper JW, De Lange P*, et al.* A polymorphism in the glucocorticoid receptor gene may be associated with and increased sensitivity to glucocorticoids in vivo. J Clin Endocrinol Metab 1998;83:144-51.
3. Van Rossum EF, Lamberts SW. Polymorphisms in the glucocorticoid receptor gene and their associations with metabolic parameters and body composition. Recent Prog Horm Res 2004;59:333-57.

4. Pui CH, Dahl GV, Rivera G, Murphy SB, Costlow ME. The relationship of blast cell glucocorticoid receptor levels to response to single-agent steroid trial and remission response in children with acute lymphoblastic leukemia. Leuk Res 1984;8:579-85.
5. Mastrangelo R, Malandrino R, Riccardi R, Longo P, Ranelletti FO, Iacobelli S. Clinical implications of glucocorticoid receptor studies in childhood acute lymphoblastic leukemia. Blood 1980;56:1036-40.
6. Homo F, Duval D, Harousseau JL, Marie JP, Zittoun R. Heterogeneity of the in vitro responses to glucocorticoids in acute leukemia. Cancer Res 1980;40:2601-8.
7. Ramdas J, Liu W, Harmon JM. Glucocorticoid-induced cell death requires autoinduction of glucocorticoid receptor expression in human leukemic T cells. Cancer Res 1999;59:1378-85.
8. Vermeer H, Hendriks-Stegeman BI, van der Burg B, van Buul-Offers SC, Jansen M. Glucocorticoid-induced increase in lymphocytic FKBP51 messenger ribonucleic acid expression: a potential marker for glucocorticoid sensitivity, potency, and bioavailability. J Clin Endocrinol Metab 2003;88:277-84.
9. Lu NZ, Cidlowski JA. Translational regulatory mechanisms generate N-terminal glucocorticoid receptor isoforms with unique transcriptional target genes. Mol Cell 2005;18:331-42.
10. Wallace AD, Cidlowski JA. Proteasome mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. J Biol Chem 2001;12:12.
11. Le Drean Y, Mincheneau N, Le Goff P, Michel D. Potentiation of glucocorticoid receptor transcriptional activity by sumoylation. Endocrinology 2002;143:3482-9.
12. Han SH, Jeon JH, Ju HR*, et al.* VDUP1 upregulated by TGF-beta1 and 1,25-dihydroxyvitamin D3 inhibits tumor cell growth by blocking cell-cycle progression. Oncogene 2003;22:4035-46.
13. Schulze PC, Yoshioka J, Takahashi T, He Z, King GL, Lee RT. Hyperglycemia promotes oxidative stress through inhibition of thioredoxin function by thioredoxin-interacting protein. J Biol Chem 2004;279:30369-74.
14. Liu Y, Min W. Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. Circ Res 2002;90:1259-66.
15. Clark AR, Lasa M. Crosstalk between glucocorticoids and mitogen-activated protein kinase signalling pathways. Curr Opin Pharmacol 2003;3:404-11.
16. Platanias LC. Map kinase signaling pathways and hematologic malignancies. Blood 2003;101:4667-79.
17. Mizuno R, Oya M, Shiomi T, Marumo K, Okada Y, Murai M. Inhibition of MKP-1 expression potentiates JNK related apoptosis in renal cancer cells. J Urol 2004;172:723-7.
18. Brown P, Meshinchi S, Levis M*, et al.* Pediatric AML primary samples with FLT3/ITD mutations are preferentially killed by FLT3 inhibition. Blood 2004;104:11841-1849.
19. Stam RW, Den Boer ML, Schneider P*, et al.* Targeting FLT3 in primary MLL-gene-rearranged infant acute lymphoblastic leukemia. Blood 2005;106:2484-90.
20. Munck A, Naray-Fejes-Toth A, *Glucocorticoid action: physiology*, in *Endocrinology*, L.J. de Groot and J.L. Jameson, Editors. 2001, W.B. Saunders Company: Philadelphia. p. 1632-46.
21. Holleman A, Cheok MH, den Boer ML*, et al.* Gene-expression patterns in drug-resistant acute lymphoblastic leukemia cells and response to treatment. N Engl J Med 2004;351:533-42.
22. Holleman A, vanderWeele DJ, Passier MMCJ, *et al*., Sensitizing effect of glycolysis inhibition on prednisolone resistance in acute lymphoblastic leukemia., in Novel genomic determinants of apoptotic defects in acute lymphoblastic leukemia, Thesis. 2005.
23. Garber K. Energy boost: the Warburg effect returns in a new theory of cancer. J Natl

Cancer Inst 2004;96:1805-6.
24. Schaeke H, Schottelius A, Doecke WD*, et al.* Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. PNAS 2004;101:227-32.
25. Einstein M, Greenlee M, Rouen G*, et al.* Selective glucocorticoid receptor nonsteroidal ligands completely antagonize the dexamethasone mediated induction of enzymes involved in gluconeogenesis and glutamine metabolism. J Steroid Biochem Mol Biol 2004;92:345-56.
26. Peterson CL, Laniel M-A. Histones and histone modifications. Curr Biol 2004;14:R546-51.
27. Ichijo T, Voutetakis A, Cotrim AP*, et al.* The SMAD6/Histone deacetylase 3 complex silences the transcriptional activity of the glucocorticoid receptor: potential clinical implications. JBC 2005, in press.
28. Goettlicher, Minucci S, Zhu P*, et al.* Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. the EMBO journal 2001;20:6969-78.
29. Stams WA, Den Boer ML, Beverloo HB*, et al.* Effect of the histone deacetylase inhibitor depsipeptide on B-cell differentiation in both TEL-AML1-positive and negative childhood acute lymphoblastic leukemia. Haematologica 2005;90:1697-9.
30. Bostrom BC, Sensel MR, Sather HN*, et al.* Dexamethasone versus prednisone and daily oral versus weekly intravenous mercaptopurine for patients with standard-risk acute lymphoblastic leukemia: a report from the Children's Cancer Group. Blood 2003;101:3809-17.
31. Di Blasio AM, van Rossum EF, Maestrini S*, et al.* The relation between two polymorphisms in the glucocorticoid receptor gene and body mass index, blood pressure and cholesterol in obese patients. Clin Endocrinol (Oxf) 2003;59:68-74.
32. Rui L, Yuan M, Frantz D, Shoelson S, White MF. SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2. J Biol Chem 2002;277:42394-8.

Samenvatting voor de leek

Acute lymfatische leukemie (ALL) wordt jaarlijks bij 100-120 kinderen vastgesteld en is daarmee de meest voorkomende vorm van kanker bij kinderen. De behandeling bestaat uit 2 jaar chemotherapie: medicijnen die als tablet of drank dan wel via een infuus worden gegeven. Desondanks lukt het bij een klein deel van de patiënten niet de leukemie weg te krijgen (complete remissie te bereiken) en komt bij een deel van de patiënten de ziekte terug (recidief), waarbij de kans op genezing aanzienlijk lager is dan bij de eerste diagnose. De laatste 35 jaar is, doordat steeds betere combinaties van medicijnen worden toegepast, de kans dat een kind een complete remissie bereikt en de ziekte niet terugkomt gestegen van bijna 0 tot 80%. Toch blijft ALL van alle vormen van kanker de belangrijkste doodsoorzaak bij kinderen. De kinderen bij wie de leukemie terugkomt zouden misschien baat hebben gehad bij een meer gerichte of zwaardere chemotherapeutische behandeling. Daarom is het van belang om bij of kort na de diagnose ALL een inschatting van de genezingskans te kunnen maken. Een van de risicofactoren voor het terugkomen van de ziekte is ongevoeligheid voor de medicijnen prednison en dexamethason. Dit zijn 2 medicijnen van hetzelfde soort, namelijk glucocorticoïden, welke zeer belangrijk zijn in de behandeling van ALL. Kinderen met ALL, die onvoldoende reageren op de eerste 7 dagen therapie waarin alleen prednison wordt gegeven, hebben een aanmerkelijk grotere kans op een recidief. Ook uit laboratorium onderzoek blijkt dat kinderen een aanmerkelijk grotere kans op een recidief hebben als hun leukemiecellen in de reageerbuis slecht doodgaan o.i.v. prednison. Ongevoeligheid, oftewel resistentie, voor prednison is dus een belangrijke voorspeller voor het terugkeren van de leukemie (en uiteindelijke overleving). Het is dan ook belangrijk de oorzaken van prednison resistentie te vinden zodat de resistente patiënten beter behandeld kunnen gaan worden. De kennis die hierover bestond bij aanvang van dit promotie onderzoek is samengevat in **hoofdstuk 2**. Aangezien er echter nog heel veel onduidelijk was, zijn de onderzoeken, zoals beschreven in dit proefschrift, verricht.

Het antileukemisch effect van prednison begint nadat prednison de leukemische cel binnendringt en aan de zogenaamde glucocorticoïd receptor bindt. Na binding worden er allerlei acties in de cel gestart, waaraan de leukemiecel uiteindelijk dood gaat. De erfelijke code voor de glucocorticoïd receptor ligt, zoals voor alle eiwitten, op het DNA en heet het glucocorticoïd receptor gen. Om het eiwit te maken wordt van het gen eerst een kopie gemaakt: het mRNA. Dit wordt daarna

gebruikt als mal om talloze kopieën van de glucocorticoïd receptor te maken.

Afwijkingen in het glucocorticoïd receptor gen

Men kan zich voorstellen dat afwijkingen in het glucocorticoïd receptor gen zouden kunnen leiden tot een veranderde functie van deze receptor. Ons onderzoek of dergelijke afwijkingen gerelateerd zijn aan prednison resistentie is beschreven in **hoofdstuk 3**. Leukemische cellen van kinderen met ALL werden onderzocht op het voorkomen van afwijkingen (mutaties) in het glucocorticoïd receptor gen. Er werden geen afwijkingen gevonden. In 67% van de leukemie samples werden wel variaties in het DNA gevonden die polymorfismen worden genoemd. Polymorfismen zijn normaal voorkomende variaties in het DNA, die niet alleen in de leukemiecellen maar ook in de andere, gezonde lichaamscellen aanwezig zijn. Van 3 van de 6 gevonden polymorfismen is echter wel bekend dat de glucocorticoïd receptor door deze veranderingen minder goed of juist beter werkt. Echter, uit onze studie bleek dat deze polymorfismen niet de oorzaak zijn voor prednison resistentie bij kinderen met ALL.

Het aantal glucocorticoïd receptoren en zijn varianten

Naast de hierboven genoemde variaties op DNA niveau is bekend dat bij het aflezen van het DNA naar het mRNA een aantal varianten van de glucocorticoïd receptor kunnen ontstaan (GR-alpha, GR-beta en GR-P), mogelijk leidend tot een verminderde functie. In **hoofdstuk 4** werd onderzocht of prednison resistentie verklaard kan worden door een veranderde expressie van deze receptor varianten. Het bleek dat prednison resistente leukemiecellen een verminderd aantal van de functionele glucocorticoïd receptor (GR-alpha) hebben. Het verschil tussen gevoelige en resistente patiënten was echter niet heel groot (ongeveer 2-voudig), zodat we betwijfelen of dit de belangrijkste oorzaak van prednison resistentie is. De verhouding tussen de functionele variant GR-alpha en de andere varianten (GR-beta en GR-P) was niet verschillend tussen gevoelige en resistente patiënten, zodat geconcludeerd werd dat deze niet-functionele receptor varianten niet bijdragen aan prednison resistentie.

Blootstelling van leukemiecellen aan prednison leidt tot een toename van het aantal glucocorticoïd receptoren. (**Hoofdstuk 5**) Dit is bijzonder, aangezien in andere soorten lichaamscellen (die niet dood gaan o.i.v. prednison) het aantal receptoren juist afneemt. De mate van toename van het aantal receptoren in leukemiecellen bleek echter niet gerelateerd aan prednison gevoeligheid. De expressie van de

niet-functionele receptor varianten (GR-beta en GR-P) nam in gelijke mate toe, zodat de verhouding van functionele en niet-functionele receptoren hetzelfde bleef. Prednison resistentie kan daarom niet verklaard worden door een onevenredige toename van niet-functionele receptor varianten. Tevens is onderzocht of resistentie verklaard kan worden door het gebruik van specifieke promoter sequenties in het DNA. Deze promoter sequenties zijn essentieel voor het opstarten van het maken van mRNA kopieën van het DNA. De glucocorticoïd receptor heeft minstens 5 verschillende promoter sequenties. Ons onderzoek liet zien dat resistentie voor prednison niet afhangt van het type promoter dat gebruikt wordt voor de synthese van glucocorticoïd receptor mRNA bij kinderen met ALL.

(Co)chaperonne eiwitten van de glucocorticoïd receptor

Om prednison goed te kunnen binden heeft de glucocorticoïd receptor een aantal eiwitten nodig, die de receptor in zo'n vorm houden dat binding goed mogelijk is. Deze eiwitten heten chaperonne en cochaperonne eiwitten. In **hoofdstuk 6** wordt het mogelijk belang van dergelijke (co)chaperonne eiwitten voor prednison resistentie onderzocht. De mRNA expressie van deze (co)chaperonne moleculen (HSP-70, HSP-40, HIP, BAG-1, HOP, HSP-90, P-23, FKBP-51, FKBP-52 and CYP-40) is zeer heterogeen en kan geen verklaring zijn voor prednison resistentie bij kinderen met ALL.

Paden langs welke prednison leukemiecellen doodt

Nadat prednison aan de glucocorticoïd receptor bindt wordt een proces in gang gezet waaraan de leukemiecel uiteindelijk dood gaat. Dit proces bestaat uit een of meer complexe cascade(s) van signalen waarover nog relatief weinig bekend is. Aangezien de studies in de **hoofdstukken 3 tot 5** lieten zien dat afwijkingen in de glucocorticoïd receptor en zijn (co)chaperonne moleculen niet de oorzaak zijn van prednison resistentie, zou resistentie mogelijk verklaard kunnen worden door afwijkingen in deze signaal-cascades. Om dit verder te onderzoeken werd een micro-array studie gedaan. Dit is een moderne techniek, waarbij van 14500 genen tegelijk het expressie niveau gemeten kan worden. Zoals beschreven in **hoofdstuk 7**, resulteerde blootstelling van leukemiecellen aan prednison in een veranderde expressie van 51 genen; de expressie van 39 genen nam toe terwijl de expressie van 12 genen juist verlaagd werd. Twee van de genen die verhoogd tot expressie kwamen o.i.v. prednison blootstelling coderen voor eiwitten die het ontstaan van tumoren kunnen onderdrukken (tumor-suppressor genen). Van de

51 genen gereguleerd door prednison zijn een aantal gerelateerd aan paden die te maken hebben met cel-overleving en -deling (MAP kinase paden en genen gerelateerd aan NF-κB) dan wel de energie voorziening van de cel. Een van de hypothesen omtrent prednison resistentie die verder onderzoek behoeft is dat resistente ALL cellen wellicht beter in staat zijn de energie productie na blootstelling aan prednison te verhogen, wat ze helpt te ontkomen aan celdood.
Een beter inzicht in de paden die uiteindelijk prednison geïnduceerde celdood veroorzaken lijkt nodig om de oorzaken van prednison resistentie te vinden. Als deze paden bekend zijn, kan mogelijk meer gerichte therapie ("targeted therapy") ontwikkeld worden, waarbij resistentie omzeild kan worden en prednison de leukemiecellen kan doden met zo min mogelijk bijwerkingen voor de gezonde cellen.

OVERWEGINGEN VOOR DE TOEKOMST

Nieuwe glucocortoid receptor varianten

Op basis van de studies zoals beschreven in dit proefschrift (**hoofdstuk 3 - 6**) concluderen we dat veranderingen van de glucocorticoïd receptor en daaraan gerelateerde moleculen slechts een beperkt effect hebben op de mate van prednison resistentie bij kinderen met ALL. De laatste tijd zijn er echter nog weer nieuwe varianten beschreven van de glucocorticoïd receptor. Het zou interessant zijn ook van deze receptor varianten de invloed op prednison resistentie te onderzoeken.

Veranderingen op eiwit niveau

In de **hoofdstukken 4 en 5** zagen we dat de hoeveelheid mRNA en eiwit van de glucocorticoïd receptor aan elkaar gecorreleerd zijn in leukemische cel lijnen. Hoewel het waarschijnlijk is dat dit ook in de leukemiecellen in de patiënt het geval is, dient dit nog wel onderzocht te worden. Het zou kunnen zijn dat ondanks gelijke hoeveelheden mRNA, het receptor-eiwit in resistente cellen sneller wordt afgebroken dan in gevoelige cellen.

Paden langs welke prednison leukemiecellen doodt

In de studie als beschreven in **hoofdstuk 7** vonden we 2 paden (MAPK paden en paden gerelateerd aan NF-κB) waarvan verschillende genen gereguleerd worden onder invloed van prednison blootstelling. De relatie van deze paden met prednison geïnduceerde celdood moet verder onderzocht worden. Een van de manieren om deze paden te onderzoeken is door de paden op bepaalde plekken te blokkeren

(door genen uit te schakelen). Als een dergelijke blokkade de gevoeligheid voor prednison vermindert, dan is dat betreffende pad mogelijk belangrijk voor het celdodend effect van prednison.
Een andere conclusie die werd getrokken na de studie als beschreven in **hoofdstuk 7**, is dat in de paden die zorgen voor de energie voorziening van de cel een aantal genen worden gereguleerd o.i.v. prednison blootstelling. Mogelijk zijn cellen die resistent zijn voor prednison beter in staat hun energie productie te verhogen, zodat ze kunnen overleven. Ander onderzoek heeft dit inmiddels nog meer aannemelijk gemaakt. Het zou daarom interessant zijn de mogelijkheden te onderzoeken om de energievoorziening van de leukemiecel te remmen, zodat de cel gevoeliger wordt voor het celdodend effect van prednison.

Bijwerkingen van prednison behandeling
Er is veel verschil tussen de ALL patiënten met betrekking tot het optreden van bijwerkingen van de prednison. De bijwerkingen die kunnen optreden zijn o.a.: hoge bloeddruk, bol, opgezet gezicht (cushingoïd uiterlijk of maangezicht), overgewicht, suikerziekte en gedragsstoornissen. Hoewel deze bijwerkingen overgaan na het stoppen van de therapie, kunnen ze wel heel lastig zijn voor de patiënt. Een van de mogelijke oorzaken voor het verschil in gevoeligheid voor deze bijwerkingen tussen patiënten zijn de verschillende polymorfismen zoals beschreven in **hoofdstuk 3**. Hoewel we hebben laten zien dat deze niet de oorzaak zijn van prednison resistentie, zouden ze wel de oorzaak kunnen zijn van het wel of niet optreden van bijwerkingen in de patient. Deze mogelijkheid wordt onderzocht in het nieuwe ALL-10 behandel protocol van de SKION (Stichting KinderOncologie Nederland).

Samenvattend hebben we in dit proefschrift laten zien dat de belangrijkste oorzaak van prednison resistentie bij kinderen met leukemie moet liggen nadat prednison is gebonden aan de glucocorticoïd receptor. Hoewel resistente leukemiecellen over minder glucocorticoïd receptoren beschikken dan gevoelige, lijkt dit toch niet de belangrijkste oorzaak van het meer dan 1000-voudige verschil in resistentie tussen prednison gevoelige en resistente leukemiecellen. Andere variaties van de glucocorticoid receptor als oorzaak van prednison resistentie (mutaties en polymorfismen, (co)chaperonne moleculen en regulatie van de receptor door prednison) werden uitgesloten. In een gen expressie studie vonden we nieuwe genen die gereguleerd worden door prednison blootstelling. Verdere studies naar

de functionele paden die leiden tot prednison geïnduceerde celdood en oorzaken van prednison resistentie bij kinderen met ALL is nodig. Inzicht in de wijze waarop prednison leukemiecellen doodt zou kunnen leiden tot meer specifieke en gerichte therapie, die hetzelfde antileukemisch effect heeft als prednison, maar met minder bijwerkingen gepaard gaat.

DANKWOORD

Naast de opleiding tot kinderoncoloog / -hematoloog is nu, met het verschijnen van dit proefschrift, ook de opleiding tot onderzoeker afgerond. Ik hoop dat ik in de toekomst beide opleidingen nog veel zal gebruiken, in mijn klinisch werk als kinderoncoloog en in het werk als onderzoeker op het zeer uitgebreide gebied in de kinderoncologie van wat we nog niet weten.

Bij het afsluiten van een periode hoort het bedanken van mensen. Ik hoop echter dat ik al tijdens de afgelopen jaren blijk heb gegeven van mijn waardering voor de mensen met wie ik samenwerkte, anders komt die waardering wel erg laat. Desalniettemin toch maar:

In de eerste plaats grote waardering voor mijn promotor: Rob Pieters. Begin 1999 kwam je als nieuw sub-hoofd van de afdeling kinderoncologie naar Rotterdam, en ik heb het afgelopen jaar aan den lijve ondervonden dat een nieuwe werkplek "wel even wennen is". Daarbij had je toen meteen een onervaren fellow, die onderzoek wilde doen. Zo kwam ik als eerste promovendus op een lab, waar behalve een post-doc (Monique den Boer) en een analist (Karin Kazemier) nog niets was, alleen wat lege lab-tafels. Dank dat je er heil in zag met deze dokter zonder onderzoekservaring te beginnen, in plaats van een bioloog met misschien meer adequate vooropleiding. Dank voor je begeleiding, je vermogen om steeds terug te vragen: "waar gaat het nu eigenlijk over" en "kan je het niet eenvoudiger formuleren", en je kennis op velerlei vlak. Na mijn promotie en door mijn vertrek naar Groningen scheiden onze wegen zich enigszins, maar ik hoop dat we in de toekomst nog veel zullen samenwerken.

Dan mijn copromotoren: Jules Meijerink en Monique den Boer. Dank voor jullie begeleiding! Jules, niet copromotor vanaf het allereerste begin, want je kwam pas naar Rotterdam nadat het project al enige tijd liep. Je nam het project over van Monique en je hebt je daar vervolgens vol overgave in gestort. Ik heb veel van je geleerd over de "hogere moleculaire biologie". Je bent in staat geweest deze klinische dokter moleculaire biologie bij te brengen, compleet met de bijbehorende technieken. Ik zal die kennis hopelijk nog lang gebruiken.
Monique, copromotor van het eerste uur, toen je begon in een lab waar nog niets was. Dank voor je kritische blik op mijn artikelen. Ik denk dat vooral jij me hebt geleerd een wetenschappelijk artikel te schrijven en je hebt me ingewijd in de diepere krochten van de statistiek, zeker ten tijde van onze array experimenten.

Dank voor al de opbouwende kritieken.

I would like to thank Scott Armstrong and Steve Sallan from the Dana-Farber Cancer Institute in Boston, USA for their collaboration in our search for glucocorticoid responsive genes. Scott, thank you for performing the gene arrays and for the stimulating discussions and visits to Boston. Steve Sallan, thank you for taking place in my promotion committee.

Professor dr. P. Sonneveld, beste Pieter. Dank voor de stimulerende discussies in de tijd dat we de polymorphisme- en splice variant studies opstartten. Dank dat je in de promotie commissie wilt zitten. Natuurlijk wil ik ook de andere leden van de promotie commissie danken: professor dr. F.H. de Jong, dr. G.J. Kaspers, professor dr. W.A. Kamps en professor dr. H.A. Büller.

Dank aan de mensen van het lab. Jullie zagen iemand komen, die nog de goede moed had "DNA te kunnen maken". Dank voor het geduld waarmee jullie me leerden de pipet vast te houden en de uitleg over weer een nieuwe techniek. De analisten die meewerkten aan het PRED project: Aart, Anita, Bas, Marli en Mathilde; zonder jullie zou ik het klinisch werk nooit hebben kunnen combineren met het onderzoek en was er niets van dit boekje terecht gekomen.
Natuurlijk ook de overige analisten (Jessica, Karin, Nathalie, Marjolein, Monique, Susan en Pauline), altijd bereid om weer iets uit te leggen of om me uit te lachen voor mijn vaak weinig moleculair biologisch verantwoorde opmerkingen.
Hetzelfde geldt voor de andere Aio's en onderzoekers: Amy, Arantza, Barbara, Esther, Henne, Inge, Judith, Marrit, Martine, Pieter, Robert, Ronald en Wendy dank voor jullie hulp en gezelligheid op het lab.

Karel Hählen. Beste Karel, in 1998 nam jij me samen met Rob aan als fellow kinderoncologie. Dank voor het in mij gestelde vertrouwen. In mijn jaren als fellow (en daarna) heb ik veel van je geleerd over de kinderoncologie. Regelmatig, als ik weer met een vraag kwam, wist je te vertellen dat dat in het verre verleden was onderzocht, waarbij je vaak ook nog het betreffende artikel in de kast had. Jij bent voor mij van grote waarde geweest voor mijn opleiding tot kinderoncoloog.

De overige collega kinderoncologen in Rotterdam: dank voor jullie kennis, gezelligheid en geduld als ik weer eens naar het lab moest. Natuurlijk wil ik Auke Beishuizen op deze plaats extra noemen: collega fellow van (bijna) het eerste uur, kamer genoot en nu paranymf. We hadden een goede en gezellige tijd op onze

kamer (eerst ver weg gestopt in de kelder als fellow, later mochten we op de gang voor "echte" stafleden). Ik mis je nog regelmatig als ik in Groningen alleen op mijn kamer zit. Dank voor je luisterend oor en je goede adviezen met betrekking tot het onderzoek.

Dank aan de "nieuwe" collega's in Groningen. Voor mij was het verfrissend om na jaren Sophie in een andere omgeving te gaan werken. Ik hoop dat het voor jullie net zo verfrissend is. Vanaf het eerste moment voelde ik me zeer welkom en stonden jullie open voor mijn ideeën. Het moet wennen zijn geweest: mijn vaak directe en snelle "Tissing-" dan wel "Rotterdamse" manier van communiceren.

Het Peizer Pediatrisch Genootschap: op eigen verzoek opgenomen in dit dankwoord. Gedurende de vele fietstochten naar het UMCG en naar huis wordt er soms over het werk gesproken, maar meestal gaat het gelukkig over andere zaken en is het gewoon gezellig. Ik denk dat het nu toch tijd wordt voor onze eerste maandelijkse evaluatie. Ik trakteer!

Rob Davelaar, dank voor je creatieve inbreng en je kennis en handigheid "tot op de pixel".

Henk, dank dat je mijn paranymf wilt zijn, een maand voordat het leven voor jullie nog drukker zal worden dan het al was. Anneke en ik zullen er voor jullie zijn in de komende tijd.

Ik had me voorgenomen een kort dankwoord te schrijven en heb een hoop mensen nog niet genoemd: de verpleging op de afdelingen, de mensen op de poli, de mensen op het lab (Mieke, Rolinda, Henk, Carla en Emmy) enzovoort. Voor jullie allen geldt wat ik hierboven al schreef: een woord van dank zou bijna te laat komen. Dank voor de prettige samenwerking in Rotterdam, en voor de Groningers: het begint nu echt!

Op de allerlaatste plaats: Anneke en de jongens. An, je wilde niet bedankt worden. Ook voor jouw geldt het bovenstaande: je weet allang wat ik van je vind, maar ik zal het mijn leven lang blijven roepen. Bas en Tim zijn ten alle tijden goed voor de nodige relativering, humor en heel veel houden van!

CURRICULUM VITAE

Wim Tissing was born on December 30th, 1965 in Lisse, the Netherlands. He finished Gymnasium Beta in Lisse in 1984. From 1984 to 1992 he studied medicine at the Leiden University and started working as a resident in Pediatrics in the Sophia Children's Hospital Rotterdam (later: ErasmusMC-Sophia) in April 1992. (successive heads professor dr. H.K.A. Visser, professor dr. H.J. Neijens and professor dr. H.A. Büller) His official training in pediatrics lasted from April 1994 till October 1998. For a short time he was *Chef the Clinique* in the department of pediatrics of the ErasmusMC-Sophia, before starting his training in Pediatric Oncology / Hematology. (head professor dr. R. Pieters) As part of this fellowship he worked for 6 months in the department of Pediatric Oncology / Hematology of the Leiden University Medical Center, in particular in the bone-marrow transplant unit (head dr. R.M. Egeler). Since October 1st, 2000 (his registration as Pediatric Oncologist / Hematologist) he was a staff member of the department of Pediatric Oncology / Hematology in the ErasmusMC – Sophia till he moved to Groningen January 1st, 2005. Since then he has been working as a staff member of the department of Pediatric Oncology / Hematology in the Beatrix Children's Hospital / University Medical Center Groningen. (head professor dr. W.A. Kamps) Since 1999 he has been working on the studies as described in this thesis in the department of Pediatric Oncology / Hematology in the ErasmusMC – Sophia. (professor dr. R. Pieters)

He is an active member of the Dutch Childhood Oncology Group (DCOG) in the committees of supportive care and rare tumors. For many years he was part of the organizing committee of "Winterkolder" (a skiing camp for children with cancer, in particular for children with amputations). He is member of the executive committee of the SKOV (foundation for holiday camps for children with cancer).

He is married to Anneke Tjassing and they have 2 children: Bas (2000) and Tim (2002).